Multiple Isoelectric Forms of Poliovirus RNA-Dependent RNA Polymerase: Evidence for Phosphorylation

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Poliovirus-specific RNA-dependent RNA polymerase (3D^{pol}) was purified to apparent homogeneity. A single polypeptide of an apparent molecular weight of 63,000 catalyzes the synthesis of dimeric and monomeric RNA products in response to the poliovirion RNA template. Analysis of purified 3D^{pol} by two-dimensional electrophoresis showed multiple forms of 3D^{pol}, suggesting posttranslational modification of the protein in virus-infected cells. The two major forms of 3D^{pol} appear to have approximate pI values of 7.1 and 7.4. Incubation of purified 3D^{pol} with calf intestinal phosphatase resulted in almost complete disappearance of the pI 7.1 form and a concomitant increase in the intensity of the pI 7.4 form of 3D^{pol}. Addition of ³²P-labeled P_i during infection of HeLa cells with poliovirus resulted in specific labeling of 3D^{pol} and 3CD, a viral protein which contains the entire 3D^{pol} sequence. Both 3D^{pol} and 3CD appear to be phosphorylated at serine residues. Ribosomal salt washes prepared from both mock- and poliovirus-infected cells contain phosphatases capable of dephosphorylating quantitatively the phosphorylated form (pI 7.1) of 3D^{pol}.

The positive-polarity, single-stranded RNA genome of picornaviruses is replicated by a virus-encoded, RNA-dependent RNA polymerase found in the cytoplasm of infected cells (2). The first RNA-dependent RNA polymerase was detected in cells infected with mengo virus, a member of the picornavirus family (3). The most studied RNA-dependent RNA polymerase, however, is that of poliovirus. The poliovirus RNA-dependent RNA polymerase (3D^{pol}) (25) results from proteolytic cleavage of a polyprotein synthesized from poliovirus RNA in cells infected with the virus (26). This enzyme is responsible for replication of the viral genome in the cytoplasm of infected cells (5, 10, 12, 13, 19, 29, 30). The input viral RNA (plus strand) is first copied by the RNA polymerase to generate a cRNA (minus strand) which serves as the template for synthesis of progeny virion RNA (2). In vitro synthesis of minus-strand RNA from a virion RNA template requires 3D^{pol} and a host cell protein called host factor (4, 9, 11). A synthetic RNA primer, oligo(U), can replace host factor in the in vitro reaction, suggesting a role of host factor in the initiation of minus-strand RNA synthesis (9).

We have purified 3D^{pol} to apparent homogeneity. We present evidence suggesting that 3D^{pol} is phosphorylated in vivo at serine residues. We also show that virus-specific protein, 3CD, which contains the entire 3D^{pol} molecule, is also phosphorylated at serine residues. The purified 3D^{pol} catalyzes oligo(U)-primed synthesis of covalently linked dimeric RNA in addition to monomeric RNA products. We discuss the possible role of phosphorylation of 3D^{pol} in the replication of the poliovirus RNA genome.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phosphocellulose was obtained from Whatman, Inc., (Clifton, N.J.). Unlabeled nucleotides were obtained from Calbiochem-Be-

hring (La Jolla, Calif.). Poly(U)-Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). Radioisotopes were purchased from Amersham Corp. (Arlington Heights, Ill.) and ICN Pharmaceuticals Inc. (Irvine, Calif.).

Cells and virus. HeLa cells were grown in Spinner cultures with Joklik modified essential medium supplemented with 6% newborn calf serum and infected with poliovirus type 1 (Mahoney strain) as previously described (10).

Labeling proteins with [³⁵S]methionine. To label poliovirus proteins with [³⁵S]methionine, cells were suspended, after virus adsorption, in Earle saline supplemented with 5% dialyzed fetal calf serum and all the amino acids except methionine. [³⁵S]methionine Trans-label (specific activity, 1,000 Ci/mmol; 1 mCi/4 × 10⁸ cells) was added to the culture after 2.5 h of infection, at which time host cell protein synthesis was inhibited so that only poliovirus-specific proteins were labeled. Cells were harvested by centrifugation 5.5 h after infection, and the pellets were washed with phosphate-buffered saline and stored at -70°C.

Labeling of infected cells with ³²P_i. The protocol for infection was the same as that described above except that the cell culture medium was phosphate free (24). At 2.5 h postinfection, ³²P_i (2 mCi/4 × 10⁸ HeLa cells) was added to the culture. Cells were harvested at 5.5 h postinfection as described above. For quantitation of phosphorylation, the polypeptide bands were excised from sodium dodecyl sulfate (SDS)-polyacrylamide gels and soaked in liquid scintillation fluid and radioactivity was measured in a liquid scintillation counter.

Purification of poliovirus polymerase. Poliovirus polymerase was purified by phosphocellulose chromatography (fraction II) as described previously (10). Fraction II polymerase was further purified by fast protein liquid chromatography on a 1-ml mono S column (Pharmacia). The enzyme was eluted with a 15-ml, 50-to-500 mM linear gradient in buffer A (50 mM Tris hydrochloride [pH 8.0], 10% glycerol, 0.1% Nonidet P-40, 5 mM 2-mercaptoethanol). Peak fractions containing poly(U) polymerase activity were dialyzed overnight in buffer B (buffer A [20% glycerol] and 50 mM KCl). Further purification of the fraction III polymerase involved

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passage through poly(U) Sepharose 4B, followed by elution of the enzyme with a 50-to-400 mM KCl gradient. Fractions containing poly(U) polymerase activity were pooled, concentrated, and dialyzed against buffer B. Fraction IV polymerase was then passed through a second poly(U) Sepharose 4B column and eluted as previously described. The enzyme (fraction V) was stored at -70°C in small aliquots. Poliovirus RNA was prepared as previously described (28).

In vitro RNA synthesis with polymerase and methylmercuric hydroxide gel electrophoresis. The standard reaction mixture of oligo(U)-dependent RNA synthesis contained the following in 50 μl : 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0); 5 mM magnesium acetate; 4 mM dithiothreitol; 10 μg of dactinomycin per ml; 30 mM KCl; 20 μM zinc chloride; 0.2 mM each ATP, GTP, and CTP; 1 μCi of [α - ^{32}P]UTP; 5 μM unlabeled UTP; 1 μg of poliovirus template RNA; and 0.1 μg of fraction V polymerase. Reactions contained 100 ng of oligo(U) per ml. Incubation was for 30 min at 37°C . For analysis of the in vitro product, the phenol-extracted, ethanol-precipitated labeled RNA was suspended in diethylpyrocarbonate-treated water, denatured with 15 mM methylmercuric hydroxide, and analyzed on a 1% agarose gel containing 10 mM methylmercuric hydroxide as described by Bailey and Davidson (1).

Phosphoamino acid analysis. 3D^{pol} was located on SDS-polyacrylamide gels by direct autoradiography, and the band was excised from multiple lanes and electro eluted as described elsewhere (27). The isolated 3D^{pol} was then acid hydrolyzed in the presence of bovine serum albumin, ovalbumin, lysozyme, and a mixture of phosphoserine, phosphotyrosine, and phosphothreonine. The phosphoamino acids were then separated by high-voltage paper electrophoresis as described elsewhere (27). The paper was dried, autoradiographed, and then stained with ninhydrin in acetone to visualize the phosphoamino acid markers.

Dephosphorylation reactions. Polymerase in buffer B was added to reaction mixtures containing 30 mM HEPES (pH 8.0), calf intestinal phosphatase (CIP), boiled CIP, or mock- or poliovirus-infected ribosomal salt wash (RSW) (24) at the indicated concentrations. Each reaction mixture also contained 5 μg of bovine serum albumin per ml as described elsewhere (18). One unit of CIP activity was defined as the amount which hydrolyzed 1.0 μM *p*-nitrophenyl phosphate to *p*-nitrophenol and P_i per min at 25°C . CIP was boiled for 15 min prior to its addition to control reactions. The reactions were prepared on ice, initiated by incubation for the appropriate amounts of time at 30°C , and then prepared for two-dimensional gel analysis.

Two-dimensional gel electrophoresis. Two-dimensional gel analysis was performed as described by O'Farrell (22). The first dimension was an isoelectric-focusing gel in the pH range of 3 to 10 or 6 to 8 as indicated previously (22). The second dimension was a 12.5% SDS-polyacrylamide gel. After separation of the proteins in two dimensions, the gel was fixed, dried, and autoradiographed with X-AR film (Eastman Kodak Co., Rochester, N.Y.) and a Cronex intensifier (Du Pont Co., Wilmington, Del.).

RESULTS

Purification of 3D^{pol} . In a previous study, we reported purification of 3D^{pol} from the soluble phase of infected HeLa cells to two polypeptides of approximate molecular weights of 63,000 and 35,000 (17). The 63,000- M_r polypeptide was 3D^{pol} , whereas the 35,000- M_r polypeptide was of host origin

FIG. 1. Purification of poliovirus polymerase. Poliovirus polymerase was purified from 2×10^9 HeLa cells infected with poliovirus as described in Materials and Methods. Fraction II, III, and V polymerase preparations were analyzed by SDS-PAGE followed by silver staining of the gel. Lanes: 1, molecular weight marker proteins (numbers on the left are molecular weights in thousands); 2 through 4, pooled and concentrated fraction II, III, and V polymerases, respectively. 3D, 3D^{pol} .

(17). This highly purified preparation of 3D^{pol} catalyzed the synthesis of covalently linked dimeric RNA products from poliovirus RNA templates in the presence of an oligo(U) primer. Experiments utilizing $5'$ - ^{32}P -labeled oligo(U) primer and all four unlabeled nucleotide triphosphates suggested that the viral RNA polymerase elongates the oligo(U) primer copying the virion RNA template (plus strand) and that the newly synthesized minus strand snaps back on itself to generate a template-primer structure. This structure is then elongated by the polymerase to form covalently linked dimeric RNA products (16, 17). To examine the possibility that the 35,000- M_r polypeptide may play a role in the oligo(U)-primed synthesis of dimeric RNA products, attempts were made to further purify the viral polymerase. We developed a modified purification scheme for 3D^{pol} which involved salt gradient elution through phosphocellulose (fraction II) and fast protein liquid-mono S chromatography (fraction III), followed by salt gradient elution through two consecutive poly(U)-Sepharose columns (fractions IV and V). Figure 1 shows polypeptide profiles of different preparations of 3D^{pol} as analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver staining. The fraction II polymerase contained several polypeptides (Fig. 1, lane 2). Subsequent purification of fraction II enzyme by mono S (Fig. 1, lane 3) and poly(U) Sepharose chromatography resulted in several-thousandfold purification of 3D^{pol} (data not shown). In fact, only one polypeptide of an approximate M_r of 63,000 could be detected in the final poly(U)-Sepharose-purified enzyme preparation (Fig. 1, lane 4). Further analysis of fraction V 3D^{pol} by two-dimensional gel electrophoresis followed by silver staining showed only one major spot at an M_r of 63,000 with a pI of 7.4 (Fig. 2B). Another faint spot of apparently the same M_r which migrated to a pI of 7.1 was also detected. Comparison of silver-stained spots in the fraction V enzyme preparation (Fig. 2B) with a partially purified ^{35}S -labeled 3D^{pol} preparation (Fig. 2A) showed that the silver-stained major (pI 7.4) and minor (pI 7.1) spots comigrated with two ^{35}S -labeled 63,000- M_r spots at pIs 7.4 and 7.1, respectively. These results suggest that highly purified preparations of the enzyme might contain at least two forms of 3D^{pol} with the same M_r but different isoelectric points.

The 63,000- M_r 3D^{pol} synthesizes dimeric RNA products. To examine the nature of RNA products synthesized by the purified 3D^{pol} enzyme, fractions II, III, and V were incubated separately with poliovirus RNA template and all four

FIG. 2. Two-dimensional gel analysis of 3D^{pol}. (A) Analysis of partially purified [³⁵S]methionine-labeled viral proteins by two-dimensional gel electrophoresis followed by fluorography. (B) Analysis of fraction V polymerase by two-dimensional electrophoresis followed by silver staining of the gel. Arrowheads indicate the positions of 3D^{pol}. IEF, Isoelectric focusing. E, Extract containing [³⁵S]methionine-labeled viral proteins.

ribonucleoside triphosphates in the presence or absence of oligo(U). Labeled RNA products were denatured with 15 mM methylmercuric hydroxide and analyzed on agarose gels containing 10 mM methyl mercury. All fraction II, III, and V enzyme preparations were dependent on oligo(U) (Fig. 3). No labeled products could be detected in the absence of oligo(U) with fraction III and V polymerases (Fig. 3, lanes 3 and 5). These results contrast those in a recent study in

FIG. 3. In vitro RNA synthesis of dimeric RNA by poliovirus polymerase. Fraction II, III, and V polymerases were used for synthesis of labeled RNA from the poliovirus RNA template in the presence of oligo(U) as described in Materials and Methods. Labeled RNAs synthesized in vitro were denatured by 15 mM methyl mercury and analyzed by agarose gel electrophoresis in the presence of 10 mM methyl mercury. Lanes: 1 and 2, 20 µg of fraction II polymerase; 3 and 4, 1 µg of fraction III polymerase; 5 and 6, 0.1 µg of fraction V polymerase; +, with oligo(U); -, without oligo(U). 1X and 2X, Monomeric and dimeric RNAs, respectively. The arrow indicates the position of an RNA species which migrates faster than 2X RNA and is not detected in the reaction containing fraction V polymerase.

which Plotch et al. observed oligo(U)-independent synthesis of dimeric RNA molecules in response to poliovirus RNA by the viral polymerase expressed in *Escherichia coli* (23). The majority of the products synthesized by the fraction II and fraction III 3D^{pol} consisted of labeled 35S RNA; however, some products of higher-molecular-weights were also synthesized. These higher-molecular-weight RNAs consisted of two bands, one migrating at approximately twice (2×) the size of single-stranded RNA and one migrating faster than the 2× RNA. In contrast, the fraction V enzyme synthesized mainly the 2× product and some monomeric RNA. The product (Fig. 3, arrow), which migrated a little faster than the 2× RNA, was not detected in the reaction containing fraction V enzyme. At present, we do not know how this product is synthesized by 3D^{pol}. However, since synthesis of this product is dependent on oligo(U), we suspect that it results from the snap-back of the newly synthesized minus-strand RNA that is not quite full length. In other words, while copying the plus-strand template during synthesis, the labeled minus strand can snap back on itself prior to reaching the 5' end of the template and prime itself for synthesis of a complementary (plus) strand. Indeed, our preliminary experiments indicate that the product does not contain sequences complementary to the 5' end of template RNA. These results suggest that highly purified 3D^{pol}, which consists of one silver-stainable polypeptide, is capable of catalyzing synthesis of covalently linked dimeric RNA molecules in response to oligo(U)-primed poliovirus RNA.

3D^{pol} is phosphorylated. Analysis of the most-purified 3D^{pol} by two-dimensional gel electrophoresis showed that there were multiple forms of the molecule which differed only by their net charges (Fig. 2). To investigate the possibility that 3D^{pol} undergoes posttranslational modification in infected HeLa cells, virus-specific proteins were labeled with [³⁵S]methionine. Labeled polymerase was then purified from the cytoplasm of infected cells as described above. [³⁵S]methionine-labeled fraction V polymerase was analyzed by two-dimensional gel electrophoresis (Fig. 4). Two forms (pIs 7.1 and 7.4) of labeled 3D^{pol} were detected again (Fig. 4A). In this particular preparation, however, the pI 7.1 form of 3D^{pol} was much more abundant than the pI 7.4 form, unlike in the preparation shown in Fig. 2. Treatment of labeled polymerase with 0.01 U (Fig. 4B) and 0.1 U (Fig. 4C) of CIP prior to two-dimensional gel analysis resulted in almost complete disappearance of the pI 7.1 form and a concomitant increase in the intensity of the pI 7.4 form. A similar result was obtained by using a different preparation of 3D^{pol} (Fig. 4D and E). However, when CIP was inactivated by heating at 100°C prior to being mixed with 3D^{pol}, the intensities of spots 1 (pI 7.1) and 2 (pI 7.4) were relatively unchanged (Fig. 4F) compared with their intensities in the control (Fig. 4D). Mixing equal amounts of each sample (Fig. 4D to F) provided further proof that the disappearance of the pI 7.1 form (spot 1) is accompanied by an increase in the intensity of the pI 7.4 form (spot 2) (Fig. 4G). These results suggest that the two different forms of 3D^{pol} could represent different phosphorylation states of the molecule.

If 3D^{pol} is phosphorylated in vivo, it should be possible to label 3D^{pol} by addition of ³²P_i during infection of cells with poliovirus. Experiments were performed in which parallel cultures of infected cells were labeled with [³⁵S]methionine or ³²P_i and 3D^{pol} was then purified from ³²P_i- and [³⁵S]methionine-labeled infected cells. Figure 5A shows analysis by SDS-PAGE of 3D^{pol} purified by phosphocellulose (lanes 1 and 4), mono S (lanes 2 and 5), and poly(U)

FIG. 4. Two-dimensional gel analysis of buffer- or CIP-treated [^{35}S]methionine-labeled fraction V polymerase. [^{35}S]methionine-labeled 3D^{pol} was treated with buffer B, CIP, or boiled CIP as indicated in Materials and Methods. (A) Buffer B; (B) 0.01 U of CIP; (C) 0.1 U of CIP; (D) buffer B; (E) buffer B plus 0.1 U of CIP; (F) boiled CIP; (G) mixture of samples from panels D, E, and F. Spots: 1, pI 7.1; 2, pI 7.4.

Sephacrose (lanes 3 and 6) column chromatographies. A ^{32}P -labeled band (lane 6) comigrated with [^{35}S]methionine-labeled 3D^{pol} (lane 3). Comparison of mono S-purified ^{35}S - and ^{32}P -labeled proteins (Fig. 5A, lanes 2 and 5, respectively) showed that a viral protein comigrating with 3CD, a putative precursor to 3D^{pol} , was also labeled by ^{32}P . Analysis of ^{35}S - and ^{32}P -labeled proteins by two-dimensional gel electrophoresis showed that the ^{32}P -labeled bands of M_r s of 63,000 and 72,000 comigrated exactly with [^{35}S]methionine-labeled 3D^{pol} and 3CD, respectively (Fig. 5B and C).

Both 3D^{pol} and 3CD are phosphorylated at serine residues. To determine which amino acids in 3D^{pol} and 3CD are phosphorylated, ^{32}P -labeled proteins were excised from an SDS gel, electro eluted, and hydrolyzed with 6 M HCl. The resulting phosphoamino acids were analyzed by high-voltage paper electrophoresis at pH 3.0. Acid hydrolysis of both 3CD and 3D^{pol} yielded spots which comigrated with phosphoserine molecules (Fig. 6).

Dephosphorylation of 3D^{pol} in cell extracts. To determine whether uninfected HeLa cells contain an activity capable of phosphorylating 3D^{pol} , crude cell extracts were prepared from mock- and poliovirus-infected cells (RSW). These extracts were then mixed with [^{35}S]methionine-labeled 3D^{pol} and unlabeled ATP. In these experiments, a shallower pH gradient was used to facilitate better separation of the two forms of 3D^{pol} . Incubation of purified 3D^{pol} with ATP alone did not change the pattern of migration of the two forms of 3D^{pol} , suggesting that fraction V polymerase did not contain a kinase capable of phosphorylating the polymerase molecule (Fig. 7, compare panel A with panel B). Incubation of an RSW prepared from mock-infected cells with 3D^{pol} and ATP resulted in complete disappearance of the acidic form (pI

FIG. 5. One- and two-dimensional gel analysis of [^{35}S]methionine- and ^{32}P -labeled polymerase. (A) SDS-PAGE analysis. Lanes: 1, [^{35}S]methionine-labeled fraction II polymerase; 2, [^{35}S]methionine-labeled fraction III polymerase; 3, [^{35}S]methionine-labeled fraction V polymerase; 4, ^{32}P -labeled fraction II polymerase; 5, ^{32}P -labeled fraction III polymerase; 6, ^{32}P -labeled fraction V polymerase; 7, same as lane 1 with 20 times the amount of protein. (B to F) Two-dimensional gel electrophoresis. (B) [^{35}S]methionine-labeled fraction III polymerase; (C) ^{32}P -labeled fraction III polymerase; (D) [^{35}S]methionine-labeled fraction V polymerase; (E) same as panel D except polymerase is ^{32}P labeled; (F) mixture of samples from panels D and E.

7.1) and a concomitant increase in the intensity of the relatively basic form (pI 7.4) of 3D^{pol} (Fig. 7C). Similar contrasting results were observed with an extract derived from poliovirus-infected cells (Fig. 7D). These results suggest that crude cell extracts contain one or more phosphatases capable of dephosphorylating 3D^{pol} and that under the conditions of the assay, phosphatase activities probably far exceed those of the kinases.

FIG. 6. Phosphoamino acid analysis of 3D^{pol} and 3CD by high-voltage paper electrophoresis of hydrolyzed 3D^{pol} and 3CD. Lanes: 1, hydrolyzed ^{32}P -labeled 3CD; 2, same as lane 1 with one-half the amount of sample; 3, hydrolyzed ^{32}P -labeled 3D^{pol} ; 4, same as lane 3 with one-half the amount of sample. An autoradiogram is shown. The positions of the marker phosphoamino acids as detected by ninhydrin staining are indicated on the right.

FIG. 7. Dephosphorylation of 3D^{pol} by cell extracts. [³⁵S]methionine-labeled fraction V polymerase was incubated with buffer B, ATP, or RSWs prepared from mock- or poliovirus-infected cells and analyzed by two-dimensional gel electrophoresis with a pH 6 to 8 gradient as described in Materials and Methods. (A) Buffer without ATP; (B) buffer with 1 mM ATP; (C) mock RSW and ATP; (D) poliovirus RSW and ATP.

DISCUSSION

We have purified 3D^{pol} to apparent homogeneity from the soluble phase of HeLa cells infected with poliovirus. The purified enzyme, which contains a single polypeptide of an apparent M_r -63,000, synthesizes both monomeric and dimeric RNA products in response to virion RNA template with an oligo(U) primer.

Two lines of evidence presented here suggest that at least a fraction of 3D^{pol} is phosphorylated in vivo. First, the purified protein exhibits at least two forms having isoelectric points of 7.1 and 7.4 when analyzed by two-dimensional gel electrophoresis. The acidic form can be partially or completely converted to the relatively basic form by treatment with CIP or phosphatases present in crude cell extracts (Fig. 4 and 7). Second, 3D^{pol} can be labeled with ³²P_i when the label is added during infection of HeLa cells with poliovirus. 3D^{pol} labeled in vivo with ³²P_i comigrated with [³⁵S]methionine-labeled 3D^{pol} when analyzed by one- and two-dimensional electrophoresis. In addition, we found that 3CD, a virus-specific precursor protein which contains the entire 3D^{pol} sequence, is also phosphorylated in vivo.

The extent of 3D^{pol} phosphorylation was approximately 0.2 mol of phosphate per mol of 3D^{pol} (Fig. 5). Conclusions from this result, however, should be considered tentative, as the amount of phosphate (³²P_i) per mol of 3D^{pol} varied considerably from one experiment to another. Likewise, the ratio of the pI 7.1 form to the pI 7.4 form of [³⁵S]methionine-labeled polymerase in different preparations varied greatly. For example, in Fig. 2A, approximately 10% of total 3D^{pol} was found to have a pI of 7.1 (phosphorylated form), whereas in Fig. 7A, almost 50% of the total 3D^{pol} had a pI of 7.1. We do not have a precise explanation for this; however, it could be due to copurification of various amounts of phosphatases in different preparations of the enzyme.

We were unable to immunoprecipitate ³²P-labeled 3D^{pol} from virus-infected cells, although [³⁵S]methionine-labeled 3D^{pol} was readily immunoprecipitated. It is possible that antibody prepared against a fusion protein (Trp-3D^{pol}) ex-

pressed in *E. coli* does not recognize the phosphorylated form of 3D^{pol}. Alternatively, it may be difficult to detect only 10 to 20% of phosphorylated 3D^{pol} in virus-infected cell extracts simply because of competition by a vast excess (80 to 90%) of unphosphorylated 3D^{pol} for immune immunoglobulin G.

The activities of a number of proteins have been shown to be affected by their phosphorylation states (for a review, see reference 8). This tendency was initially observed in studies of enzymes involved in respiration and metabolism and has since been seen in RNA polymerase II (7), simian virus 40 large T antigen (18), vesicular stomatitis virus phosphoprotein NS (6), and interferon-induced, double-stranded-RNA-activated protein kinase (15). Phosphorylation also plays a major role in the regulation of protein synthesis. Initiation factors, ribosomal proteins, messenger ribonucleoprotein particles, and amino acyl-tRNA synthetases have been shown to be modified by phosphorylation (for a review, see reference 21). Whether phosphorylation or dephosphorylation of 3D^{pol} plays any role in the initiation of RNA replication is not yet known. Our preliminary results suggest that dephosphorylation of 3D^{pol} by CIP enhances its RNA chain elongation activity. A recent publication from the laboratory of E. Wimmer suggested that 3D^{pol} may have a direct role in the initiation of RNA synthesis, most probably via its involvement in uridylylation of VPg (29). In light of this, it is interesting to speculate as to how the phosphorylation state of 3D^{pol} may affect its ability to replicate the poliovirion RNA templates in vivo. Gillis-DeWalt and Semler have described a poliovirus mutant which is defective in the protease responsible for processing precursor proteins (14). Consequently, the mutant virus is unable to produce 3D^{pol} in sufficient quantities. However, despite this defect, the overall RNA synthesis by this mutant virus is similar to that seen with the wild-type virus. It is conceivable that a small fraction of the total pool of 3D^{pol} in the infected cells may represent the active form of the polymerase. This active form could be one of the two isoelectric forms observed in the studies presented here.

Previous results from this laboratory demonstrated that hydrolysis of ATP was required for in vitro RNA synthesis of minus-strand RNA from a poliovirion template in a reaction catalyzed by 3D^{pol} and host factor (20). Morrow et al. also demonstrated that host factor could be a 67,000- M_r protein with a possible protein kinase activity (19). The obvious question is whether the kinase present in host factor preparations is capable of phosphorylating 3D^{pol}. Because we purified host factor from the RSW of HeLa cells, we asked whether RSWs could phosphorylate 3D^{pol} in vitro. Such crude preparations as those seen in Fig. 7 contain phosphatases and cannot be used to address this question. Experiments in the near future will determine whether highly purified host factor is capable of phosphorylating 3D^{pol}.

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