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THE PRIMER PROTEIN OF BACTERIOPHAGE M2 INHIBITS ELONGATION ACTIVITY OF ITS DNA POLYMERASE

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The primer protein (PP) and DNA polymerase (Pol) of bacteriophage M2, both of which are essential for the protein-primed replication of the genome, were purified from *Escherichia coli* cells that harbored the recombinant plasmid pIKM23, on which the genes for PP and Pol are under the control of the *tac* promoter. The purified PP and Pol formed a heterodimeric complex at a molar ratio of 1 to 1. Maximal activity of Pol for M2 DNA replication was attained when an equimolecular amount of PP, relative to that of Pol, was added to the replication system in vitro. When excess amount of PP was added, however, the replication activity was reduced. The activity of Pol for the priming reaction in M2 DNA replication was not reduced even by the presence of a five-fold molar excess of PP over Pol. By contrast, the activities for DNA chain elongation with templates of primed M2 DNA and with poly(dA) : oligo (dT) were inhibited by 70% and by 80%, respectively, on the addition of PP in an equimolecular amount to that of Pol. Therefore, the reduction in M2 DNA replication appears to be caused by an inhibitory effect of PP on Pol in the elongation process.

The *Bacillus subtilis* phage M2 has a linear double-stranded genome with terminal protein (TP) covalently linked to both 5′ termini of the DNA strands (*33*). Replication of M2 DNA is initiated at the termini by a protein-priming mechanism followed by displacement of a parental single-strand (*20, 21*), as in the replication in bacteriophages ϕ29 and PRD1, and in adenovirus (for reviews, see *10, 16, 27*). The replication of M2 DNA requires three viral components: primer

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protein (PP), DNA polymerase (Pol) and TP-linked DNA (*21*). In the protein-priming reaction, Pol catalyzes covalent linking of dNMP to the hydroxyl residue of an amino acid of PP. In the replication of ϕ29 and adenovirus genomes, PP forms a complex with Pol and the replication is considered to be catalyzed by the complex (*18,23,29,31,32*). In the case of M2, formation of a complex of PP and Pol has been shown, but the constitution of the complex has not been clarified yet (*13,14*). Furthermore, we have shown that PP is involved in the interaction of Pol with TP-DNA and that the PP which is linked with dAMP (PP-dAMP) does not form the complex with Pol (*13*). These observations indicate the importance of the PP-Pol complex in the protein-primed replication of these phage and viral genomes.

In the present study, we have characterized the protein-primed replication of M2 DNA by examining the optimal ratio of PP versus Pol by use of the in vitro DNA replication system with purified PP and Pol. These two proteins were prepared from *Escherichia coli* cells that harbored a plasmid on which both the genes for PP and Pol were under the control of the *tac* promoter. We show that the maximal activity of Pol for M2 DNA replication is attained when an equimolecular amount of PP versus Pol is added to the system, and suggest that the replication is catalyzed by the heterodimeric complex of PP and Pol. Furthermore, we have found that the replication activity of Pol is inhibited by an excess amount of PP over Pol.

MATERIALS AND METHODS

Phage and bacterial strain. Bacteriophage M2 strain H6 which is considered identical to Nf (*10,26*) was used. *Escherichia coli* JM109 strain was provided from Takara Shuzo (Kyoto).

Chemicals and enzymes. [α-^{35}S] dATP and [methyl-^{3}H] dTTP were purchased from New England Nuclear (Boston). Unlabeled nucleotides and plasmid pKK223-3 DNA were obtained from Pharmacia P-L Biochemicals (Uppsala). The Klenow fragment of DNA polymerase I from *E. coli* was purchased from Takara Shuzo. Low Molecular Weight (LMW) Protein Calibration Kit was purchased from Pharmacia P-L Biochemicals. Isopropyl-β-D-thiogalactoside (IPTG) was from Boehringer Mannheim (Mannheim).

Template DNAs. Poly(dA) : oligo(dT)$_{12-16}$ was from Pharmacia P-L Biochemicals. M2 TP-DNA was extracted from purified phage particles by centrifugation in a 5–20% sucrose gradient containing 4 M guanidium hydrochloride, 10 mM Tris-HCl (pH 8.0), and 1 mM ethylenediaminetetraacetic acid at 21,000 rpm for 17 h at 4°C. Treatment of TP-DNA with proteinase K was performed as described elsewhere (*21*). Partially replicated DNA was prepared as follows. M2 TP-DNA (100 μg/ml) was incubated in the standard reaction buffer that contained three kind of dNTPs (100 μM each) without dCTP, Pol (11.2 μg/ml) and PP (6.0 μg/ml) for 30 min at 30°C. After digestion of the products with proteinase K (50 μg/ml) for 1 h at 30°C, the digest was treated with phenol. The DNA molecules obtained had

been replicated from its termini up to the residue that preceded the first cytosine at the 9th and 12th nucleotide (*23*, *32*).

Overproduction of PP and Pol. To construct the plasmid for overproduction of PP and Pol, the *Eco*RI-*Hae*II fragment of M2 DNA, which contains genes E and G (*19*, *22*), was cloned on pKK223-3 using the *Eco*RI and *Hae*II sites downstream of the *tac* promoter. The resultant plasmid pIKM23 (Fig. 1) was introduced into JM109 cells by calcium treatment. The transformed cells were grown at 37°C in 8 *l* of M9 medium supplemented with 2 mg/ml Casamino Acids (Difco, Detroit), 1 mM thiamine and 50 μg/ml ampicillin. At 120 Klett units, IPTG (2 mM) was added to induce overproduction of the phage proteins, and incubation was further continued for 2 h. Cells were collected by centrifugation and suspended in TSD buffer [50 mM Tris-HCl (pH 7.5), 10% sucrose, and 1 mM dithiothreitol] and stored at −80°C.

Purification of PP and Pol. The frozen cells from the 8 *l* culture were thawed and suspended at 0°C in 150 ml of TSD buffer that contained lysozyme (1.1 mg/ml), 1 mM phenylmethylsulfonyl fluoride and 0.1 mM EDTA. After 60 min of incubation at 0°C, 1/100 volume of Brij 58 (10%, v/v) was added and the cells were lysed by three cycles of freezing and thawing. After addition of NaCl (4 M solution) to a final concentration of 0.8 M, cell debris was removed by ultracentrifugation at 28,000 rpm for 90 min at 2°C.

To the cleared extract, a 10% solution (w/v) of polyethyleneimine (BDH Chemicals Ltd.) was added dropwise to a final concentration of 0.15%. The mixture was stirred for 60 min and the precipitate was removed by centrifugation. The supernatant was then fractionated by ammonium sulfate precipitation between 40% and 60% saturation. This precipitate was dissolved in buffer A [Tris-HCl (pH 7.5) 60 mM ammonium sulfate, and 1 mM dithiothreitol] and dialyzed for 4 h against buffer A. The dialyzed fraction was then applied to a column (4.4 cm I.D. × 15 cm) of phosphocellulose (P11; Whatman, Clifton, NJ) equilibrated with buffer A. After washing with buffer A, a 1,000-ml linear gradient from 0 to 1.0 M NaCl in buffer A was applied. The fractions active in M2 DNA replication, that contained both PP and Pol, were eluted at 0.6 M NaCl. The active fractions were pooled and dialyzed for 12 h against buffer A that contained 0.2 M NaCl. The dialyzate was applied to a column (2.2 cm I.D. × 12 cm) of Heparin Sepharose CL-6B (Pharmacia). After washing with buffer A that contained 0.2 M NaCl, a 200-ml linear gradient from 0.2 to 0.8 M NaCl was applied. Pol was eluted at 0.6 M NaCl and PP was eluted at 0.4 M NaCl. The fractions containing Pol were pooled and subjected to rechromatography on Heparin Sepharose CL-6B. The fractions containing PP that were eluted at 0.4 M NaCl from the first column chromatography on Heparin Sepharose were pooled and dialyzed against buffer A, and then applied to a column (1.6 cm I.D. × 12 cm) of DEAE-Toyopearl (Tosoh, Tokyo) equilibrated with buffer A. After washing, a 100-ml linear gradient from 0 to 1.0 M NaCl in buffer A was applied. PP was eluted at 0.4 M NaCl. The purified PP and Pol proteins were dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5), 40 mM ammonium sulfate and 10% glycerol and stored at −80°C. Concentrations of

the proteins were determined by the method of Bradford (*9*) according to the manual supplied by Bio-Rad (California). The amounts of the final preparations of PP and Pol were 2.2 mg and 4.3 mg, respectively.

Assay conditions. The standard reaction mixture (50 μl) contained 50 mM Hepes-KOH (pH 7.5), 10 mM $MgCl_2$, 2.4 mM dithiothreitol, 1 mM spermidine, 10% glycerol, 0.02% bovine serum albumin and 40 mM ammonium sulfate. All incubations were conducted at 30°C for 10 min. *Replication activity*: to the standard reaction mixture, 0.1 mM each of [^{3}H]dTTP (0.1 Ci/mmol), dATP, dGTP, dCTP and 1 μg of M2 TP-DNA were added. *Priming activity*: 0.4 μM [^{35}S]dATP (10 μCi) and 1 μg of TP-DNA were added to the standard reaction mixture. *Elongation activity*: 0.1 mM each of dNTPs, including [^{3}H]dTTP, and 5 μg of partially replicated M2 DNA were added to the standard reaction mixture. When the poly(dA) : oligo(dT)$_{12-16}$ template (2 μg) was used, [^{3}H]dTTP alone was added at 0.1 mM. For quantification of the priming activity, the incubated mixture was passed through a spun column that had been equilibrated with the sample buffer used for SDS-polyacrylamide gel electrophoresis (PAGE). Excluded samples were heated at 95°C for 3 min and were then subjected to SDS-PAGE. The gel was dried and the radioactivity of the band of [^{35}S]-labeled 32-kDa protein was measured. In the other assays, trichloroacetic acid-insoluble materials were collected on GF/C filters (Whatman) and the radioactivity was measured.

RESULTS

In order to overproduce PP and Pol of bacteriophage M2, we constructed the recombinant plasmid pIKM23 that contained both the genes E and G, the genes encoding PP and Pol, respectively (*19*, *22*), downstream from the *tac* promoter of

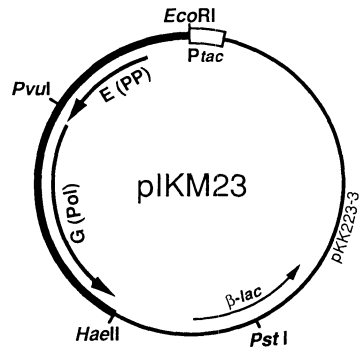

Fig. 1. Structure of the plasmid constructed for expression of the genes for PP and Pol of bacteriophage M2.

The *Eco*RI-*Hae*II DNA fragment, which contains both genes E and G, was isolated from the *Eco*RI-B fragment of the M2 genome (*19*, *22*) and inserted into the pKK223-3 vector using the *Eco*RI and *Hae*II sites just downstream from the *tac* promoter. The thick arrows indicate the reading frames of the genes E and G.

pKK223-3 (Fig. 1). After addition of 2 mM IPTG, the *E. coli* JM109 cells transformed with pIKM23 efficiently overproduced both PP and Pol (Fig. 2A). However, no Pol was detected in the cases of the cells harboring the recombinant plasmid that contained gene G alone, while the cells harboring the recombinant plasmid that contained gene E alone produced PP efficiently (Fig. 2A). Deletion derivatives of pIKM23 which lacked the intact gene E did not overproduce Pol (data not shown). Therefore, the presence of intact gene E upstream of gene G seems to be required for the efficient overproduction of M2 Pol.

After fractionation of the lysate of IPTG-induced cells by ammonium sulfate precipitation and successive chromatography using phosphocellulose, Heparin Sepharose, and DEAE-Toyopearl (see MATERIALS AND METHODS), we have suc-

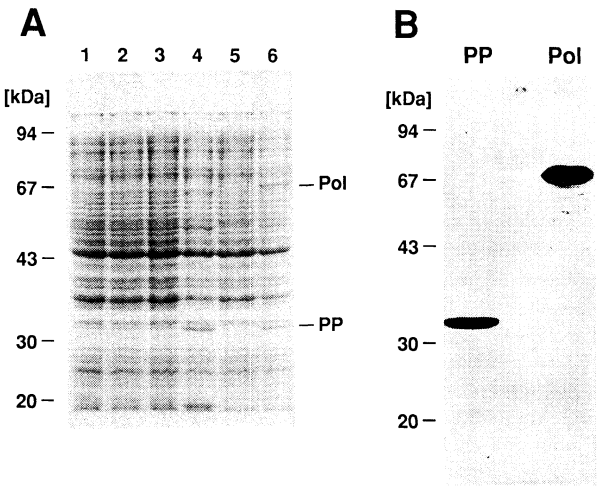

Fig. 2. Efficient expression of PP and Pol of bacteriophage M2 and SDS-PAGE of the purified PP and Pol.

(A) Freshly transformed single colonies were inoculated into LB medium (*28*) in the presence of 50 μg/ml ampicillin and incubated at 37°C. At about 100 Klett units, IPTG was added to a final concentration of 2 mM and the cultures were further incubated for 2 h. Then, 1 ml samples were withdrawn, cells were centrifuged and suspended in the sample buffer for SDS-PAGE and subjected to SDS-PAGE on a 12.5% gel. The proteins were stained with Coomassie Brilliant Blue. Lanes 1 and 2 are the cells transformed with the recombinant plasmid of pKK223-3 containing Pol gene alone (*Pvu*I-*Hae*II fragment). Lanes 3 and 4 are the cells transformed with the recombinant plasmid of pKK223-3 containing PP gene alone (*Eco*RI-*Pst*I fragment). Lanes 5 and 6 are the cells transformed with pIKM23 (containing both the genes PP and Pol) shown in Fig. 1. Lanes 1, 3, and 5 are the uninduced cells and lanes 2, 4, and 6 are the IPTG-induced cells. (B) The final fractions from the purifications described in MATERIALS AND METHODS were subjected to SDS-PAGE on a 12.5% gel and the proteins were stained with Coomassie Brilliant Blue. Amounts of PP and Pol loaded on the gel were 15 and 20 μg, respectively. Numbers on the left indicate the molecular weights in kDa of the reference proteins.

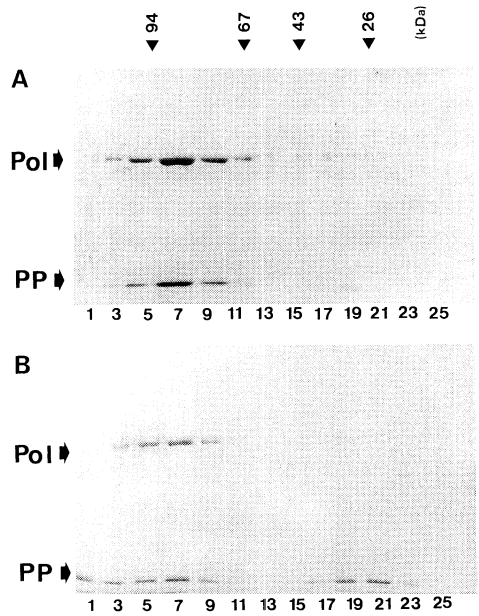

Fig. 3. Formation of a complex between PP and Pol.

(A) 60μg of PP was incubated with 112μg of Pol for 30 min at 0°C in 0.2 ml of a buffer that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 40 mM ammonium sulfate and 10% glycerol. The incubated mixture was subjected to density gradient centrifugation in 15–30% glycerol at 55,000 rpm for 24 h at 0°C with Hitachi RPS65T rotor. After 0.2-ml fractions were collected from the bottom of the gradient, 50μl of each alternate fraction (odd numbers) were subjected to SDS-PAGE on a 10% gel. (B) PP at twice the molar ratio (120μg) was mixed with Pol (112μg). After 0.2-ml fractions had been collected from the bottom, 25μl of each alternative fraction were subjected to gel electrophoresis. As molecular-weight markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and α-chymotrypsinogen (25 kDa) were centrifuged in parallel with the experimental gradient, and they are indicated by arrowheads.

ceeded in obtaining homogeneous preparations of PP and Pol. Homogeneity of the purified proteins was confirmed by SDS-PAGE (Fig. 2B). Only single bands of proteins of 32 kDa and 67 kDa were detected, which corresponded to the molecular weights of the products deduced from the nucleotide sequences of gene E (*17*) and gene G (*22*).

To examine whether Pol and PP could form a stable complex, the two proteins, PP (60μg) and Pol (112μg), were mixed at an equimolecular ratio and incubated for 30 min at 0°C in the presence of 40 mM ammonium sulfate and then subjected to centrifugation in a 15–30% glycerol gradient. The fractions collected from the bottom were subjected to SDS-PAGE (Fig. 3). Most of the PP and Pol proteins were detected in the fractions close to the marker protein of 94 kDa. When PP and Pol were analyzed in separate gradients, they were sedimented at the positions

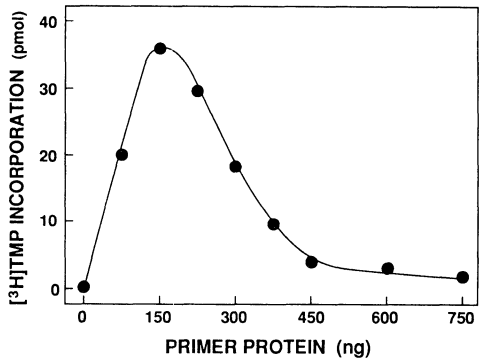

Fig. 4. Effects of the concentration of PP on replication activity.

The activity of Pol for the replication was carried out in the conditions described under MATERIALS AND METHODS. M2 TP-DNA (1 μg) was incubated with Pol (280 ng), dNTPs (0.1 mM each) that included [^{3}H]dTTP (0.1 Ci/mmol), and the indicated amounts of PP. After 10 min incubation at 30°C, trichloroacetic acid was added and the radioactivities of acid-insoluble materials were measured.

corresponding to 32 kDa and 67 kDa (data not shown). These results indicate that PP and Pol are present as a heterodimeric complex (PP : Pol, 1 : 1). When twice the amount of PP was mixed with Pol, the complex was also found in the fractions close to the 94-kDa marker protein, and excess PP remained separate in monomeric form (Fig. 3B). Rapidly sedimenting material observed in fraction number 1 may be an aggregated form of PP.

To determine the optimal molar ratio of PP to Pol for M2 DNA replication, the activity of replication was examined with various amounts of the two proteins (Fig. 4). In the presence of a constant amount (280 ng) of Pol, the maximal activity was attained with an amount of PP that was slightly over that equimolecular to Pol (at the molar ratio of about 1.2). The maximal replication activity appeared, therefore, to be achieved when PP and Pol were in the heterodimeric complex. With increase in the amount of PP beyond the equimolecular, an apparent reduction in the activity was observed. When 560 ng of Pol was used, maximal replication activity (68 pmol) was observed with 300 ng of PP that was at the molar ratio of about 1.1. Further addition of PP also reduced the degree of replication with a similar pattern to that shown in Fig. 4.

To examine the effect of excess PP on the priming and elongation reactions in M2 DNA replication, respective reactions were assayed separately with varying amounts of PP. The priming activity of Pol increased in proportion to the amount of PP added, and the priming activity reached a plateau value at a molar ratio of PP to Pol of about 2.5. Further addition of PP, even a five-fold molar excess over Pol, did not reduce the priming activity at all (Fig. 5).

The elongation activity of Pol was examined next, by using partially replicated M2 DNA as template. With the addition of PP, marked reduction in the elongation

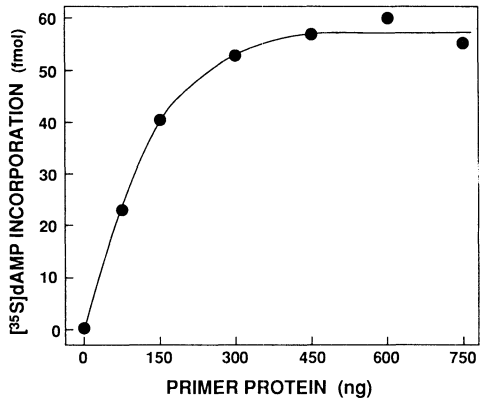

Fig. 5. Effects of the concentration of PP on priming activity.

Priming activity assay was carried out in the conditions described under MATERIALS AND METHODS. M2 TP-DNA (1 μg) was incubated with Pol (280 ng), 0.4 μM [α-^{35}S]dATP (10 μCi), and indicated amounts of PP. After 10 min incubation at 30°C EDTA was added to stop the priming reaction. Each reaction mixture was then passed through a spun column of Sephadex G50 that had been equilibrated with the sample buffer used for SDS-PAGE. Excluded fractions were subjected to SDS-PAGE, and the radioactivity of the band of the [^{35}S]-labeled 32-kDa protein was measured.

activity was observed. At an equimolecular amount of PP, the activity was inhibited by 70% (Fig. 6A). The partially replicated DNA was inert as a template for Klenow fragment of DNA polymerase I of *E. coli* (Fig. 6A); therefore, we could not examine the effect of PP on this template. Furthermore, the activity of M2 Pol on poly(dA) : oligo(dT)$_{12-16}$ template was also inhibited by 80% upon the addition of an equimolecular amount of PP (Fig. 6B). With this template, the activity of Klenow fragment was not affected by the addition of PP. Heat-treated PP did not affect the elongation (data not shown). When ammonium sulfate was eliminated from the system, the inhibition by PP was diminished to 25% (Fig. 6B). The inhibition by PP thus requires the presence of 40 mM ammonium sulfate; at this concentration of ammonium sulfate both M2 DNA replication and the priming reaction showed maximum activities (data not shown).

DISCUSSION

Using purified PP and Pol proteins of bacteriophage M2, we have shown that maximal activity of Pol for M2 DNA replication is attained when an equimolecular amount of PP versus Pol is added to the replication system, and have found that addition of an excess amount of PP inhibits the activity by PP for the replication. The inhibition of the replication activity of Pol appears to occur at the step of elongation rather than priming reaction. Because the activity of Klenow fragment

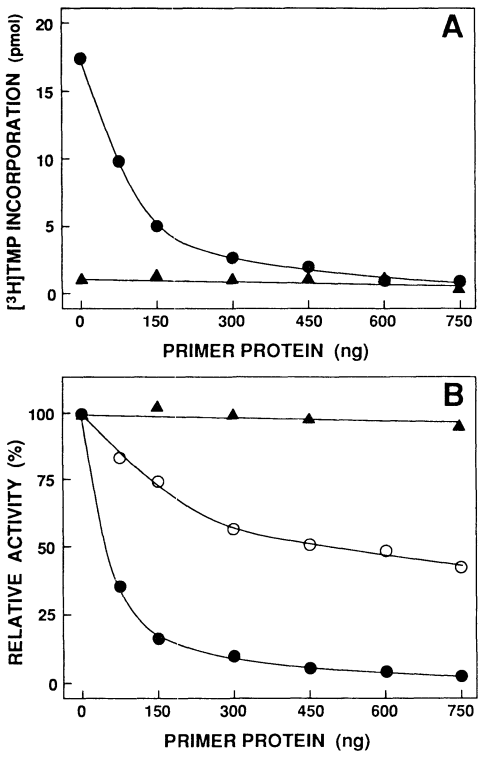

Fig. 6. Effects of the concentration of PP on elongation activity.

Elongation activity assay was carried out in the conditions described under MATERIALS AND METHODS using partially replicated M2 DNA and poly(dA) : oligo $(dT)_{12-16}$ templates. (A) Pol (280 ng; ●) or the Klenow fragment of DNA polymerase I (0.4 unit; ▲) was incubated with partially replicated M2 DNA (see MATERIALS AND METHODS; 5 μg), dNTPs (0.1 mM each) that included [^{3}H]dTTP (0.1 Ci/mmol), and indicated amounts of PP. The incubation was for 10 min at 30°C. (B) Pol (280 ng) was incubated with poly(dA) : oligo $(dT)_{12-16}$ (2 μg) and 0.1 mM [^{3}H] dTTP in the presence (●) or absence (○) of 40 mM ammonium sulfate. The Klenow fragment (0.4 unit; ▲) was included in incubations under the same conditions as those in the case of Pol with 40 mM ammonium sulfate. Values taken as 100% were 25.6 pmol (●), 52.0 pmol (○), and 32.9 pmol (▲), respectively.

of DNA polymerase I from *E. coli* was not inhibited by PP (Fig. 6B), PP presumably exerts the inhibitory action through interaction with Pol. The inhibition of M2 DNA replication by PP was observed in the presence of a slight excess of PP over an amount equimolecular to that of Pol (Fig. 4). The elongation activity on partially replicated M2 DNA and on poly(dA) : oligo$(dT)_{12-16}$ templates was reduced, in proportion to the amount of PP added (Fig. 6). Therefore, it appears that a single molecule of PP is fully effective to inactivate a molecule of Pol engaged in elongation. These results suggest a specific and direct interaction of PP to Pol on elongation. PP may block the sliding of Pol on the template DNA or it

may render Pol unable to transfer a nucleoside monophosphate to the 3′OH residue of DNA. In ϕ29 DNA replication system, inhibition by PP has not been described. The experiments previously reported were carried out at either high molar ratios (about 10–80) or low ratios (about 0.2–0.5) of PP versus Pol (*2*, *4*, *7*). The inefficient replication observed (*2*, *7*) might be due to the presence of excess or small amount of PP.

In addition to the role of PP in the priming reaction, the results presented herein suggest that PP might have some role in controlling the elongation activity of Pol. Recent homology searches on conserved segments among DNA polymerases and analyses of the mutation sites of aphidicolin-resistant and temperature-sensitive mutants of ϕ29 and M2 suggest that protein priming and polymerase activities are catalyzed by a common functional domain of Pol (*1*, *8*, *11*, *12*, *20*, *22*, *24*, *25*). Thus, for catalysis of the apparently different reactions of priming and elongation of M2 Pol, the structure and function of this common domain would be changed in response to the reaction engaged. Indeed, the K_m value of Pol for nucleoside triphosphate in the protein-priming reaction is lower than that in the subsequent elongation (*6*), and the latter reaction is much more sensitive to aphidicolin than the former (*3*). In addition, by forming the complex with PP, ϕ29 Pol is able to covalently link any of four dNTPs to PP in the absence of template DNA (*5*). Formation of the complex of Pol with PP could be responsible for these catalytic differences between priming and elongation reactions. We have found that M2 Pol binds to PP but to neither PP-dAMP (i.e., primed PP) nor DNA-linked TP (*13*, *15*). Thus, it appears that, after linking the first molecule of dAMP to PP, Pol can no longer bind to the primed PP. In other words, after the priming reaction, by dissociating itself from the primed PP, the Pol molecule regains its elongation activity. Taken together, these results suggest that PP plays an important role in rendering the function of Pol specific for the protein-priming reaction.

In order to overproduce PP and Pol of M2, we have constructed the recombinant plasmid pIKM23 that contained both the genes for PP and Pol downstream of the *tac* promoter of pKK223-3. However, deletions of the PP gene from pIKM23 rendered the clone unstable and no deletion derivative which stably overproduced M2 Pol was established. This inefficient expression of M2 Pol was also the case with the T7ϕ10 promoter vector system (pGEM vectors: Promega) which is good for expression of a toxic gene (*30*) (Matsumoto et al., unpublished observation). This result suggests that even a background level expression of M2 Pol is harmful for the host cells. Thus, the formation of the complex with PP, which result in inhibition of the elongation activity, could explain the successful establishment of pIKM23 system which efficiently overproduce Pol of M2. PP could act to sequester excess Pol, and thereby to limit certain harmful effect of excess Pol.

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