Replication of phage ϕ 29 DNA *in vitro*: role of the viral protein p6 in initiation and elongation

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ABSTRACT

The ϕ 29 protein p6 stimulates the formation of the protein p3-dAMP initiation complex when added to a minimal system containing the terminal protein p3, the ϕ 29 DNA polymerase p2 and ϕ 29 DNA-protein p3 complex, by decreasing about 5 fold the K_m value for dATP. In addition, protein p6 stimulates elongation of the p3-dAMP initiation complex. Whereas the effect of protein p6 on initiation is similar with protein p3-containing fragments from the right or left ϕ 29 DNA ends, the stimulation of elongation is higher with the right than with the left ϕ 29 DNA terminal fragment, suggesting DNA sequence specificity. The stimulation by protein p6 of the initiation and elongation steps of ϕ 29 DNA replication does not require the presence of the parental protein p3 at the ϕ 29 DNA ends. No effect of protein p6 was obtained on the elongation of the template-primer poly(dT)-(dA)₁₂₋₁₈ by the ϕ 29 DNA polymerase.

INTRODUCTION

Bacteriophage ϕ 29 from *Bacillus subtilis* has a linear, double-stranded DNA of $\sim 18,000$ base pairs (1) with a protein, the product of the viral gene 3 (2), covalently linked to each 5' end through a phosphodiester bond between serine and dAMP, the terminal nucleotide at both 5' ends (3). Replication of ϕ 29 is initiated at either DNA end (4-6) by the covalent attachment of 5'-dAMP to the serine residue 232 (7) of a free molecule of the terminal protein p3 (8,9). The formation of the p3-dAMP initiation complex is catalyzed by the ϕ 29 DNA polymerase p2 (10,11) and requires the presence of a ϕ 29 DNA specific DNA template (12,13). Proteins p2 and p3 have been purified (10,11, 14) and, when added to the ϕ 29 DNA-protein p3 template, the p3-dAMP initiation complex is formed and further elongated to produce full-length ϕ 29 DNA (15), indicating that the ϕ 29 DNA

polymerase is the only one required for the in vitro synthesis of ϕ 29 DNA. However, under these conditions the rate of elongation is low, suggesting the need of accessory proteins for the efficient replication of ϕ 29 DNA. In fact, in vivo ϕ 29 DNA replication requires, besides proteins p2 and p3, the products of genes 5, 6 and 17 (16-18). By shift-up experiments using B. subtilis infected with ϕ 29 ts mutants, the products of genes 5 and 6 were shown to be involved in elongation, although the possibility that they were also involved in initiation could not be ruled out (19). Gene 6 has been recently cloned and protein p6 has been highly purified. The purified protein p6 was shown to stimulate the formation of the p3-dAMP initiation complex when added to purified proteins p2 and p3 and ϕ 29 DNA-p3 complex as template (20).

In this paper we show that the effect of protein p6 on the initiation reaction is due to a decrease in the K_m value for dATP. In addition, protein p6 stimulates the elongation reaction. The effect of protein p6 on elongation, but not on initiation, seems to be sequence-dependent, since the elongation proceeding from the right ϕ 29 replication origin is stimulated to a higher extent than that from the left replication origin.

MATERIALS AND METHODS

Materials

The ϕ 29 DNA-protein p3 complex was isolated as described by Peñalva and Salas (8). Left and right ϕ 29 DNA fragments, 5.9 and 12.1 kb long, respectively, were obtained by treatment of ϕ 29 DNA-protein p3 with the restriction nuclease ClaI (New England Biolabs) followed by centrifugation for 27 h at 130,000 x g in a 5-20% (w/v) sucrose gradient in 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 M NaCl. The fragment-containing fractions were pooled and precipitated with ethanol. The recombinant plasmid pID13, containing the two replication origins of ϕ 29 DNA, and the isolation of AhaIII fragments from the latter were as described (13). The template-primer poly(dT)-(dA)₁₂₋₁₈ was obtained from PL Biochemicals.

Assay for formation of the p3-dAMP initiation complex

The standard incubation mixture for the initiation reaction contained, in 25 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM

DTT, 1 mM spermidine, 0.25 μM $\{\alpha\text{-}^{32}\text{P}\}$ dATP (1–2.5 μCi ; Amersham International); and purified proteins p2 (10) and p3 (14), and $\phi 29$ DNA-protein p3 template (8), in amounts of 5 ng, 160 ng and 0.5 μg respectively, unless otherwise stated. When indicated, purified protein p6, before or after glycerol gradient centrifugation (20), was used. After incubation for 10–20 min at 30°C the reaction was stopped by addition of EDTA to 10 mM and heating for 10 min at 68°C . The samples were treated with micrococcal nuclease and subjected to electrophoresis in 0.1% SDS/10% acrylamide gels (8), either directly or after precipitation with 10% trichloroacetic acid in the presence of yeast RNA (100 μg). Quantitation was done by excising from the gel the radioactive band corresponding to the p3-dAMP complex and counting the Cerenkov radiation or by densitometry of the autoradiographs.

When indicated, the samples, without micrococcal nuclease treatment, were filtered through Sephadex G-50 spun columns (21) in the presence of 0.1% SDS; the excluded volume was precipitated with 10% trichloroacetic acid in the presence of 100 μg of yeast RNA and the precipitate was dissolved in 0.5 M piperidine and incubated for 2 h at 37°C . After removal of the piperidine, the samples were subjected to electrophoresis in 20% acrylamide/8 M urea denaturing gels as described (22). The reaction products from a chemical sequence analysis of a DNA fragment labelled at one of the 3' ends were run in parallel as size markers.

Truncated elongation of the p3-dAMP initiation complex

For the truncated elongation reaction, the incubation mixture was as described above except that 10 μM or the indicated amounts of $\{\alpha\text{-}^{32}\text{P}\}$ dATP (2.5 μCi) and 10 μM dGTP, 10 μM dTTP and 100 μM ddCTP were used to stop elongation at nucleotides 9 and 12 from the left and right DNA ends, respectively, according to the $\phi 29$ DNA sequence (23,24). After incubation for 20 min at 30°C the reaction was stopped as described above and the samples were subjected to electrophoresis in 0.1% SDS/20% acrylamide gels, either directly or after precipitation with 10% trichloroacetic acid in the presence of 100 μg of yeast RNA.

Replication assay with $\phi 29$ DNA-protein p3 or poly(dT)-(dA)₁₂₋₁₈ as templates.

The incubation mixture was as described above for the initia-

tion reaction except that it contained 15 μM each $\{\alpha\text{-}^{32}\text{P}\}$ dATP (1 μCi), dGTP, dCTP and dTTP. When poly(dT)-(dA)₁₂₋₁₈ was used as template, protein p3, $\phi 29$ DNA-protein p3, dGTP, dCTP and dTTP were not added. After incubation for the indicated times at 30°C, the reaction was stopped by addition of EDTA to 10 mM and SDS to 0.1% and heating for 10 min at 68°C. The samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS and the excluded fractions were counted by Cerenkov radiation.

When indicated, the DNA labelled in the replication assay described above, was denatured by treatment with 0.3 N NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels as described (25) alongside with DNA length markers (1 Kb ladder from Bethesda Research Lab.). After electrophoresis, DNA length markers were detected with ethidium bromide and then, the gel was dried and autoradiographed with intensifying screens at -70°C.

RESULTS

Effect of protein p6 on the K_m value for dATP in the initiation reaction.

When the effect of purified protein p6 on the formation of the protein p3-dAMP initiation complex was studied at different dATP concentrations, it was observed that, at low dATP concentrations (1 μM or smaller) there was a large stimulation by protein p6 whereas the stimulation decreased when the dATP concentration increased (Fig. 1A). This result suggested that protein p6, when added to the in vitro system containing proteins p2 and p3 and the $\phi 29$ DNA-protein p3 complex, decreases the K_m value for dATP. Indeed, it was found that, in the absence of protein p6, the K_m value for dATP was 6 μM , whereas in its presence, a K_m value of 1.2 μM was obtained. As shown in the Lineweaver-Burk plot, the same value for the V_{max} was obtained in the absence or presence of p6 (Fig. 1A).

Effect of protein p6 on the limited elongation of the p3-dAMP complex

Using extracts from $\phi 29$ infected B. subtilis it had been previously found that, at 0.5 μM dATP concentration, only the p3-dAMP initiation complex was formed (8) instead of the expected

Figure 1. Effect of protein p6 on the formation of the p3-dAMP initiation complex and limited elongation as a function of the dATP concentration. A. ϕ 29 DNA-protein p3 complex was incubated with protein p3 and protein p2 in the presence of the indicated concentrations of $\{\alpha\text{-}^{32}\text{P}\}$ dATP (1 μCi), in the absence or presence of 1.2 μg of purified protein p6. After incubation for 20 min at 30°C, the samples were digested with micrococcal nuclease, subjected to electrophoresis and the amount of p3-dAMP, in fmol, was represented in a Lineweaver-Burk plot. o---o, -p6; ●—●, +p6. B. The incubation was as described in A in the presence of the indicated concentrations of $\{\alpha\text{-}^{32}\text{P}\}$ dATP (2.5 μCi) in the absence (a-d) or presence (e-h) of 2 μg of protein p6. After 10 min at 30°C the samples were processed, treated with piperidine and subjected to electrophoresis on a denaturing gel as described in Materials and Methods. The reaction products from a chemical sequence analysis of a labelled DNA fragment were run in parallel as size markers (lane i). The amount of dAMP incorporated, determined before the treatment with piperidine, was the following, in fmol : a, 1.3; b, 5.7; c, 17.8; d, 34.5; e, 4.5; f, 15; g, 33.1; h, 48.9.

p3-pdApdApdA complex, taking into account the nucleotide sequence at the two ends of ϕ 29 DNA (23,24). Elongation required a concentration of dNTPs 10 μM or higher (8,26). To determine the dATP concentration required to incorporate the second and third dAMP residues, the product of the initiation reaction with purified proteins p2 and p3 and ϕ 29 DNA-protein p3 complex, in the absence or presence of protein p6, was treated with piperidine to hydrolyze the linkage between protein p3 and the nucleotide residue and then subjected to electrophoresis in a polyacrylamide denatu-

Figure 2. Effect of protein p6 on the truncated elongation of the p3-dAMP complex. Ø29 DNA-protein p3 complex (0.5 µg) was incubated with 160 ng of protein p3 and 5 ng of protein p2, 10 µM dGTP and dTTP and 100 µM ddCTP with the indicated concentrations of {α-³²P} dATP (1 µCi) in the absence (a-e) or presence (f-j) of 2 µg of purified protein p6. After incubation for 20 min at 30°C the samples were processed and subjected to electrophoresis as described in Materials and Methods. B. The radioactivity present in the p3-dAMP band and that present in the p3-(dNMP)₉ and p3-(dNMP)₁₂ bands together was determined by counting the Cerenkov radiation. o---o, initiation -p6; ●—●, initiation +p6; Δ---Δ, truncated elongation -p6; ▲—▲, truncated elongation +p6.

ring gel alongside with the nucleotides resulting from chemical sequencing reactions as size markers. As shown in Fig. 1B, in the absence of protein p6, from 0.25 μ M to 10 μ M dATP concentration, most of the reaction product corresponds to protein p3 linked to dAMP (lanes a-d). In the presence of protein p6, it can be seen that, at 0.25 μ M and 1 μ M dATP concentration, most of the reaction product corresponds also to p3 linked to dAMP (lanes e and f), whereas at 4 μ M and 10 μ M dATP, radioactivity corresponding to the position of the dinucleotide appears (lanes g and h). In addition, a small band corresponding to the trinucleotide position is present at 10 μ M dATP. Therefore, protein p6 stimulates the elongation of p3-dAMP to p3-pdApdA and p3-pdApdApdA, but only above a minimal dATP concentration.

When the elongation of the p3-dAMP complex is carried out in the presence of dATP, dGTP, dTTP and ddCTP, a truncated elongation

product is formed, namely protein p3 linked to oligonucleotides 9 and 12 bases long from the left and right Ø29 DNA ends, respectively (8). The effect of protein p6 on the truncated elongation reaction as a function of the dATP concentration is shown in Fig. 2. In the absence of protein p6, essentially no elongation took place except a small amount at 10 µM dATP (Fig. 2A, lanes a-e). In the presence of protein p6, elongation took place with the appearance of the truncated elongation products, but only at high dATP concentration (Fig. 2A, lanes f-j). Moreover, at 10 µM dATP concentration (lane j), the amount of elongation product corresponding to the right Ø29 DNA end (12 bases long) was greater than that corresponding to the left end (9 bases long), whereas at 4 µM dATP (lane i) the same amount of elongation product was obtained for both ends (the same number of A residues is present in the two oligonucleotides). When the radioactivity present in the bands corresponding to the p3-dAMP initiation complex and to the truncated elongation products was quantitated and plotted it was clearly seen that, whereas the initiation reaction took place at even very low dATP concentration (0.1 µM), there was a lag in the appearance of the elongation products, a concentration higher than 1 µM dATP being required to get elongation (Fig. 2B).

Cosedimentation of protein p6 and the initiation and truncated elongation activities

It was previously shown that the activity that stimulates the initiation reaction cosediments in a glycerol gradient with the protein p6 peak (20). To control that the activity stimulating the truncated elongation reaction is due to p6 and not to a minor contaminant, the purified protein p6 was subjected to glycerol gradient centrifugation. As shown in Fig. 3, the stimulating activities for the formation both of the p3-dAMP initiation complex and of the truncated elongation products cosediment at the p6 position. It can be also seen that, in the p6 peak fractions (17 to 19), the amount of elongation product corresponding to the right end is greater than that corresponding to the left end, whereas in the remaining fractions with some p6 activity, the same amount of elongation product was obtained for both ends, or even more elongation from the left end than from

Figure 3. Cosedimentation of the initiation and truncated elongation stimulating activities of protein p6. Purified protein p6 (1 mg), labelled with (^{35}S)-methionine, was subjected to glycerol gradient centrifugation as described (20). Fractions were taken and a sample from each was used to determine the position of protein p6, indicated by an arrow, by determining the radioactivity and by SDS-polyacrylamide gel electrophoresis. A sample from each fraction was also used to determine the stimulation of the formation of the p3-dAMP initiation complex (a) as well as that of the truncated elongation reaction (b) when added to purified proteins p3 and p2 in the presence of $\phi 29$ DNA-protein p3 complex as template. The bands corresponding to the p3-dAMP initiation complex and to the truncated elongation products were cut from the gel and counted by Cerenkov radiation. The values obtained in the absence of p6 were subtracted for the representation. ●—●, initiation reaction; o---o, truncated elongation reaction.

the right end was seen (fractions 15 and 20).

Effect of protein p6 on $\phi 29$ DNA-protein p3 replication

Fig. 4A shows that highly purified protein p6 strongly

Figure 4. Effect of protein p6 on Ø29 DNA-protein p3 replication. A. Ø29 DNA-protein p3 was incubated with protein p3, protein p2 and 15 μM each dGTP, dTTP, dCTP and $\{\alpha\text{-}^{32}\text{P}\}$ dATP (1 μCi) in the absence or presence of increasing concentrations of protein p6 purified by glycerol gradient centrifugation. After 10 min at 30°C the samples were processed as described in Materials and Methods. B. The incubation conditions were as described in A except that the dGTP concentration was changed from 1 to 15 μM , as indicated, and the amount of protein p6 was 1.5 μg . o---o, -p6; ●—●, +p6. The inset shows the Lineweaver-Burk plot.

stimulates the replication of Ø29 DNA-protein p3 under conditions in which the initiation reaction is essentially not stimulated, indicating an effect of p6 on elongation. Figure 4B shows the stimulation of Ø29 DNA-protein p3 replication by protein p6 when different concentrations of dGTP were used, keeping constant the other three dNTPs. From this experiment, a K_m value for dGTP in the elongation reaction was calculated to be about 9 μM , both in the absence or presence of protein p6, whereas the V_{max} was increased about 4 fold in the presence of protein p6. To determine whether or not the effect of protein p6 on Ø29 DNA-protein p3 replication is due to an increase in the rate of elongation, the size of the DNA synthesized in the absence or presence of protein p6 was determined by alkaline agarose gel electrophoresis. The rate of elongation increased from 16 nucleotides/second to 26 nucleotides/second in the presence of

Figure 5. Effect of protein p6 on the initiation, truncated elongation and replication reactions using as templates the right or left ClaI fragments of ϕ 29 DNA-containing protein p3. **A.** The initiation reaction was as described in Materials and Methods and contained proteins p3 and p2 and the separated ClaI A (0.33 μ g) (a and b) or ClaI B (0.17 μ g) (c and d) fragments from the right and left ϕ 29 DNA ends, respectively, in the absence (a and c) or presence (b and d) of protein p6 purified by glycerol gradient centrifugation (1.5 μ g). After 20 min at 30°C, the samples were digested with micrococcal nuclease and subjected to electrophoresis as described in Materials and Methods. The amount of p3-dAMP formed was the following, in fmol: a, 1.0; b, 5.3; c, 0.8; d, 2.4. **B.** The truncated elongation reaction in the presence of ddCTP was carried out as described in Materials and Methods, other details being as in A. The amount of p3-dAMP formed was the following in fmol: a, 20.9; b, 23.2; c, 15.3; d, 16.9 and that of the truncated elongation products was: a, 4.6; b, 19.6; c, 3.9; d, 6.7. **C.** The replication assay with the ClaI A (1 μ g) or ClaI B (0.5 μ g) fragments as templates was carried out at 30°C for the indicated times as described in Materials and Methods and contained proteins p3 and p2 in the absence or presence of 4 μ g of protein p6. ○---○, ClaI A, -p6; ●—●, ClaI A, +p6; △---△, ClaI B, -p6; ▲—▲, ClaI B, +p6.

protein p6 (data not shown).

The activity of the ϕ 29 DNA polymerase itself, in the absence or presence of protein p6, was tested on the template-primer poly(dT)-(dA)₁₂₋₁₈. No effect of protein p6 on the dAMP incorporation at different dATP concentrations was observed (results not shown).

Protein p6 stimulates preferentially elongation from the right end of Ø29 DNA

Fig. 2A and 3b showed the existence of polarity in the stimulation of the truncated elongation reaction by protein p6, the right DNA end being stimulated to a greater extent than the left end. To confirm those results, the Ø29 DNA-protein p3 ClaI fragments, about 5.9 and 12.1 Kb long, from the left and right Ø29 DNA ends, respectively (27), each containing one replication origin, were used as templates in the formation of the p3-dAMP initiation complex and in the truncated elongation reaction indicated above. Figure 5A shows that protein p6 stimulated to a similar extent the initiation reaction with the right (lanes a and b) or the left (lanes c and d) p3-containing ClaI fragments, being the stimulation similar to that obtained with Ø29 DNA-protein p3 undigested or digested with ClaI (not shown). On the contrary, as shown in Fig. 5B, the stimulation by protein p6 of the formation of the truncated elongation product in the presence of ddCTP was higher with the right p3-containing ClaI fragment (lanes a and b) than with the left one (lanes c and d). In agreement with the above results, when the incorporation of $\{\alpha\text{-}^{32}\text{P}\}$ dATP was determined in the replication assay with the p3-containing ClaI fragments from the right and left Ø29 DNA ends, protein p6 stimulated to a greater extent the replication of the ClaI fragment from the right end than that from the left end (Fig. 5C).

Effect of protein p6 on initiation and truncated elongation using protein-free terminal Ø29 DNA fragments as templates.

Recently we have cloned the terminal fragments of Ø29 DNA in such a way that treatment of the recombinant plasmid pID13 with the restriction nuclease AhaIII releases the two origins of replication, each one at the end of one restriction fragment, 2956 and 993 bp long, respectively. These two protein-free restriction fragments were active as templates in the initiation and truncated elongation reactions in the presence of terminal protein p3 and Ø29 DNA polymerase p2 (13). To find out whether the parental terminal protein is needed for the stimulating activity of protein p6 in initiation and elongation, we have carried out experiments similar to those presented in Fig. 5

(A and B), using the protein-free fragments as templates. The results (not shown) establish that p6 stimulates both initiation and elongation, just as it does in the presence of parental terminal p3.

DISCUSSION

We have recently shown that the formation of the p3-dAMP initiation complex in a purified system with the terminal protein p3, the ϕ 29 DNA polymerase p2 and ϕ 29 DNA-protein p3 as template was strongly stimulated by highly purified protein p6, product of the viral gene 6 (20). In this paper we show that the stimulation of the initiation reaction by protein p6 is due to a decrease in the K_m value for dATP from 6 μ M to 1.2 μ M. This result could be explained by interaction of protein p6 with the ϕ 29 DNA polymerase-terminal protein complex enhancing the affinity for dATP. The stimulation by protein p6 of the formation of the p3-dAMP complex was similar using the right or left protein p3-containing ϕ 29 DNA ClaI fragments as templates. The initiation reaction was also stimulated by protein p6 to a similar extent when protein-free fragments containing the left or right terminal sequences of ϕ 29 DNA at the ends were used as templates. Although the K_m value for dATP with protein-free fragments was 11 μ M (not shown), about 2 fold higher than that with ϕ 29 DNA-protein p3, protein p6 decreased this value to an extent similar to that of protein p3-containing DNA. These results indicate that the parental terminal protein p3 is not required for the action of protein p6 in initiation.

Protein p6 is also shown to stimulate the truncated elongation reaction in the presence of ddCTP with ϕ 29 DNA-protein p3 as template. In this reaction, protein p3 linked to oligonucleotides 9 and 12 bases long was obtained under conditions of high dATP concentration in which there is essentially no effect of protein p6 on the initiation reaction. This result indicates that protein p6 stimulates, in addition to the initiation reaction, a limited elongation of the p3-dAMP complex. When further elongation of ϕ 29 DNA-protein p3 was allowed to occur, it was found that protein p6 stimulated this reaction by a factor similar to that of the truncated elongation reaction. Since the K_m value

for dGTP in ϕ 29 DNA-protein p3 replication was not affected by p6 and the rate of elongation was increased only from 16 to 26 nucleotides/second, the main function of protein p6 in elongation could be to stimulate the incorporation of the first nucleotide(s) to the p3-dAMP initiation complex. It is also interesting to note the fact that, in order to incorporate the second dAMP residue using ϕ 29 DNA-protein p3 as template, a concentration of dATP above 1 μ M is required, even in the presence of p6, whereas the initiation reaction occurs at very low dATP concentration (0.1 μ M). This result may suggest that the ϕ 29 DNA polymerase p2 has two active sites, one for initiation and another for elongation, or that the interaction with the terminal protein p3 during initiation produces some change in the ϕ 29 DNA polymerase to allow activity at very low dATP concentration.

The effect of protein p6 on the elongation reaction is dependent on the template, since there was stimulation with ϕ 29 DNA terminal fragments, with or without parental protein p3, but not with poly(dT)-(dA)₁₂₋₁₈. In addition, the effect of protein p6 on the elongation reaction, unlike its effect on initiation, seems to be sequence-dependent since the replication of the right end of ϕ 29 DNA-protein p3 was preferentially stimulated by protein p6 over that of the left end. The fact that replication of protein-free fragments containing the left or right ϕ 29 replication origins were also stimulated by protein p6 (data not shown) suggests that the parental terminal protein is not required for the p6 effect on elongation, as also in initiation.

Protein p6 is one of the major ϕ 29 early proteins synthesized in ϕ 29-infected *B. subtilis* (17), in agreement with a possible role as a DNA-binding protein (unpublished results). The use of p6 mutants available (28,29) as well as the proteins corresponding to p6 from ϕ 29-related phages (30) could be useful to determine the active center(s) of the protein in the initiation and elongation steps in the replication of ϕ 29 DNA.

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