Effect of NH_4^+ Ions on $\phi 29$ DNA-Protein p3 Replication: Formation of a Complex between the Terminal Protein and the DNA Polymerase

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Ammonium ions stimulated the formation of the $\phi 29$ protein p3-dAMP initiation complex by decreasing the K_m value for dATP in a purified system containing the viral terminal protein p3, the viral DNA polymerase p2, and the $\phi 29$ DNA-protein p3 complex as a template. In addition, NH_4^+ ions stimulated the amount of p3-dAMP complex elongation and increased by about twofold the rate of elongation. The stimulatory effect of NH_4^+ ions on in vitro $\phi 29$ DNA replication is probably related to the formation of a stable complex between the terminal protein and the DNA polymerase, which was detected only in the presence of NH_4^+ ions.

The *Bacillus subtilis* bacteriophage $\phi 29$ has a linear, double-stranded DNA of 19,285 base pairs (bp) (10, 29, 33) with a 6-bp-long inverted terminal repeat (AAAGTA) (7, 32) and a terminal protein, p3, covalently linked to the two 5' ends (reviewed in reference 26) by a phosphodiester bond between the OH group of serine residue 232 and dAMP (14). Initiation of replication starts at either end of the DNA (13, 15, 28) by a protein-priming mechanism in which a covalent complex between a free molecule of terminal protein p3 and 5' dAMP is formed in a reaction that is catalyzed by $\phi 29$ DNA polymerase p2 (4, 30) and that requires the presence of templates containing the $\phi 29$ replication origins (9, 11, 12). The p3-dAMP initiation complex formed in vitro is further elongated by the viral DNA polymerase to produce full-length $\phi 29$ DNA (5). The viral protein p6, required for $\phi 29$ DNA replication in vivo (20), stimulates both the initiation and elongation steps of $\phi 29$ DNA replication in vitro (3, 23).

When purified proteins p2 and p3 were used in the in vitro replication system as the only proteins, with $\phi 29$ DNA-protein p3 complex as the template, the initiation reaction, as well as the incorporation of deoxynucleoside triphosphates, was strongly stimulated by the addition of NH_4^+ ions (5). This is a report of (i) the effect of NH_4^+ ions in the initiation and elongation reactions on $\phi 29$ DNA replication and (ii) the formation of a stable complex between the terminal protein and the DNA polymerase.

MATERIALS AND METHODS

Materials. The $\phi 29$ DNA-protein p3 complex was isolated as described by Peñalva and Salas (24). When indicated, the p3-DNA complex was treated with *Clal* or *HindIII* (Boehringer Mannheim Biochemicals) in the presence of bovine serum albumin (1 mg/ml), and the mixture of fragments was used as the template in the initiation and replication assays. The recombinant plasmid pID13, which contains terminal fragments from the right and left ends of $\phi 29$ DNA, was as described elsewhere (11).

Assay for formation of the p3-dAMP initiation complex or for limited elongation. The standard incubation mixture for the initiation reaction contained, in 25 μl , 50 mM Tris hydrochloride (pH 7.5), 10 mM MgCl_2 , 1 mM dithiothreitol, 1 mM spermidine, $\phi 29$ DNA-protein p3 template (0.5 μg), and [$\alpha\text{-}^{32}\text{P}$]dATP (Amersham International), purified proteins p2 (4) and p3 (25), and NaCl as indicated in each case. When indicated, $(\text{NH}_4)_2\text{SO}_4$ or protein p6 purified by glycerol gradient centrifugation (23) was added. After incubation for 15 to 20 min at 30°C, the reaction was stopped and the samples were treated with micrococcal nuclease and subjected to polyacrylamide gel electrophoresis as described elsewhere (3). Quantitation was done by excising the radioactive band corresponding to the p3-dAMP complex from the gel and by counting the Cerenkov radiation.

In the limited elongation assays, the samples without micrococcal nuclease treatment were filtered through Sephadex G-50 spun columns (16) in the presence of 0.1% sodium dodecyl sulfate, (SDS), treated with piperidine as described elsewhere (3), and subjected to electrophoresis in 20% acrylamide-8 M urea denaturing gels (18). The reaction products from a chemical sequence analysis (18) of a DNA fragment labeled at one of the 3' ends were run in parallel as size markers.

Replication assay. The incubation mixture was the same as that described above for the initiation reaction, except that it contained 15 to 20 μM each dGTP, dCTP, dTTP, and [$\alpha\text{-}^{32}\text{P}$]dATP (1 μCi). $\phi 29$ DNA-protein p3 (0.5 μg) was used either untreated or treated with *HindIII*. When indicated, the recombinant plasmid pID13 (20 ng; 11), which contains the $\phi 29$ DNA replication origins, was used after treatment with *DraI*. After incubation for the indicated times at 30°C, the reaction was stopped and the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. The excluded fraction was counted by Cerenkov radiation.

When indicated, the DNA labeled in the replication assay described above was either subjected to electrophoresis in 1% agarose gels after digestion with proteinase K or denatured by treatment with 0.3 N NaOH and electrophoresed in alkaline 0.7% agarose gels (19) alongside DNA length mark-

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FIG. 1. Effect of $(NH_4)_2SO_4$ on the formation of the p3-dAMP initiation complex and limited elongation as a function of the dATP concentration. (A) $\phi 29$ DNA-protein p3 complex was incubated with protein p3 (160 ng) and protein p2 (5 ng) at a final NaCl concentration of 46 mM and with the indicated concentrations of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (2.5 μCi) in the absence (a) (see also reference 3) or presence (b) of 20 mM $(NH_4)_2SO_4$. After incubation for 20 min at 30°C , the samples were digested with micrococcal nuclease and subjected to electrophoresis, and the amount (in femtomoles) of p3-dAMP was determined and represented in a Lineweaver-Burk plot. Symbols: ○, without $(NH_4)_2SO_4$; ●, with $(NH_4)_2SO_4$. (B) The incubation was done as described above with the indicated concentrations of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (2.5 μCi) in the presence of 20 mM $(NH_4)_2SO_4$ without protein p6 (lanes b to e) or with 2 μg of protein p6 (lanes f to i). After 10 min at 30°C , the samples were processed, treated with piperidine, and subjected to electrophoresis in a denaturing gel, as described in Materials and Methods. The reaction products from a chemical sequence analysis of a labeled DNA fragment were run in parallel as size markers (lane a). dAMP runs close to the dinucleotide position (24). The amount of dAMP incorporated, determined before the treatment with piperidine (in femtomoles) was 3.6 (lane b), 15.5 (lane c), 35 (lane d), 54.8 (lane e), 25.8 (lane f), 38.8 (lane g), 55.8 (lane h), and 86.5 (lane i).

ers (1-kilobase ladder; Bethesda Research Laboratories). After electrophoresis, DNA length markers were detected with ethidium bromide and the gel was dried and autoradiographed with intensifying screens at -70°C .

RESULTS

Effect of ammonium sulfate on the K_m value for dATP in the initiation reaction. When the formation of the protein p3-dAMP initiation complex with purified proteins p2 and p3 and $\phi 29$ DNA-protein p3 as the template was studied at different concentrations of dATP, it was found that the addition of 20 mM $(NH_4)_2SO_4$, previously shown to be the optimal concentration (5), decreased the K_m value for dATP from 6 μM (3) to 1.2 μM (Fig. 1A). As shown in the Lineweaver-Burk plot, the same value for $V_{\max}$ was obtained in the absence and presence of NH_4^+ ions (Fig. 1A). When both protein p6 and NH_4^+ ions were added to the in vitro system, the K_m value for dATP in the formation of the p3-dAMP complex showed no further change (results not shown). Treatment of $\phi 29$ DNA-protein p3 with *Cla*I produced two fragments, each containing one replication origin. When either fragment was used as the template, the initiation reaction was stimulated to a similar extent by $(NH_4)_2SO_4$ (results not shown).

Effect of ammonium sulfate on the limited elongation of the p3-dAMP complex. To determine the effect of NH_4^+ ions on the limited elongation of the p3-dAMP initiation complex to p3-pdApdApdA, which is the expected product with dATP as the only nucleotide, the product of the reaction with purified proteins p2 and p3 and $\phi 29$ DNA-protein p3 as the template, at different concentrations of dATP and in the presence or absence of 20 mM $(NH_4)_2SO_4$, was treated with

piperidine to hydrolyze the linkage between protein p3 and the nucleotide residue (14). The product was then subjected to electrophoresis in a polyacrylamide sequencing gel, alongside size markers. As previously shown, in the absence of $(NH_4)_2SO_4$, essentially all the reaction products corresponded to protein p3 linked to dAMP (3). In the presence of NH_4^+ ions, in addition to the dAMP band, radioactivity corresponding to the dinucleotide position appeared, and this product increased at the higher dATP concentrations (Fig. 1B, lanes b to e), as was the case when protein p6 was added (3). A very small amount of trinucleotide appeared either in the presence of NH_4^+ ions (Fig. 1B, lanes b to e) or in the presence of protein p6 (3). However, when both $(NH_4)_2SO_4$ and protein p6 were added, radioactivity corresponding to the trinucleotide position appeared; this product also increased at the higher dATP concentrations (Fig. 1B, lanes f to i).

Effect of ammonium sulfate on $\phi 29$ DNA-protein p3 replication. Figure 2 shows the stimulation of $\phi 29$ DNA-protein p3 replication by 20 mM $(NH_4)_2SO_4$ when different concentrations of dGTP were used and the other three deoxynucleoside triphosphates were kept at a concentration of 15 μM . Under these conditions, there was essentially no effect of $(NH_4)_2SO_4$ on initiation (Fig. 1A). The K_m value for dGTP in the elongation reaction with purified proteins p2 and p3, which was 9 μM (3) (Fig. 2), was reduced to 4.4 μM in the presence of NH_4^+ ions, and $V_{\max}$ increased fivefold, thus indicating an effect of NH_4^+ ions on elongation.

The stimulation of $\phi 29$ DNA replication by $(NH_4)_2SO_4$ was dependent on the amount of $\phi 29$ DNA-protein p3 (Fig. 3A) or protein p3 (Fig. 3B) added, but not on the amount of protein p2 added (Fig. 3C). Taking into account the fact that the interaction of protein p3 with DNA has been observed (I.

FIG. 2. Effect of $(\text{NH}_4)_2\text{SO}_4$ on ϕ 29 DNA-protein p3 replication at different concentrations of dGTP. ϕ 29 DNA-protein p3 was incubated for 20 min at 30°C with protein p3 (160 ng) and protein p2 (5 ng) at final concentrations of 46 mM NaCl and 15 μM each dTTP, dATP, and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (1 μCi) and the indicated concentrations of dGTP in the absence (○) (see also reference 3) or presence (●) of 20 mM $(\text{NH}_4)_2\text{SO}_4$. After incubation, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the radioactivity was determined. The inset shows the Lineweaver-Burk plot.

Prieto, Ph.D. thesis, Universidad Autónoma de Madrid, Madrid, Spain), these results could be explained if NH_4^+ ions prevented the unspecific binding of protein p3 to the DNA. In agreement with this, the replication activity of ϕ 29 DNA-protein p3 remaining when a fivefold excess of linearized M13 DNA was added in the absence of $(\text{NH}_4)_2\text{SO}_4$ was 15%, whereas in the presence of NH_4^+ ions, it was 60% (results not shown). In addition, the binding of protein p3 to DNA fragments, as determined by filter binding assay, required a greater amount of protein p3 in the presence of $(\text{NH}_4)_2\text{SO}_4$ than in its absence (results not shown).

To determine whether the stimulation by NH_4^+ ions was due to an increase in the rate of elongation, the DNA synthesized at different incubation times was analyzed by alkaline agarose gel electrophoresis (Fig. 4A). The rate of elongation increased twofold when $(\text{NH}_4)_2\text{SO}_4$ was added, whereas the addition of protein p6 had essentially no effect, either in the absence (see also reference 3) or in the presence of $(\text{NH}_4)_2\text{SO}_4$ (Fig. 4). The increase in the rate of elongation relative to incubation time was probably due to the existence

of an initial lag in the incorporation of nucleotides. Taking into account the strong inhibition of ϕ 29 DNA-protein p3 replication by NaCl (unpublished results), the elongation rate was also determined at a final NaCl concentration of 20 mM, sixfold lower than the one used in the experiment shown in Fig. 4, with no significant effect (results not shown).

Specificity of the ammonium sulfate effect. To test the effect of ammonium sulfate on elongation proceeding from each DNA end, ϕ 29 DNA-protein p3 was treated with *Hind*III. The incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ in the presence of purified proteins p2 and p3 was then studied. Figure 5A shows that the presence of protein p3 stimulated incorporation only in the two terminal *Hind*III B and L fragments, 2,899 and 269 bp long, from the left and right ϕ 29 DNA ends, respectively (lane e), with respect to a control without protein p3 (lane a). Therefore, the protein p3-dependent labeling was due to replication of the ϕ 29 DNA origin-containing fragments, whereas the protein p3-independent labeling was due to the filling of the cohesive ends of the fragments by protein p2.

FIG. 3. Effect of (NH₄)₂SO₄ on ϕ29 DNA-protein p3 replication as a function of template, protein p3, and protein p2. (A) ϕ29 DNA-protein p3 at the indicated amounts was incubated for 10 min at 30°C with protein p3 (21 ng) and protein p2 (80 ng) at final concentrations of 30 mM NaCl and 20 μM each dCTP, dGTP, dTTP, and [α-³²P]dATP (1 μCi) in the absence (○) or presence (●) of 20 mM (NH₄)₂SO₄. After incubation, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the radioactivity was determined. (B) As for panel A, except that 0.5 μg of ϕ29 DNA-protein p3 and the indicated amounts of protein p3 were incubated for 5 min at 30°C. (C) As for panel A, except that 0.5 μg of ϕ29 DNA-protein p3 and the indicated amounts of protein p2 were added.

The addition of 20 mM (NH₄)₂SO₄, only in the presence of protein p3, stimulated the incorporation directed by the two terminal fragments, but not that directed by the internal fragments (lanes c and g). Taking into account the length of the *Hind*III B and L fragments, the efficiency of replication from the left terminal *Hind*III B fragment in the presence of (NH₄)₂SO₄ was 1.5-fold greater than that from the right one, as was the case in the absence of the NH₄⁺ ions. On the contrary, as previously shown for the *Cla*I fragments (3), protein p6 stimulated replication from the two terminal *Hind*III fragments only in the presence of p3, with replication from the right *Hind*III L fragment being about 2.7-fold greater than that from the left *Hind*III B fragment and with no effect on the replication of the internal fragments (lanes b and f). The presence of both NH₄⁺ ions and protein p6 further stimulated replication from the two terminal *Hind*III fragments in the presence of p3 (lane h), with replication from the right terminal *Hind*III L fragment being 1.4-fold greater than that from the left one. Again, there was no effect on the replication of the internal fragments (lanes d and h).

To test whether the (NH₄)₂SO₄ effect was dependent on the presence of the parental terminal protein, the recombinant plasmid pID13, which contains the ϕ29 replication origins, was digested with *Dra*I, producing fragments 2,956 and 993 bp long that contained 73 and 269 bp from the left and right ϕ29 DNA ends, respectively, and fragments 692 and 19 bp long that did not contain ϕ29 sequences (Fig. 5C). Incorporation of [α-³²P]dATP into the origin-containing fragments (Fig. 5B) was greatly stimulated by the addition of 20 mM (NH₄)₂SO₄ in the presence (lanes c and d) but not in the absence (lanes a and b) of protein p3. However, contrary to what happens with the terminal fragments of ϕ29 DNA-protein p3, the incorporation from the fragment containing the right replication origin was 4.6-fold greater than that from the fragment containing the left one. This effect was not

due to the initiation reaction, since the formation of the p3-dAMP complex was similar with right or left origin-containing fragments (11). No incorporation into the blunt-ended *Dra*I C fragment, which does not contain ϕ29 DNA terminal sequences, was obtained (Fig. 5B).

Interaction of the terminal protein p3 and the DNA polymerase p2 in the presence of ammonium sulfate. In the presence of 20 mM (NH₄)₂SO₄, proteins p2 and p3 formed a complex identified by glycerol gradient centrifugation (Fig. 6). About 50% of protein p3 that by itself sedimented at the position corresponding to a monomer (31 kilodaltons; Fig. 6C), in the presence of protein p2 and (NH₄)₂SO₄ sedimented together with protein p2 (Fig. 6A), with an apparent molecular weight of 86 kilodaltons, which was close to the expected molecular weight (99 kilodaltons). This 86-kilodalton complex was able to replicate the ϕ29 DNA-protein p3 template without the addition of either protein p2 or p3 (results not shown). The formation of the p2-p3 complex stabilized the DNA polymerase activity, since protein p2, when it was centrifuged alone through the glycerol gradient, even in the presence of (NH₄)₂SO₄, lost its activity (Fig. 6B). This loss of activity was probably due to the low ionic strength used in the experiment. When proteins p2 and p3 were incubated and centrifuged in the absence of (NH₄)₂SO₄, no p2-p3 complex was detected; all protein p3 was present at the position of the monomer, and the DNA polymerase activity was lost (results not shown), as was the case when protein p3 was not added.

Interaction of the terminal protein p3-DNA polymerase p2 complex with ϕ29 DNA-protein p3. Proteins p2 and p3, in the presence of 20 mM (NH₄)₂SO₄, interacted with ϕ29 DNA-protein p3, as determined by glycerol gradient centrifugation (Fig. 7A). The formation of a complex of proteins p2 and p3 and ϕ29 DNA-protein p3 was indicated not only by the fact that the p2 and p3 activities cosedimented with the ϕ29

FIG. 4. Analysis of the products of ϕ 29 DNA-protein p3 replication in the presence of $(\text{NH}_4)_2\text{SO}_4$, protein p6, or both by alkaline agarose gel electrophoresis. ϕ 29 DNA-protein p3 was incubated for the indicated times at 30°C with protein p3 (40 ng) and protein p2 (5 ng) at final concentrations of 120 mM NaCl, 15 μM each dGTP, dTTP, dCTP, and $[\alpha\text{-}^{32}\text{P}]\text{dATP}$, and, when indicated, 20 mM $(\text{NH}_4)_2\text{SO}_4$ (AS) or 3 μg of protein p6. After incubation, the samples were filtered through a Sephadex G-50 spun column in the presence of 0.1% SDS and subjected to alkaline agarose gel electrophoresis, as described in Materials and Methods. (A) Autoradiography of the gels. Samples a and b are the same, but the exposure time for b was six times longer than that for a and the other samples. The mobilities of the size markers, run in parallel, are indicated at the left. kb, Kilobases. The amounts of dAMP incorporated (in picomoles) were as follows for the times indicated: 0.04, 0.08, 0.33, and 0.93 (with the addition of p2 and p3); 0.12, 0.48, 3.17, and 17.25 (with the addition of p2, p3, and p6); 0.20, 0.60, 2.60, and 5.72 [with the addition of p2, p3, and $(\text{NH}_4)_2\text{SO}_4$]; and 2.87, 8.62, 25.76, and 65.87 [with the addition of p2, p3, p6, and $(\text{NH}_4)_2\text{SO}_4$]. (B) From the results shown in panel A, the rate of elongation was calculated at the points of maximal length at the time intervals indicated. When needed, longer exposure times were used for the calculations. Symbols: $\bullet$, p2 and p3; $\circ$, p2, p3, and p6; $\blacktriangle$, p2, p3, and $(\text{NH}_4)_2\text{SO}_4$; $\triangle$, p2, p3, p6, and $(\text{NH}_4)_2\text{SO}_4$.

DNA-protein p3 peak but also by the fact that replicating activity in the DNA peak was detected in the *in vitro* assay without the addition of protein p2, protein p3, or ϕ 29 DNA-protein p3. No such interaction was seen in the absence of $(\text{NH}_4)_2\text{SO}_4$, although protein p3 interacted with p3-DNA (Fig. 7B), or in the absence of protein p3 (Fig. 7C). The centrifugation time in this experiment was short enough to maintain the activity of protein p2, even in the absence of protein p3 or $(\text{NH}_4)_2\text{SO}_4$ (Fig. 7B and C). The additional peak of protein p3 activity shown in Fig. 7B in the absence of NH_4^+ ions could correspond to the nonspecific interaction of the protein with some aggregated form of the ϕ 29 DNA-protein p3 template (22). These results indicate that the DNA polymerase does not form a stable complex with ϕ 29 DNA-protein p3 and that it probably requires the formation of a complex with protein p3 for stable interaction. Nevertheless, by using a gel retardation assay (8), interaction of the ϕ 29

DNA polymerase with single- and double-stranded DNAs has been detected (unpublished results).

DISCUSSION

We recently showed that ϕ 29 DNA-protein p3 replication, in a purified system with the terminal protein p3 and the DNA polymerase p2, was strongly stimulated by NH_4^+ ions and, to a lesser extent, by K^+ ions but not by Na^+ ions (5). The formation of the p3-dAMP initiation complex was similarly stimulated by NH_4^+ ions (5). In the present study, we showed that the presence of 20 mM $(\text{NH}_4)_2\text{SO}_4$ decreased fivefold the K_m value for dATP in the initiation reaction, as was the case when purified protein p6 was added (3). As shown in the present study, NH_4^+ ions were needed for the *in vitro* formation of a stable complex between the ϕ 29 terminal protein and the DNA polymerase. This stable

FIG. 5. Effect of $(\text{NH}_4)_2\text{SO}_4$ on elongation from the left and right ends of $\phi 29$ DNA-protein p3 and protein-free $\phi 29$ DNA fragments. (A) $\phi 29$ DNA-protein p3 (0.5 μg) was digested with *Hind*III and incubated for 10 min at 30°C with $20 \mu\text{M}$ each dGTP, dTTP, dCTP, and $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (2.5 μCi) and proteins p3 (250 ng) and p2 (6 ng) (lanes e to h) or protein p2 alone (lanes a to d) at a final NaCl concentration of 20 mM without further additions (lanes a and e) or with 3 μg of protein p6 (lanes b and f), 20 mM $(\text{NH}_4)_2\text{SO}_4$ (lanes c and g), or both (lanes d and h). The samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, treated with proteinase K, and subjected to agarose gel electrophoresis, as described in Materials and Methods. (B) Plasmid pID13 (20 ng) was digested with *Dra*I and incubated for 10 min at 30°C with $20 \mu\text{M}$ each dGTP, dCTP, dTTP, and $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (2.5 μCi) and proteins p3 (43 ng) and p2 (6 ng) (lanes c and d) or protein p2 alone (lanes a and b) at a final NaCl concentration of 30 mM without additions (lanes a and c) or with 20 mM $(\text{NH}_4)_2\text{SO}_4$ (lanes b and d). The samples were

FIG. 6. Interaction of the terminal protein and the DNA polymerase in the presence of $(\text{NH}_4)_2\text{SO}_4$. Proteins p2 (0.6 μg) and p3 (1.3 μg) (A), protein p2 (0.6 μg) (B), and protein p3 (1.3 μg) (C) were each incubated for 1 h at 4°C in a final 0.2-ml volume of a buffer containing 50 mM Tris hydrochloride (pH 7.5), 44 mM NaCl, and 20 mM $(\text{NH}_4)_2\text{SO}_4$ and centrifuged in a 15 to 30% glycerol gradient in the above buffer for 28 h at $290,000 \times g$ at 0°C . Cytochrome c (100 μg), bovine serum albumin (140 μg), and 6-phosphogluconate dehydrogenase (40 μg) were included in each gradient as molecular weight markers. The positions of the markers, indicated by arrows, were determined by the method of Bradford (6) or by silver staining (1) after SDS-polyacrylamide gel electrophoresis. The positions of proteins p2 and p3 were determined by assaying the in vitro replication of the $\phi 29$ DNA-protein p3 complex by the addition of protein p3 or p2, respectively. Symbols: ●, p2 activity; ○, p3 activity. Sedimentation was from right to left. K, Kilodaltons.

complex might have a higher affinity for dATP than the free DNA polymerase has. In addition, since NH_4^+ ions decreased the nonspecific binding of protein p3 to DNA, the amount of protein p3 available for binding to the replication origins may increase. On the other hand, protein p6, which does not seem to interact with protein p2 or p3 (Prieto, Ph.D. thesis), interacts with terminal fragments of $\phi 29$ DNA, probably inducing a conformational change in the latter (27) that might facilitate the interaction of the p2-p3 complex with the DNA to initiate replication. Therefore, protein p6 and NH_4^+ ions seem to stimulate the initiation of $\phi 29$ DNA

treated as described for panel A. (C) Plasmid pID13. $\phi 29$ DNA fragments are shown with solid bars. The names of the *Dra*I fragments are indicated. The sizes of the fragments were as follows: A, 2,596 bp; B, 993 bp; and C, 692 bp.

FIG. 7. Interaction of DNA polymerase p2 and terminal protein p3 with ϕ 29 DNA-protein p3 in the presence of $(\text{NH}_4)_2\text{SO}_4$. ϕ 29 DNA-protein p3 (2 μg) was incubated for 1 h at 4°C with proteins p2 (0.13 μg) and p3 (1.8 μg) at a 0.2-ml volume in the buffer described in the legend to Fig. 6 in the presence (A) or absence (B) of 20 mM $(\text{NH}_4)_2\text{SO}_4$ or with protein p2 alone (0.13 μg) in the presence of 20 mM $(\text{NH}_4)_2\text{SO}_4$ (C) and subjected to glycerol gradient centrifugation as described in the legend to Fig. 6 for 4 h at $260,000 \times g$ at 4°C . The position of ϕ 29 DNA-protein p3 was determined by agarose gel electrophoresis, and the positions of proteins p2 (p2 activity) and p3 (p3 activity) were determined by assaying the in vitro replication of exogenously added ϕ 29 DNA-protein p3 and protein p3 or p2, respectively. The presence of the complex of proteins p2 and p3 and ϕ 29 DNA-protein p3 was determined by assaying the incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ without the addition of the proteins and template. Symbols: $\circ$, p3 activity; $\bullet$, p2 activity; $\blacktriangle$, p2, p3, and p3-DNA activity. Sedimentation was from left to right.

replication through a different mechanism. The formation of a complex between the ϕ 29 terminal protein and the DNA polymerase is also suggested by the fact that the two proteins copurify (17, 31). Interestingly, the buffers used by Watabe et al. (31) throughout the purification procedure contained $(\text{NH}_4)_2\text{SO}_4$. The fact that the two genes are linked (21, 33) and that they are transcribed from the same promoter (2) is also in agreement with the idea that the two proteins form a complex in vivo.

Ammonium ions also stimulated ϕ 29 DNA-protein p3 replication under conditions of high dATP concentration in which there was very little effect on the initiation reaction, indicating an effect of the NH_4^+ ions on the elongation step itself. The K_m for dGTP was reduced about twofold in the

presence of 20 mM $(\text{NH}_4)_2\text{SO}_4$, and V_{max} was increased fivefold, suggesting a possible effect on the elongation rate. When the latter was calculated, it was found that the NH_4^+ ions increased the rate of elongation about twofold. Alternatively, the possibility that, in the presence of NH_4^+ ions, less protein p3 nonspecifically binds to the DNA could explain the stimulation of the elongation rate by NH_4^+ ions. However, the stimulation of the rate of elongation does not account for all of the NH_4^+ effect. Since the formation of the p3-pdApdA complex from the p3-dAMP initiation complex was stimulated by NH_4^+ ions, an additional effect of the latter could be to facilitate the transition from initiation to elongation.

As shown in this study, only replication which was initi-

ated at the $\phi 29$ origins and was dependent on free protein p3 was stimulated by the NH_4^+ ions. Moreover, the elongation of the template primer poly(dT)-oligo(dA) by the $\phi 29$ DNA polymerase was not stimulated by NH_4^+ ions and did not require protein p3 (unpublished results). On the other hand, the presence of the parental terminal protein in the DNA fragments was not needed for the NH_4^+ stimulation. However, there was a change in the polarity, since in the absence of the parental protein p3, the incorporation into the right terminal fragment was stimulated to a higher extent than that into the left one, contrary to what happened in the presence of the parental terminal protein. These results suggest a different interaction of the p2-p3 complex with $\phi 29$ DNA-protein p3 and with protein-free $\phi 29$ DNA or the possibility that some sequences present in the terminal $\phi 29$ DNA *Hind*III fragments that are not present in the cloned left replication origin influence the ability of the fragments to be replicated.

The $\phi 29$ DNA polymerase does not seem to interact strongly with DNA, since a protein p2-DNA complex was seen only by using a gel retardation assay but not by using a filter-binding assay (unpublished results) or glycerol gradient centrifugation, as shown in this study. The terminal protein binds strongly to DNA and seems to interact with the parental protein p3 (Prieto, Ph.D. thesis), and through its interaction with the DNA polymerase in the presence of NH_4^+ ions, it might facilitate the location of the latter at the DNA ends to initiate replication and further elongation of the p3-dAMP complex. As shown in this study, NH_4^+ ions keep the $\phi 29$ DNA polymerase-terminal protein complex in a stable form *in vitro*. Whether the p2-p3 complex is stabilized by NH_4^+ or K^+ ions *in vivo* remains an open question.

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LITERATURE CITED

1. Ansorge, W. 1982. Fast visualization of protein bands by impregnation in potassium permanganate and silver nitrate, p. 235-242. In D. Stathakos (ed.), *Proceedings of electrophoresis*. Walter de Gruyter & Co., Berlin.
2. Barthelemy, I., M. Salas, and R. P. Mellado. 1986. *In vivo* transcription of bacteriophage $\phi 29$ DNA: transcription initiation sites. *J. Virol.* **60**:874-879.
3. Blanco, L., J. Gutiérrez, J. M. Lázaro, A. Bernad, and M. Salas. 1986. Replication of phage $\phi 29$ DNA *in vitro*: role of the viral protein p6 in initiation and elongation. *Nucleic Acids Res.* **14**:4923-4937.
4. Blanco, L., and M. Salas. 1984. Characterization and purification of a phage $\phi 29$ -encoded DNA polymerase required for the initiation of replication. *Proc. Natl. Acad. Sci. USA* **81**:5325-5329.
5. Blanco, L., and M. Salas. 1985. Replication of phage $\phi 29$ DNA with purified terminal protein and DNA polymerase: synthesis of full-length $\phi 29$ DNA. *Proc. Natl. Acad. Sci. USA* **82**:6404-6408.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
7. Escarmis, C., and M. Salas. 1981. Nucleotide sequence at the termini of the DNA of *Bacillus subtilis* phage $\phi 29$. *Proc. Natl. Acad. Sci. USA* **78**:1446-1450.
8. Fried, M. G., and D. M. Crothers. 1981. Equilibria and kinetics of lac repressor-operator interacting by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9**:6505-6525.
9. García, J. A., M. A. Peñalva, L. Blanco, and M. Salas. 1984. Template requirements for initiation of phage $\phi 29$ DNA replication *in vitro*. *Proc. Natl. Acad. Sci. USA* **81**:80-84.
10. Garvey, K. J., H. Yoshikawa, and J. Ito. 1985. The complete sequence of the *Bacillus* phage $\phi 29$ right early region. *Gene* **40**:301-309.
11. Gutiérrez, J., J. A. García, L. Blanco, and M. Salas. 1986. Cloning and template activity of the origins of replication of phage $\phi 29$ DNA. *Gene* **43**:1-11.
12. Gutiérrez, J., J. Vinós, I. Prieto, E. Méndez, J. M. Hermoso, and M. Salas. 1986. Signals in the $\phi 29$ DNA-terminal protein template for the initiation of phage $\phi 29$ DNA replication. *Virology* **155**:474-483.
13. Harding, N. E., and J. Ito. 1980. DNA replication of bacteriophage $\phi 29$: characterization of the intermediates and location of the termini of replication. *Virology* **104**:323-338.
14. Hermoso, J. M., E. Méndez, F. Soriano, and M. Salas. 1985. Location of the serine residue involved in the linkage between the terminal protein and the DNA of phage $\phi 29$. *Nucleic Acids Res.* **13**:7715-7728.
15. Inciarte, M. R., M. Salas, and J. M. Sogo. 1980. Structure of replicating DNA molecules of *Bacillus subtilis* bacteriophage $\phi 29$. *J. Virol.* **34**:187-199.
16. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*, p. 464-465. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. Matsumoto, K., T. Saito, C. I. Kim, T. Ando, and H. Hirokawa. 1984. Bacteriophage $\phi 29$ DNA replication *in vitro*: participation of the terminal protein and the gene 2 product in elongation. *Mol. Gen. Genet.* **196**:381-386.
18. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
19. McDonnell, M. W., M. N. Simon, and F. W. Studier. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* **110**:119-146.
20. Mellado, R. P., M. A. Peñalva, M. R. Inciarte, and M. Salas. 1980. The protein covalently linked to the 5' termini of the DNA of *Bacillus subtilis* phage $\phi 29$ is involved in the initiation of DNA replication. *Virology* **104**:84-96.
21. Moreno, F., A. Camacho, E. Viñuela, and M. Salas. 1974. Suppressor-sensitive mutants and genetic map of *Bacillus subtilis* bacteriophage $\phi 29$. *Virology* **62**:1-16.
22. Ortín, J., E. Viñuela, M. Salas, and C. Vázquez. 1971. DNA-protein complex in circular DNA from phage $\phi 29$. *Nature (London)* **234**:275-277.
23. Pastrana, R., J. M. Lázaro, L. Blanco, J. A. García, E. Méndez, and M. Salas. 1985. Overproduction and purification of protein p6 of *Bacillus subtilis* phage $\phi 29$: role in the initiation of DNA replication. *Nucleic Acids Res.* **13**:3083-3100.
24. Peñalva, M. A., and M. Salas. 1982. Initiation of phage $\phi 29$ DNA replication *in vitro*: formation of a covalent complex between the terminal protein p3 and 5'-dAMP. *Proc. Natl. Acad. Sci. USA* **79**:5522-5526.
25. Prieto, I., J. M. Lázaro, J. A. García, J. M. Hermoso, and M. Salas. 1984. Purification in a functional form of the terminal protein of *Bacillus subtilis* phage $\phi 29$. *Proc. Natl. Acad. Sci. USA* **81**:1639-1643.
26. Salas, M. 1983. A new mechanism for the initiation of replication of $\phi 29$ and adenovirus DNA: priming by the terminal protein. *Curr. Top. Microbiol. Immunol.* **109**:89-106.
27. Salas, M., I. Prieto, J. Gutiérrez, L. Blanco, A. Zaballo, J. M. Lázaro, G. Martín, A. Bernad, C. Garmendia, R. P. Mellado, C. Escarmis, and J. M. Hermoso. 1987. Replication of phage $\phi 29$

- DNA primed by the terminal protein. UCLA Symp. Mol. Cell. Biol. **47**:215–225.
28. Sogo, J. M., J. A. García, M. A. Peñalva, and M. Salas. 1982. Structure of protein-containing replicative intermediates of *Bacillus subtilis* phage ϕ 29 DNA. Virology **116**:1–18.
29. Vlček, C., and V. Pačes. 1986. Nucleotide sequence of the late region of *Bacillus* phage ϕ 29 completes the 19285-bp sequence of ϕ 29 genome. Comparison with the homologous sequence of phage PZA. Gene **46**:215–225.
30. Watabe, K., M. Leusch, and J. Ito. 1984. Replication of bacteriophage ϕ 29 DNA *in vitro*: the roles of terminal protein and DNA polymerase. Proc. Natl. Acad. Sci. USA **81**:5374–5378.
31. Watabe, K., M. F. Shih, and J. Ito. 1983. Protein-primed initiation of phage ϕ 29 DNA replication. Proc. Natl. Acad. Sci. USA **80**:4248–4252.
32. Yoshikawa, H., T. Friedmann, and J. Ito. 1981. Nucleotide sequences at the termini of ϕ 29 DNA. Proc. Natl. Acad. Sci. USA **78**:1336–1340.
33. Yoshikawa, H., and J. Ito. 1982. Nucleotide sequence of the major early region of bacteriophage ϕ 29. Gene **17**:323–335.