JOURNAL OF VIROLOGY, Nov. 1988, p. 4167–4172
0022-538X/88/114167-06$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 62, No. 11

Transition from Initiation to Elongation in Protein-Primed ϕ29 DNA Replication: Salt-Dependent Stimulation by the Viral Protein p6

LUIS BLANCO, ANTONIO BERNAD, AND MARGARITA SALAS*

Centro de Biología Molecular, Consejo Superior de Investigaciones Cientificas, Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Received 29 April 1988/Accepted 2 August 1988

The transition step from the p3-dAMP initiation complex to the first elongated products, p3-$(dAMP)_2$ and p3-$(dAMP)_3$, requires a dATP concentration higher than that needed for the initiation reaction or for the further elongation of the p3-$(dAMP)_3$ complex. The elongation in ϕ29 DNA-protein p3 replication in vitro was strongly inhibited by salt. Under inhibitory salt concentration, the viral protein p6 greatly stimulated ϕ29 DNA-protein p3 replication. The effect of protein p6 was not on the rate of elongation but on the amount of elongated product, stimulating the transition from initiation to formation of the first elongation products.

Bacteriophage ϕ29 has a linear, double-stranded DNA genome which is 19,285 base pairs (bp) long (16), and the viral protein p3 is covalently linked to both 5′ ends (14). ϕ29 replication starts at either DNA end by a protein-priming mechanism and proceeds toward the opposite end by strand displacement (for a review, see reference 13). An in vitro replication system using purified proteins has been developed. A free molecule of the terminal protein p3, in the presence of the ϕ29 DNA polymerase p2 and dATP and with ϕ29 DNA-protein p3 as the template, is deoxyadenylylated (3, 11, 17) in a reaction which is probably catalyzed by the DNA polymerase. The protein p3-dAMP initiation complex formed is further elongated by the DNA polymerase activity of protein p2 to produce full-length ϕ29 DNA (4). Whereas the initiation reaction occurs at a low dATP concentration (0.1 μM), a truncated elongation reaction in the presence of ddCTP, which gives rise to p3-(deoxynucleoside monophosphate)$_9$ and p3-(deoxynucleoside monophosphate)$_{12}$, requires a dATP concentration higher than 1 μM (1). Both the formation of the initiation complex and its elongation are stimulated by NH_4^+ ions, shown to be needed for the formation in vitro of a stable complex between the terminal protein p3 and the DNA polymerase p2 (2, 4). The viral protein p6, which binds to double-stranded DNA (12), stimulates both the initiation and the elongation steps in ϕ29 DNA-protein p3 replication (1, 9). However, there is not an absolute requirement for protein p6 in vitro, although mutants with mutations in gene 6 are unable to synthesize ϕ29 DNA in vivo (5, 8). In addition, protein p6 inhibits transcription from specific early promoters located at the right end of ϕ29 DNA (18; I. Barthelemy, R. P. Mellado, and M. Salas, submitted for publication).

In this paper, we show that protein p6 greatly stimulates the amount of DNA synthesized under salt conditions that decrease the replication of ϕ29 DNA-protein p3 dependent on the terminal protein p3 and the DNA polymerase p2. In addition, we show that the effect of protein p6 is on the transition from the p3-dAMP initiation complex to p3-$(dAMP)_2$ and p3-$(dAMP)_3$ and that this step requires a higher dATP concentration than the initiation reaction and the elongation from p3-$(dAMP)_3$.

* Corresponding author.

MATERIALS AND METHODS

Materials. The ϕ29 DNA-protein p3 complex was isolated as described by Peñalva and Salas (10). The p3-DNA complex was treated with *Hin*dIII, *Acc*I, or *Hin*fI (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 replication assays. Poly(dT) · poly$(dA)_{12-18}$ and poly(dA) · poly$(dT)_{12-18}$ were obtained from P-L Biochemicals. Proteins ssb from *Escherichia coli*, gp32 from phage T4, and DBP from adenovirus were gifts from A. Kornberg, J. J. Toulmé, and P. van der Vliet, respectively.

Assay for formation of the p3-dAMP initiation complex or for limited elongation. The standard incubation mixture for the initiation reactions contained (in 25 μl): 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM spermidine, 20 mM $(NH_4)_2SO_4$, ϕ29 DNA-protein p3 (0.5 μg), and various amounts of [α-^{32}P]dATP (Amersham International), purified proteins p2 (3) and p3 (11), and NaCl. For some experiments, highly purified protein p6 (9) was also added. After incubation at 30°C for various times, the reactions were stopped and the samples were treated with micrococcal nuclease and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (1). Quantitation was done by excising the radioactive band corresponding to the p3-dAMP complex from the gel and counting the Cerenkov radiation.

In the limited elongation assays, with dATP as the only nucleotide, the samples without micrococcal nuclease treatment were filtered through Sephadex G-50 spun columns (6) in the presence of 0.1% SDS, treated with piperidine as described previously (1), and subjected to electrophoresis in 20% acrylamide–8 M urea denaturing gels (7). The reaction products from a chemical sequence analysis (7) were run in parallel as size markers.

Replication assay. The incubation mixture was as described above for the initiation reaction, except that it contained various amounts of the deoxynucleoside triphosphates (dNTPs) and [α-^{32}P]dATP (1 μCi). ϕ29 DNA-protein p3 was used either untreated or treated with *Hin*dIII, *Acc*I, or *Hin*fI. For some experiments, the template primer poly(dT) · poly$(dA)_{12-18}$ or poly(dA) · poly$(dT)_{12-18}$ (0.25 μg) was added instead of ϕ29 DNA-protein p3. In this case, [α-^{32}P]dATP or [^{3}H]dTTP, respectively, (2.5 μCi at various concentrations) was the only nucleotide added and protein p3 was omitted. After incubation for various times at 30°C,

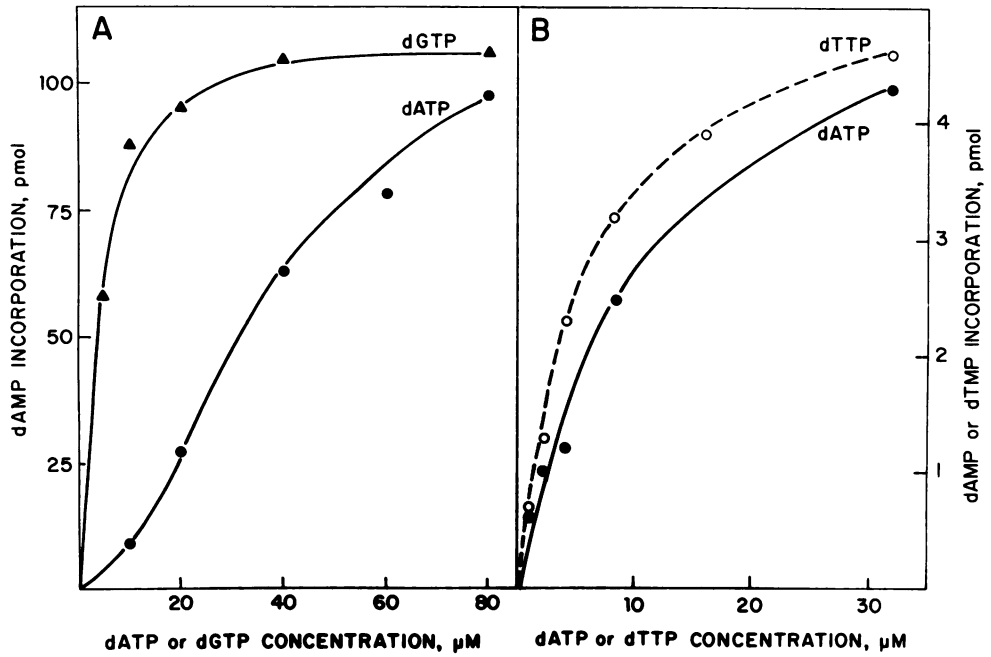

FIG. 1. High dATP requirement for the replication of ϕ29 DNA-protein p3. (A) ϕ29 DNA-protein p3 complex was incubated for 2.5 min at 30°C with protein p3 (21 ng) and protein p2 (80 ng) at a final NaCl concentration of 30 mM in the presence of [α-^{32}P]dATP (2 μCi) at the indicated concentrations of either dGTP or dATP, with the other dNTPs at 80 μM each. After incubation, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the excluded radioactivity was determined. (B) poly(dA) · poly(dT)$_{12\text{-}18}$ (○) or poly(dT) · poly(dA)$_{12\text{-}18}$ (●) was incubated for 10 min at 30°C with [^{3}H]dTTP (2.5 μCi) or [α-^{32}P]dATP (2.5 μCi), respectively, at the indicated concentrations in the presence of protein p2 (80 ng) at a final NaCl concentration of 4 mM. After incubation, the trichloroacetic acid-insoluble radioactivity was determined.

the reaction was stopped and the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. Cerenkov radiation in the excluded fraction was counted. For some experiments, the labeled DNA from the excluded fraction was treated with proteinase K and subjected to electrophoresis in either 1% agarose (for the *Hin*dIII fragments) or 3.5% (for the *Acc*I fragments) or 8% (for the *Hin*fI fragments) acrylamide gels. After electrophoresis, the gel was dried and autoradiographed with intensifying screens at −70°C.

In some cases, after the initiation reaction in the presence of dATP, immunoglobulin G anti-p3 (16 μg) was added to stop further initiation, and after 2.5 min at 30°C, elongation was allowed to proceed by addition of the remaining dNTPs.

RESULTS

Requirement of high dATP concentration in ϕ29 DNA-protein p3 replication. To investigate whether the high dATP requirement for the elongation of ϕ29 DNA-protein p3 replication was unique for this nucleotide, the effect of changing the concentration of each dNTP while keeping the concentrations of the other three dNTPs constant on the replication of ϕ29 DNA-protein p3 was studied. When the dGTP concentration was changed, the reaction was essentially saturated at 20 μM, whereas when the dATP concentration was changed, a sigmoid kinetics curve was obtained and saturation did not occur up to around 80 μM (Fig. 1A). Changing the dCTP or dTTP concentration gave results similar to those obtained in the case of dGTP (results not shown).

To determine whether dATP hydrolysis accounts for the high dATP concentration required, the hydrolysis of dATP (or of dCTP as a control) was determined in the presence of the remaining dNTP in each case concomitant with dATP or dCTP incorporation. Neither dATP nor dCTP hydrolysis was detected (results not shown).

To find out whether the high requirement for dATP was a property of the ϕ29 DNA polymerase or whether it was dependent on the template, the effect of dATP or dTTP concentration with the template primer poly(dT) ·poly (dA)$_{12\text{-}18}$ or poly(dA) · poly(dT)$_{12\text{-}18}$, respectively, was studied. Similar kinetics in the incorporation of either dATP or dTTP with the corresponding template were found, with the reaction being essentially saturated with a nucleotide concentration of 30 μM (Fig. 1B).

High dATP concentration is required for the transition from the p3-dAMP initiation complex to p3-(dAMP)$_3$. To determine the step in elongation that has a high dATP concentration requirement, the replication of ϕ29 DNA-protein p3 was started with either 0.25 or 20 μM dATP in the absence of the remaining dNTPs to allow the formation of p3-dAMP or p3-(dAMP)$_3$, respectively. After 7.5 min at 30°C, immunoglobulin G anti-p3 was added to stop further initiations, and then, after a fourfold dilution to decrease the initial dATP concentration, the elongation was allowed to proceed in the presence of increasing dATP concentration, with the remaining dNTPs kept at 60 μM. When the dATP concentration in the first step was 0.25 μM, the lag in the elongation step was seen, whereas when the dATP concentration in the first step was 20 μM, the lag disappeared (Fig. 2). This result strongly suggests that the step responsible for the high dATP requirement is the transition from the p3-dAMP initiation complex to the p3-(dAMP)$_3$ partially elongated product. This was confirmed when the formation of p3-dAMP, p3-(dAMP)$_2$,

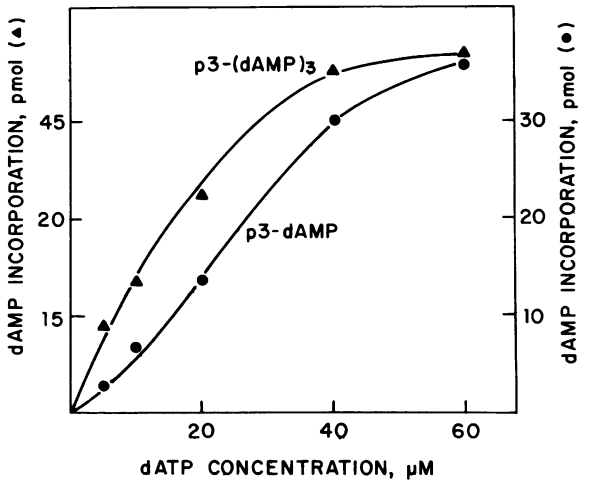

FIG. 2. Effect of the dATP concentration on the elongation of p3-dAMP or p3-$(dAMP)_3$ in φ29 DNA-protein p3 replication. φ29 DNA-protein p3 complex was incubated with protein p3 (21 ng) and protein p2 (80 ng) at a final NaCl concentration of 41 mM with either 0.25 (●) or 20 (▲) μM [α-^{32}P]dATP (2.5 μCi) to allow the formation of p3-dAMP or p3-$(dAMP)_3$, respectively. After incubation for 7.5 min at 30°C, immunoglobulin G anti-p3 (16 μg) was added, and after 2.5 min at 30°C the reaction mixture was diluted fourfold and 60 μM dGTP, dCTP, and dTTP and the indicated concentrations of dATP were added. After 2.5 min at 30°C, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS and the excluded radioactivity was determined.

and p3-$(dAMP)_3$ was determined as a function of the dATP concentration. The intensity of the radioactive band corresponding to the p3-dAMP initiation complex decreased with increasing cold dATP concentration, whereas no such decrease in intensity of bands occurred for the p3-$(dAMP)_2$ or p3-$(dAMP)_3$ complex (Fig. 3).

The stimulation of φ29 DNA-protein p3 replication by protein p6 is dependent on the salt concentration. Figure 4A shows the salt inhibition of the initiation reaction in φ29 DNA-protein p3 replication and the stimulation by protein p6 at all of the NaCl concentrations tested. The replication of φ29 DNA-protein p3 was strongly inhibited by salt (Fig. 4B). An increase in the NaCl concentration from 20 to 125 mM produced a large decrease in the dAMP incorporation. In the

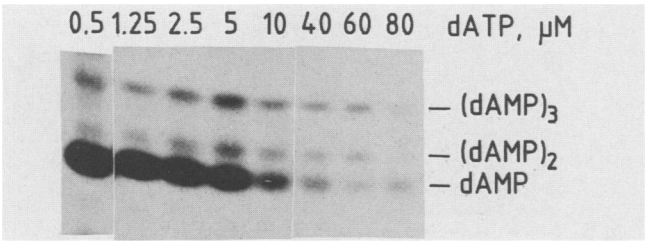

FIG. 3. Limited elongation in φ29 DNA-protein p3 replication as a function of dATP concentration. φ29 DNA-protein p3 was incubated for 2.5 min at 30°C with protein p3 (21 ng) and protein p2 (80 ng) at a final NaCl concentration of 30 mM and the concentrations of [α-^{32}P]dATP (5 μCi) indicated at the top. After incubation, 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 (not shown). The dAMP has been previously shown to run close to the dinucleotide position (10).

presence of protein p6, no inhibition was observed at up to 70 mM NaCl; an increase in the NaCl concentration up to 125 mM produced a small inhibition, much lower than that obtained in the absence of protein p6. It is clear from the above results that the stimulation of φ29 DNA-protein p3 replication in vitro by protein p6 is dependent on the NaCl concentration. Taking this fact into account and the fact that protein p6 does not affect the initiation step of φ29 DNA-protein p3 replication (1) at the high dATP concentration used in the replication assay, the above results indicate that the stimulation by protein p6 seen in Fig. 4B is due to an effect in the elongation step of replication. Other proteins tested, such as ssb from *E. coli*, gp32 from phage T4, DBP from adenovirus, and bovine serum albumin, had no effect.

To determine whether the salt was decreasing the rate of elongation and whether protein p6 was maintaining the elongation rate at the higher salt concentrations, the elongation rates at 25 and 100 mM NaCl in the absence or presence of protein p6 were measured. When the DNA synthesized was subjected to alkaline agarose gel electrophoresis, the results shown in Fig. 5 were obtained. It can be seen that an increase in the NaCl concentration from 25 to 100 mM decreased the rate of elongation about twofold and that protein p6 essentially had no effect on the elongation rate but increased the amount of DNA synthesized. Therefore, the stimulation by protein p6 obtained at 100 mM NaCl must be affecting some specific step of the viral DNA elongation.

Salt-dependent stimulation by protein p6 of the transition from p3-dAMP to p3-$(dAMP)_2$ and p3-$(dAMP)_3$. The effect of protein p6 on the transition from p3-dAMP to p3-$(dAMP)_2$ and p3-$(dAMP)_3$ as a function of the salt concentration is shown in Fig. 6. In the absence of protein p6, the increase in the NaCl concentration from 20 to 150 mM reduced the amounts of p3-dAMP initiation complex, p3-$(dAMP)_2$, and p3-$(dAMP)_3$ formed. When protein p6 was added, no inhibition was observed when NaCl concentration was increased. These results indicate that the elongation step affected by protein p6 at high salt concentrations is the transition from the p3-dAMP initiation complex to the first elongation products, p3-$(dAMP)_2$ and p3-$(dAMP)_3$; this is in addition to its effect in the initiation reaction.

Effect of salt concentration on the stimulation by protein p6 of φ29 DNA-protein p3 replication from the right or left end. It has been previously shown that protein p6 stimulates replication from the right φ29 DNA end to a higher extent than it does that from the left end (1, 2). The effect of protein p6 on the replication from the left or right end as a function of the salt concentration was studied by digesting φ29 DNA-protein p3 with *Hin*dIII and analyzing the incorporation of [α-^{32}P]dATP in the absence or presence of purified protein p6 by gel electrophoresis. At 100 mM NaCl, the specific incorporation of [α-^{32}P]ATP into the terminal φ29 *Hin*dIII B and L fragments (2,899 and 269 bp long [2]) was reduced to 15 and 8%, respectively, of the values obtained at 60 mM NaCl (Fig. 7A). As was expected from the previous results, in the presence of protein p6 the incorporation of [α-^{32}P]ATP from the φ29 DNA-protein p3 terminal fragments was more salt resistant than it was in the absence of the protein. Thus, at 110 mM NaCl, the incorporation into the *Hin*dIII B and L terminal fragments was 98 and 55%, respectively, of the values obtained at 70 mM NaCl. In addition, at the lower salt concentration (70 mM NaCl), the stimulation of replication from the right-end *Hin*dIII L fragment by protein p6 was higher than that from the left one. However, the reverse result was found at a higher salt concentration (135 mM NaCl) due to the fact that the

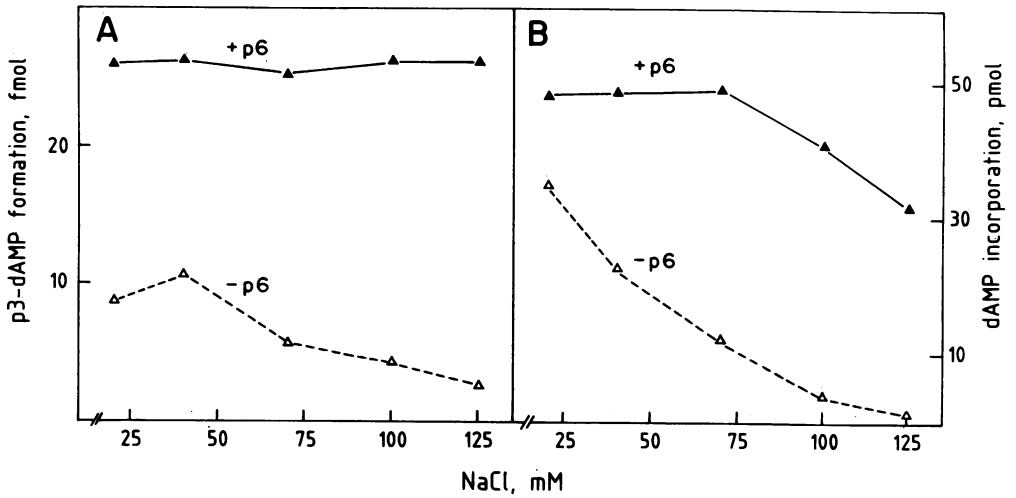

FIG. 4. Effect of the salt concentration on the stimulation by protein p6 of φ29 DNA-protein p3 replication. (A) φ29 DNA-protein p3 was incubated for 10 min at 30°C with protein p3 (125 ng), protein p2 (6.2 ng), and 0.25 μM [α-^{32}P]dATP (2 μCi) at the indicated NaCl concentrations in the absence (−p6) or presence (+p6) of protein p6 (3 μg). After incubation, the samples were digested with micrococcal nuclease, filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and subjected to electrophoresis, and the amount of p3-dAMP formation was determined. (B) Same as in panel A, except that 20 μM dATP, dCTP, dGTP, and dTTP were added. After incubation for 10 min at 30°C, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS and the excluded radioactivity was determined.

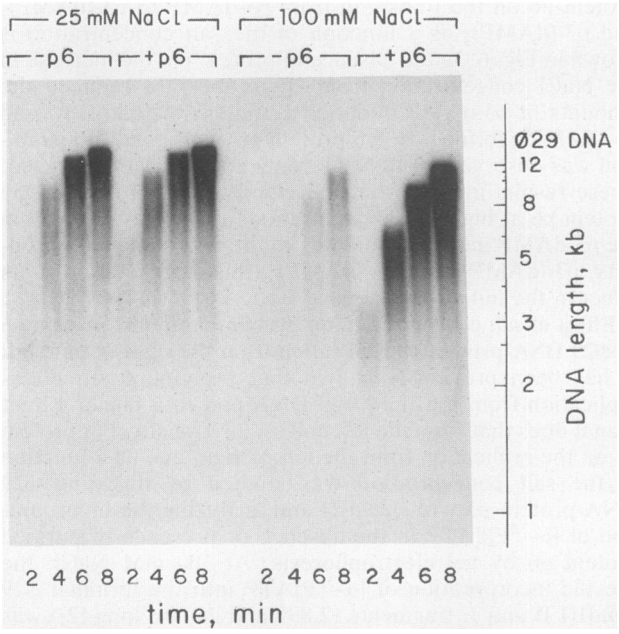

FIG. 5. Analysis by alkaline agarose gel electrophoresis of the products of φ29 DNA-protein p3 replication at different salt concentrations in the absence or presence of protein p6. φ29 DNA-protein p3 was incubated for the times indicated below the lanes at 30°C with protein p3 (21 ng), protein p2 (70 ng), and 80 μM each dCTP, dGTP, dTTP, and [α-^{32}P]dATP (2.5 μCi) at a final NaCl concentration of 25 or 100 mM in the absence (−p6) or presence (+p6) of protein p6 (3 μg), as indicated above the lanes. After incubation, the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS and subjected to alkaline agarose gel electrophoresis as described in Materials and Methods. The mobilities of size markers, which were run in parallel, are indicated at the right.

incorporation from the left-end *Hin*dIII B fragment was more resistant to salt than that from the right-end *Hin*dIII L fragment.

To determine the influence of the size of the terminal fragment on the stimulation by protein p6 at high salt concentration, φ29 DNA-protein p3 was digested with either *Acc*I, which gives rise to fragments 1,025 and 125 bp long from the left and right ends, respectively, or with *Hin*fI, which produces terminal fragments 158 and 59 bp long from the left and right ends, respectively. Addition of protein p6 stimulated the p3-dependent replication of the *Acc*I right terminal fragment (125 bp long) (Fig. 7B) and that of the left one (1,025 bp long) (not shown). Figure 7C shows the stimulation by protein p6 of the p3-dependent replication of the *Hin*fI terminal fragment (158 bp long) from the left φ29 DNA end. No specific incorporation dependent on protein p3 was seen in the 59-bp-long *Hin*fI fragment from the right

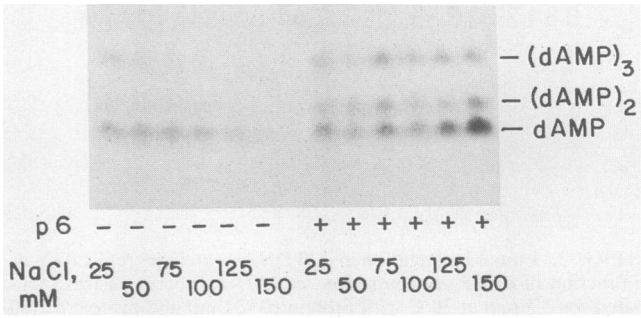

FIG. 6. Limited elongation in φ29 DNA-protein p3 replication as a function of the salt concentration in the absence or presence of protein p6. φ29 DNA-protein p3 (0.5 μg) was incubated for 2.5 min at 30°C with protein p3 (10 ng) and protein p2 (47 ng) in the presence of 20 μM [α-^{32}P]dATP (5 μCi) at the indicated concentrations of NaCl, in the absence (−) or presence (+) of protein p6 (2 μg). After incubation, the samples were processed and subjected to electrophoresis as described for Fig. 3.

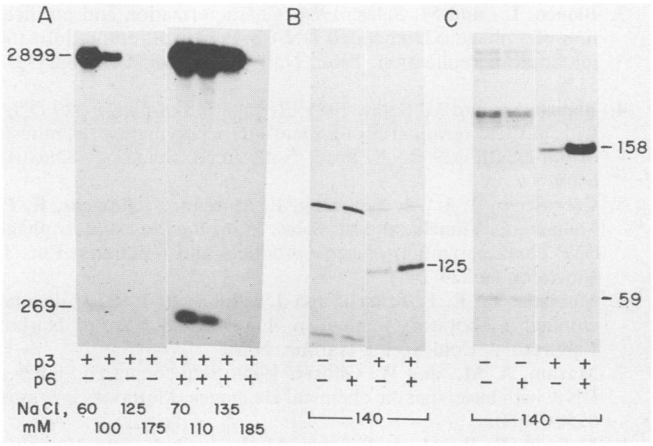

FIG. 7. Effect of the salt concentration and protein p6 on the replication of terminal fragments of φ29 DNA. (A) φ29 DNA-protein p3 was digested with *Hin*dIII and incubated for 15 min at 30°C with protein p3 (11 ng), protein p2 (5 ng), and 40 μM each dCTP, dGTP, dTTP, and [α-^{32}P]dATP (2.5 μCi) at the indicated concentrations of NaCl in the absence (−) or presence (+) of protein p6 (3 μg). The samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, treated with proteinase K, and subjected to electrophoresis in 1% agarose gels. Quantitation was done by densitometry of different exposures of the autoradiograms. The radioactivity in the *Hin*dIII B fragment in the absence of protein p6 at 100 and 125 mM NaCl was 15 and 8%, respectively, of that at 60 mM NaCl, and that in the *Hin*dIII L fragment at 100 mM NaCl was 8% of that at 60 mM NaCl. In the presence of protein p6, the radioactivity in the *Hin*dIII B fragment at 110 and 135 mM NaCl was 98 and 67%, respectively, of that at 70 mM NaCl, and that in the *Hin*dIII L fragment at 110 and 135 mM NaCl was 55 and 9%, respectively, of that at 70 mM NaCl. Numbers to the left indicate restriction fragment sizes in base pairs. (B) φ29 DNA-protein p3 was digested with *Acc*I and incubated for 15 min at 30°C with protein p3 (44 ng), protein p2 (7.5 ng), and 20 μM each dCTP, dGTP, dTTP, and [α-^{32}P]dATP (2.5 μCi) at 140 mM NaCl in the absence (−) or presence (+) of protein p6 (3 μg). The samples were processed as in panel A and subjected to electrophoresis in 3.5% acrylamide gels. Number to the right indicates restriction fragment size in base pairs. (C) φ29 DNA-protein p3 was digested with *Hin*fI and incubated for 15 min at 30°C as in panel B. Electrophoresis was in 8% acrylamide gels. Numbers at the right indicate terminal restriction fragment sizes in base pairs.

DNA end, probably due either to the small size of the fragment or to interference by an internal fragment of the same size (16). It can be also seen in Fig. 7B and C that the p3-independent incorporation corresponding to filling in of the cohesive ends by the φ29 DNA polymerase was not affected by the presence of protein p6 and that the presence of protein p3 decreased this filling-in reaction, probably due to its DNA-binding activity.

DISCUSSION

The replication of φ29 DNA-protein p3 in vitro only requires two purified viral proteins, the DNA polymerase p2 and the terminal protein p3, to produce full-length φ29 DNA (4). The replication of φ29 DNA in vivo requires, in addition to the gene 2 and 3 products, those of genes 5, 6, and 17 (5, 8, 15).

Protein p6 was purified and shown to stimulate the initiation reaction, i.e., the formation of the p3-dAMP complex (9), as well as the elongation of p3-dAMP (1). The effect of protein p6 on initiation was to decrease the K_m value for dATP; therefore, at high dATP concentrations, only the effect of the protein on the elongation reaction was seen (1). In this paper we have described the effect of protein p6 on φ29 DNA-protein p3 replication in vitro as a function of the salt concentration. Whereas the initiation reaction at low dATP concentrations was stimulated by protein p6 at NaCl concentrations from 20 to 125 mM, the stimulation of the elongation reaction by protein p6 was very much dependent on the NaCl concentration used. Thus, at 20 mM NaCl, there was a small stimulation by protein p6 that became larger with increasing NaCl concentration. At 100 or 125 mM NaCl, close to physiological salt concentrations, the replication of φ29 DNA-protein p3 became essentially dependent on the addition of protein p6. This result is mainly due to the fact that φ29 DNA-protein p3 replication with only proteins p2 and p3 is very strongly inhibited by salt, and protein p6 avoids this inhibition. Although an increase in the NaCl concentration from 25 to 100 mM produced about a twofold decrease in the elongation rate, addition of protein p6 had no effect on the rate of elongation. On the other hand, an increase in the NaCl concentration decreased the amount of p3-$(dAMP)_2$ and p3-$(dAMP)_3$ in the absence but not in the presence of protein p6, indicating that the elongation step stimulated by protein p6 is the transition from the p3-dAMP initiation complex to the first elongated products, p3-$(dAMP)_2$ and p3-$(dAMP)_3$, as has already been suggested (2). Protein p6 has been shown to interact with double-stranded DNA, specifically with φ29 DNA terminal fragments, producing a characteristic DNase I footprinting pattern of hypersensitive bands spaced 24 nucleotides apart flanking protected regions all along the terminal fragments used (~300 bp long) (12). In agreement with the salt-dependent stimulation by protein p6 of the φ29 DNA-protein p3 replication is the fact that the interaction of protein p6 with the φ29 DNA terminal fragments, but not with internal or heterologous fragments, is resistant to 100 mM NaCl. It is likely that the interaction of protein p6 with the φ29 DNA ends produces some conformational change that could facilitate replication. In agreement with this, for stimulation of φ29 DNA replication, protein p6 had to be added before the p3-dAMP complex was formed (unpublished results). The fact that replication with φ29 DNA terminal fragments 158 and 125 bp long from the left and right ends, respectively, was stimulated by protein p6 suggests that the sequences recognized by the protein are located within these fragments. The negative results obtained with the right 59-bp-long fragment could indicate the lack of these sequences. A precise mapping of specific DNA sequences required for stimulation by protein p6 is being carried out.

It was previously shown that, although the initiation reaction in φ29 DNA-protein p3 replication could take place at a very low dATP concentration (0.1 μM), a minimal dATP concentration higher than 1 μM was needed for elongation to occur (1). We show here that the requirement for dATP concentration in elongation is higher than that for the remaining dNTPs. This high dATP requirement is not a property of the φ29 DNA polymerase, since when the template-primer poly(dT) · $poly(dA)_{12-18}$ is used, the dATP requirement is similar to that of dTTP with poly(dA) $poly(dT)_{12-18}$. In fact, we showed that the high dATP requirement is for the transition from the p3-dAMP initiation complex to the first elongation products, p3-$(dAMP)_2$ and p3-$(dAMP)_3$, which, as indicated above, seems to be the step in which protein p6 stimulates φ29 DNA-protein p3 replication.

From the above results, it seems that φ29 DNA-protein p3

replication has three differentiated steps: (i) the formation of the p3-dAMP initiation complex, clearly identified at low dATP concentrations; (ii) the elongation of p3-dAMP to p3-$(dAMP)_2$ and p3-$(dAMP)_3$, which requires a high dATP concentration; and (iii) the elongation of p3-$(dAMP)_3$ to p3-DNA in a highly processive way (L. Blanco, A. Bernad, J. M. Lázaro, and M. Salas, manuscript in preparation). The ϕ29 DNA polymerase p2 seems to be the only polymerase involved in ϕ29 DNA-protein p3 replication, and therefore, it should be able to catalyze the three steps indicated above. The differences in dATP requirements for these steps could be due to the different natures of the residues involved in the phosphodiester linkage; dAMP has to be covalently linked to the OH group of a serine residue in the initiation reaction instead of the OH group of a nucleotide, as occurs in the elongation of a DNA chain. Alternatively, the differences in dATP requirement could reflect conformational changes in the dNTP-binding site of the DNA polymerase, probably due to the protein-protein interaction between the terminal protein and the DNA polymerase (2), which is likely to be required for the initiation of protein p3-DNA replication versus the protein-DNA interaction required for the elongation of a DNA primer. In the transition step, the DNA polymerase has to recognize the OH group of a nucleotide covalently linked to the terminal protein and, probably, dissociation of the complex formed by the two proteins has to occur; this may lead to a conformational change that could affect the interaction with dATP. Finally, the elongation from the p3-$(dAMP)_3$ complex is probably similar to the polymerizing activity of any DNA polymerase. The results presented in this paper suggest that the viral protein p6, when assayed at salt conditions close to in vivo values, plays an important role in the coupling of the initiation and elongation steps in ϕ29 DNA replication.

ACKNOWLEDGMENTS

This investigation has been aided by Public Health Service research grant 5 R01 GM27242-08 from the National Institutes of Health, by grant 3325 from Comisión Asesora para la Investigación Científica y Técnica, and by a grant from Fondo de Investigaciones Sanitarias. A.B. was a Fellow from Caja de Ahorros de Madrid.

LITERATURE CITED

1. **Blanco, L., J. Gutiérrez, J. M. Lázaro, A. Bernad, and M. Salas.** 1986. Replication of phage ϕ29 DNA *in vitro*: role of the viral protein p6 in initiation and elongation. Nucleic Acids Res. **14:** 4923–4937.
2. **Blanco, L., I. Prieto, J. Gutiérrez, A. Bernad, J. M. Lázaro, J. M. Hermoso, and M. Salas.** 1987. Effect of NH_4^+ ions on ϕ29 DNA-protein p3 replication: formation of a complex between the terminal protein and the DNA polymerase. J. Virol. **61:** 3983–3991.
3. **Blanco, L., and M. Salas.** 1984. Characterization and purification of a phage ϕ29-encoded DNA polymerase required for the initiation of replication. Proc. Natl. Acad. Sci. USA **82:**5325–5329.
4. **Blanco, L., and M. Salas.** 1985. Replication of phage ϕ29 DNA with purified terminal protein and DNA polymerase: synthesis of full-length ϕ29 DNA. Proc. Natl. Acad. Sci. USA **82:**6404–6408.
5. **Carrascosa, J. L., A. Camacho, F. Moreno, F. Jiménez, R. P. Mellado, E. Viñuela, and M. Salas.** 1976. *Bacillus subtilis* phage ϕ29: characterization of gene products and functions. Eur. J. Biochem. **66:**229–241.
6. **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.
7. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
8. **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 ϕ29 is involved in the initiation of DNA replication. Virology **104:**84–96.
9. **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 ϕ29: role in the initiation of DNA replication. Nucleic Acids Res. **13:**3083–3100.
10. **Peñalva, M. A., and M. Salas** 1982. Initiation of phage ϕ29 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.
11. **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 ϕ29. Proc. Natl. Acad. Sci. USA **81:**1639–1643.
12. **Prieto, I., M. Serrano, J. M. Lázaro, M. Salas, and J. M. Hermoso.** 1988. Interaction of the bacteriophage ϕ29 protein p6 with double-stranded DNA. Proc. Natl. Acad. Sci. USA **85:** 314–318.
13. **Salas, M.** 1983. A new mechanism for the initiation of replication of ϕ29 and adenovirus DNA: priming by the terminal protein. Curr. Top. Microbiol. Immunol. **109:**89–106.
14. **Salas, M., R. P. Mellado, E. Viñuela, and J. M. Sogo.** 1978. Characterization of a protein covalently linked to the 5′ termini of the DNA of *Bacillus subtilis* phage ϕ29. J. Mol. Biol. **119:** 269–291.
15. **Talavera, A., M. Salas, and E. Viñuela.** 1972. Temperature-sensitive mutants affected in DNA synthesis in phage ϕ29 of *Bacillus subtilis*. Eur. J. Biochem. **31:**367–371.
16. **Vlček, C., and V. Pačes.** 1986. Nucleotide sequence of the late region of *Bacillus* phage ϕ29 completes the 19285-bp sequence of ϕ29 genome. Comparison with the homologous sequence of phage PZA. Gene **46:**215–225.
17. **Watabe, K., M. Leusch, and J. Ito.** 1984. Replication of bacteriophage ϕ29 DNA *in vitro*: the roles of terminal protein and DNA polymerase. Proc. Natl. Acad. Sci. USA **81:**5374–5378.
18. **Whiteley, H. R., W. D. Ramey, G. B. Spiegelman, and R. D. Holder.** 1986. Modulation of *in vivo* and *in vitro* transcription of bacteriophage ϕ29 early genes. Virology **155:**392–401.