*Proc. Natl. Acad. Sci. USA*
Vol. 80, pp. 4248–4252, July 1983
Biochemistry

# Protein-primed initiation of phage $\phi$29 DNA replication

**(linear DNA replication/$\phi$29 terminal protein/genome-linked protein/DNA initiation)**

KOUNOSUKE WATABE, MENG-FU SHIH, AND JUNETSU ITO

Department of Molecular and Medical Microbiology, College of Medicine, University of Arizona, Tucson, Arizona 85724

*Communicated by C. S. Marvel, April 4, 1983*

**ABSTRACT** **We recently reported the development of an *in vitro* replication system for bacteriophage $\phi$29 DNA. We have used this system for the isolation of replication activity associated with gene 3 protein (terminal protein) from $\phi$29-infected *Bacillus subtilis* cells. We utilized two assay systems: (*i*) DNA replication dependent on $\phi$29 DNA with the 5′ end covalently linked to terminal protein (DNA-protein) and (*ii*) the formation of complex between the terminal protein and dAMP. The DNA-replication and the complex-forming activities were purified together through all steps. The complex of terminal protein and dAMP formed in the purified fraction was shown to serve as an effective primer for successive chain elongation in the presence of dNTPs by a pulse–chase experiment. The protein fraction purified from cells infected with a temperature-sensitive $\phi$29 mutant in gene 3 was thermolabile compared to the wild-type activity in the assay system for complex formation. This shows that the purified fraction having replication activity includes the gene 3 product of $\phi$29. Both the DNA replication and the complex formation activities are highly specific for $\phi$29 DNA-protein as template. The product analysis of elongated DNA revealed that the replication starts at both termini of the $\phi$29 genome. These results are consistent with the basic elements of the protein-priming model for the initiation of linear DNA synthesis.**

Recently, the genomes of the small *Bacillus* phages became of considerable interest because they provide unique systems to study the "protein-priming" mechanism for the initiation of linear DNA replication (1). We have developed an *in vitro* DNA replication system for phage $\phi$29 (2). Cell-free extracts prepared from $\phi$29-infected *Bacillus subtilis* catalyze the initiation and the semiconservative replication of the $\phi$29 genome. This cell-free system also catalyzes the formation of a covalent complex between $\phi$29 terminal protein and dAMP, the 5′-terminal nucleotide of the $\phi$29 genome (3). Our *in vitro* $\phi$29 DNA replication system specifically requires $\phi$29 DNA covalently linked to a 30-kilodalton (kDa) protein at each 5′ terminus ($\phi$29 DNA-protein) as template, the four dNTPs, and $Mg^{2+}$ (2, 3). A similar cell-free system has recently been described independently by others (4).

The $\phi$29 genome is a linear duplex DNA molecule of 18,000 base pairs possessing a 30-kDa terminal protein covalently linked to the 5′ terminus (1). The $\phi$29 DNA terminal protein is encoded by gene 3 and is essential for the initiation of $\phi$29 DNA replication (5). Studies on $\phi$29 DNA replication *in vivo* have shown that the initiation takes place at either end of the linear DNA molecule and proceeds in the 5′-to-3′ direction by an asymmetric strand-displacement mechanism (6, 7). The development of an *in vitro* DNA replication system facilitated the functional characterization of the $\phi$29 DNA terminal protein (3, 4) and will enable the factors involved in $\phi$29 DNA replication to be purified and their functions to be determined.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Ever since it was discovered that DNA polymerases are incapable of initiating the synthesis of new chains, replication of linear DNA molecules has been an intriguing problem (8, 9). Although various models have been proposed for replication of linear DNA molecules, such as adenovirus DNA (10) and $\phi$29 DNA (6, 7), the protein-priming model proposed by Rekosh *et al.* (11) is most consistent with the experimental results described to date (2, 7). In this model, the terminal protein binds covalently with the first 5′ nucleotide and thus provides the 3′-OH end that can be used as primer for subsequent chain elongation by DNA polymerase (11).

In the present communication, we describe the isolation and characterization of a protein fraction that catalyzes both terminal protein-dAMP complex formation and subsequent chain elongation reaction. Our results verify conclusively the protein-priming mechanism for the initiation of $\phi$29 DNA replication.

## MATERIALS AND METHODS

**Materials.** Most of the preparations of cell extracts, phage $\phi$29, $\phi$29 DNA-protein, and $\phi$29 DNA have been described (2). Phage $\phi$29 K132 (*ts3*) has been described (12) and was obtained from M. Salas. *N*-Ethylmaleimide and 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) were purchased from Sigma, and denatured calf thymus DNA-cellulose was from P-L Biochemicals. $N^2$-(*p*-*n*-Butylphenyl)guanine and immune serum against *B. subtilis* DNA polymerase III were kindly provided by N. Brown.

**Assay for DNA Synthesis.** The conditions of the DNA synthesis assay were the same as described in the previous paper (2) with slight modification. The reaction mixture (50 $\mu$l) contained 50 mM Tris·HCl, pH 7.6; 10 mM $MgCl_2$; 1 mM ATP; 40 $\mu$M each of dATP, dCTP, dGTP, and [*methyl*-$^3$H]dTTP (600–800 cpm/pmol); 1 mM spermidine·3HCl; 1 mM dithiothreitol; 5% (vol/vol) glycerol; 25 $\mu$g of bovine serum albumin; 0.8 $\mu$g (2.5 nmol of nucleotide residues) of $\phi$29 DNA-protein; and 3–6 $\mu$l of fractions at various stages of purification. The mixture was incubated at 30°C for 30 min unless otherwise stated. The DNA products were precipitated with trichloroacetic acid. The acid-insoluble materials were collected on GF/C glass fiber discs and their radioactivities were measured as described (2). One unit catalyzes the incorporation of 1 nmol of [$^3$H]dTMP in 30 min.

**Assay for Complex Formation of Terminal Protein-dAMP.** The conditions for complex formation were basically the same as described previously (3) with the following modifications. The standard reaction mixture (50 $\mu$l) contained 25 mM Hepes at pH 7.6, 2 mM ATP, 0.3 $\mu$M [$\alpha$-$^{32}$P]dATP (3,200 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ Bq), 60 mM $(NH_4)_2SO_4$, 25 $\mu$g of bovine serum albumin, 0.8 $\mu$g of $\phi$29 DNA-protein, and 3–6 $\mu$l of

Abbreviations: kDa, kilodalton(s); dd-, 2′,3′-dideoxy-; Ad-pTP, adenovirus terminal protein.

fraction at various purification steps. The mixture was incubated at 30°C for 25 min. The mixture was processed and electrophoresed through a sodium dodecyl sulfate/10% polyacrylamide gel as described (3). The band was cut from the gel and its radioactivity was measured with a liquid scintillation counter. One unit catalyzes incorporation of 1 pmol of [$^{32}$P]dAMP into terminal protein-dAMP complex in 25 min.

**Product Analysis of DNA.** For the analysis of DNA product, [$^{32}$P]dATP was used as a labeled nucleotide in the reaction mixture for DNA replication. The reaction was stopped by adding 1% sodium dodecyl sulfate and the mixture was treated with proteinase K (200 $\mu$g/ml) for 30 min at 37°C. The DNA was then extracted with phenol, precipitated with ethanol, and suspended in *Msp* I buffer. After digestion of the DNA by *Msp* I, it was electrophoresed through a 1.4% agarose gel in Tris/borate/EDTA buffer. The gels were dried and each band was cut out and its radioactivity was measured.

## RESULTS

**Isolation of the Replication Activity Associated with the Terminal Protein.** The enzymatic activity of terminal protein can be assayed by using a system to detect the formation of complex between the terminal protein and dAMP (3), the first 5′-terminal nucleotide of the $\phi$29 genome. During the course of the purification of the $\phi$29 terminal protein, we found that the fraction active in the formation of complex between the terminal protein and dAMP is also active in DNA replication. Thus, we have been able to isolate this DNA replication activity by using both assay systems: $\phi$29 DNA-synthesizing activity and complex-forming activity between the terminal protein and dAMP. Elution profiles for both the $\phi$29 DNA-synthesizing activity and terminal protein-dAMP complex-forming activity as well as total protein from a phosphocellulose column chromatography are shown in Fig. 1. As indicated, both activities have rather high affinity for phosphocellulose and are eluted with 0.5 M NaCl in the same fraction. In contrast, host DNA polymerase III was eluted near the void volume and host DNA polymerase II was eluted with 0.2 M NaCl (data not shown). Some residual amount of DNA-synthesizing activity using calf thymus DNA as template also coincided with the $\phi$29 DNA replication activity. Generally, the $\phi$29 DNA replication activity increased significantly after phosphocellulose column chromatography, suggesting the removal of some inhibitors or proteolytic enzyme activities. The results of a typical purification are summarized in Table 1. Throughout these purifications, both $\phi$29 DNA replication activity and complex-forming activity were copurified. The purified fraction was stable at 0°C for at least 2 weeks, although dilution of the fraction resulted in the rapid loss of activity.

**Thermosensitivity of the Complex-Forming Activity Purified from *B. subtilis* Infected with a Temperature-Sensitive Gene 3 Mutant.** Gene 3 of $\phi$29 encodes the terminal protein (1). To examine whether gene 3 protein is indeed responsible for the complex-forming activity in the purified fraction, we prepared the active fraction through phospho- and DNA-cellulose column chromatography from cells infected with $\phi$29 temperature-sensitive mutant *ts*3. As a control, the active fraction was also prepared from cells infected with $\phi$29 wild type. Fractions were incubated at various temperatures and were then examined for their ability to support the complex formation between the terminal protein and dAMP at 25°C. Fig. 2 shows temperature inactivation of complex-forming activity in the purified fraction prepared from cells infected with *ts*3 and wild-type $\phi$29, respectively. The fraction purified from the *ts*3-infected cells was far less stable at 35°C than that from wild-type-infected cells. These results indicate that protein labeled with dAMP is indeed the gene 3 protein, the same gene product previously shown to be the terminal protein of the $\phi$29 DNA-protein (14).

**Properties of the Purified Fraction.** The properties of the purified fraction V are shown in Table 2. Both DNA-synthesizing activity and complex-forming activity depended on the addition of $Mg^{2+}$ and $\phi$29 DNA-protein. ATP is not a necessary factor for this fraction.

The activities in both reactions are specific for template. The

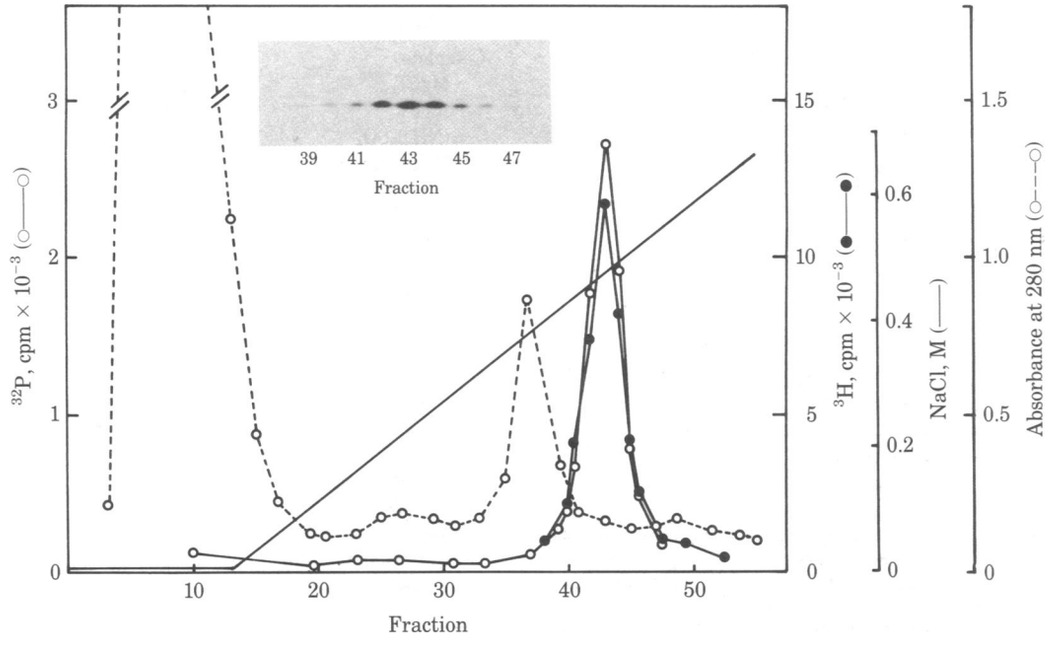


FIG. 1. Phosphocellulose chromatography of Fraction II. (*Inset*) Autoradiogram of product of complex formation between terminal protein and $^{32}$P-labeled dAMP. The band was cut and radioactivity was measured (○——○). ●——●, Replication activity for $\phi$29 dependent on DNA-protein as template.

Table 1. Purification of the protein with DNA-replication and complex-formation activity

| Fraction | Purification step | Total protein, mg | Complex-forming activity: Total units | Complex-forming activity: Specific activity (*A*), units/mg | Replication activity: Total units | Replication activity: Specific activity (*B*), units/mg | Ratio of *A* to *B* |
|---|---|---|---|---|---|---|---|
| I | Crude extract | 2,365 | — | — | — | — | — |
| II | $(NH_4)_2SO_4$ precipitate | 1,340 | 1,136 | 0.8 | 63.0 | 0.05 | — |
| III | Phosphocellulose | 171 | 1,732 | 10.1 | 36.6 | 0.21 | 48 |
| IV | DEAE-cellulose | 2.0 | 1,234 | 617 | 26.0 | 13.0 | 47 |
| V | Denatured DNA-cellulose | 0.9 | 472 | 514 | 7.8 | 8.6 | 59 |

A crude extract (fraction I) was prepared from a 12-liter culture of *B. subtilis* cells infected with $\phi$29 as described (2). After the extract had been passed through the DEAE-cellulose column, ammonium sulfate was added to 50% saturation and the resultant precipitate was suspended with 20 ml of buffer I [20 mM Tris·HCl, pH 7.0/20% (vol/vol) glycerol/5 mM $MgCl_2$/60 mM $(NH_4)_2SO_4$/1 mM 2-mercaptoethanol]. The suspension was dialyzed against the same buffer for 5 hr (fraction II, 23.5 ml). Fraction II was diluted by the same volume of buffer I and applied to a phosphocellulose column (100 ml) activated as described in ref. 13. The column was washed and eluted with a 600-ml linear gradient of NaCl (0–0.7 M) in buffer I. Fractions containing the activity (0.5 M NaCl) were pooled, adjusted to pH 8.0 with NaOH, and dialyzed against buffer II [20 mM Tris·HCl, pH 8.0/20% glycerol/5 mM $MgCl_2$/60 mM $(NH_4)_2SO_4$/1 mM dithiothreitol] for 3 hr (fraction III, 55 ml). Fraction III was applied to a DEAE-cellulose column (6 ml) equilibrated with buffer II. The column was washed and then eluted with a 30-ml linear gradient of NaCl (0–0.6 M) in buffer II. Active fractions (0.4–0.45 M NaCl) were pooled and dialyzed against buffer I for 3 hr (fraction IV, 4.4 ml). Fraction IV was applied to denatured DNA-cellulose column (1.2 ml) equilibrated with buffer I. The column was washed extensively with buffer I containing 0.2 M NaCl. The activity was eluted in a stepwise fashion with buffer I containing 0.7 M NaCl (fraction V, 1.4 ml).

$\phi$29 DNA-protein could not be replaced by deproteinized DNA even though the DNA was heat denatured. Single- and double-stranded DNA of the *Escherichia coli* phage M13 had no activity as template. Likewise coliphage $\phi$X174 DNA did not work as template (data not shown).

To examine if the whole genome structure of $\phi$29 DNA-protein is necessary, the DNA-protein was cleaved with *Bst*N-I, which cuts $\phi$29 DNA only once and generates two fragments, 15.3-kilobase-pair fragment A (right end) and 2.7-kilobase-pair fragment B (left end) (15). These fragments containing the terminal protein at one end were purified by sucrose gradient centrifugation and then tested for their template activity. Both fragments showed a significant amount of activity, though the efficiency of complex formation appeared to be lower than that of replication. These results suggest that the intact structure of the $\phi$29 DNA-protein may not be necessary for the template activity.

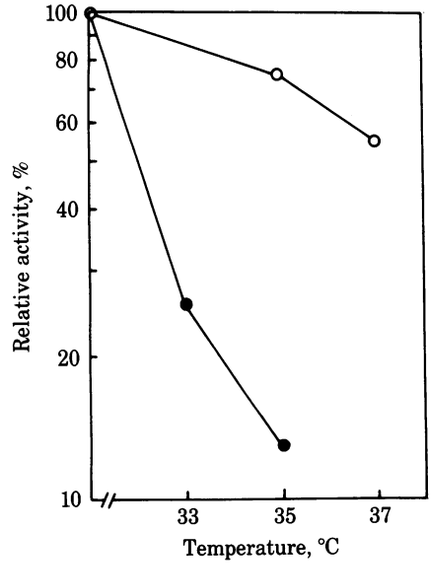


FIG. 2. Thermolability of gene *3* product purified from cells infected with a temperature-sensitive mutant. The activity for complex formation was purified through phospho- and DNA-cellulose chromatography as described in Table 1 from cells infected with $\phi$29 wild type or the temperature-sensitive mutant *ts3*. Each fraction was incubated at various temperatures for 10 min and then tested for its ability to support the formation of the complex of terminal protein and dAMP at 25°C. Actual radioactivity at 100% is 6,202 cpm for wild type (○) and 2,497 cpm for *ts*3 (●).

It was of considerable interest to determine whether DNA polymerase III of the host cell is involved in $\phi$29 DNA synthesis. Therefore, using the purified fraction, we have tested anti-DNA polymerase III serum and $N^2$-(*p-n*-butylphenyl)-guanine, a specific inhibitor of *B. subtilis* DNA polymerase III (16). Neither inhibited the activity, suggesting the DNA polymerase III of the host cell is not involved in $\phi$29 DNA replication *in vitro*.

**Can the $\phi$29 Terminal Protein-dAMP Complex Serve as a Primer for Replication?** The following experiments were designed to test the protein-primer model for the initiation of DNA

Table 2. Properties of the purified fraction

| Assay mixture | Complex formation: fmol | Complex formation: % | DNA replication: pmol | DNA replication: % |
|---|---|---|---|---|
| Complete | 2.0 | 100 | 11.2 | 100 |
| −$MgCl_2$ | 0.08 | 4 | 0.7 | 6 |
| −ATP | 0.88 | 44 | 12.1 | 107 |
| −$(NH_4)_2SO_4$ | 0.86 | 43 | — | — |
| −DNA-protein | 0.02 | 1 | 1.0 | 8 |
| −DNA-protein +DNA* | 0.1 | 5 | 2.0 | 17 |
| −DNA-protein +heat-denatured DNA* | 0.1 | 5 | — | — |
| −DNA-protein +M13 RF DNA | 0.04 | 2 | 0.8 | 7 |
| −DNA-protein +M13 ss DNA | 0.02 | 1 | 0.8 | 7 |
| −DNA-protein +*Bst*NI fragment A | 0.22 | 11 | 6.7 | 60 |
| −DNA-protein +*Bst*NI fragment B | 0.28 | 14 | 3.9 | 35 |
| +MalNEt (10 mM) | 1.2 | 60 | 5.4 | 48 |
| +BuPhGua (0.2 mM) | 2.6 | 130 | 10.7 | 95 |
| +Antiserum to DNA polymerase III | 1.9 | 95 | 9.7 | 80 |

RF, replicative form (double-stranded, closed circular); ss DNA, single-stranded DNA; MalNEt, *N*-ethylmaleimide; BuPhGua, $N^2$-(*p-n*-butylphenyl)guanine.

* Deproteinized $\phi$29 DNA was used as DNA template.

replication directly. The $\phi$29 DNA sequences are 5′-A-A-A-G-T-A-A-G-C (left end) and 5′-A-A-A-G-T-A-G-G-G-T-A-C (right end) (17, 18). Accordingly, if the terminal protein-dAMP complex serves as primer, the addition of chain-termination analogue ddGTP instead of dGTP in the reaction mixture for replication should result in the termination of chain elongation at the fourth nucleotide. Similarly, ddTTP should produce a complex between the terminal protein and pentanucleotides, and ddCTP should yield two species of complexes: terminal protein-nonanucleotide (left end) and terminal protein-dodecanucleotide (right end).

In the first stage, the terminal protein-dAMP complex was formed in the standard assay mixture for DNA replication in the absence of dNTPs with the purified protein fraction. Then, in the second stage, one ddNTP together with the remaining three dNTPs was added and the reaction mixture was further incubated. The formation of products was analyzed by acrylamide gel electrophoresis as shown in Fig. 3. When ddCTP was added in the second stage (lane 2), more than 90% of the terminal protein-dAMP complex was elongated into a 35-kDa product as expected. The 31.5-kDa product was obtained when ddGTP or ddTTP was used in the second stage (lanes 3 and 4). Moreover, we chased the elongation product for a short time (10–80 sec) after addition of dNTP (without ddNTP) in the second stage (lanes 5–8). When the time of second incubation was increased, the 30-kDa band was decreased and shifted to relatively high molecular mass position. As can be seen from Fig. 3, the amount of the high molecular mass band (35–40 kDa) increased with time. These results indicate that the complexes of terminal protein-dAMP formed in the purified protein fraction serve as primers for successive chain elongation of $\phi$29 DNA *in vitro*.

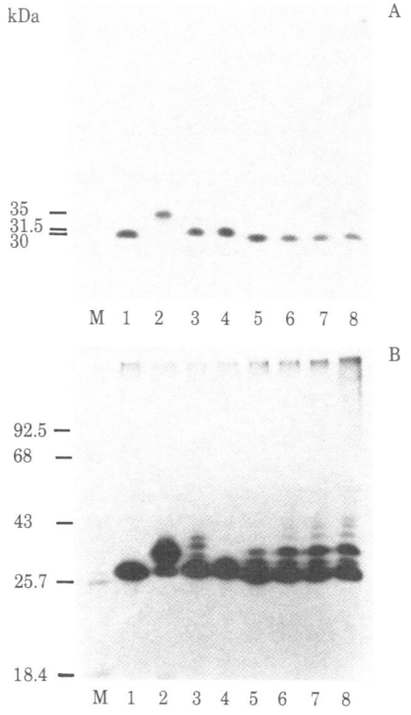


FIG. 3. Function of the terminal protein-dAMP complex as a primer for chain elongation. Purified fraction (3 $\mu$l) was incubated in the reaction mixture for DNA replication without dNTPs in the presence of 0.5 $\mu$M [$^{32}$P]dATP for 20 min at 30°C (stage I). Then 40 $\mu$M each dGTP, dTTP, and ddCTP and 0.8 nM dATP (lane 2); 40 $\mu$M each dCTP, dGTP, and ddTTP and 0.8 nM dATP (lane 3); or 40 $\mu$M each dCTP, dTTP, and ddGTP and 0.8 nM dATP (lane 4) were added in the reaction mixture. After the addition of 3 $\mu$l of fresh protein fraction, each reaction mixture was further incubated at 30°C for 30 min (stage II). Lane 1 is the control only for stage I. For the pulse–chase experiment without using a ddNTP, 40 $\mu$M each dGTP, dCTP, and dTTP, 0.8 nM dATP, and 3 $\mu$l of protein fraction were added in the reaction mixture after the reaction of stage I (lanes 5–8). Then, reaction mixtures were incubated at 25°C for 10 sec (lane 5), 20 sec (lane 6), 40 sec (lane 7), or 80 sec (lane 8), respectively. After the reaction of stage II, all the reaction mixtures were processed and subjected to sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis and autoradiographed for 2 hr (*A*) or 6 hr (*B*) with intensifying screen. Lane M shows the protein markers: phosphorylase *b* (92.5 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), $\alpha$-chymotrypsinogen (25.7 kDa), and $\beta$-lactoglobulin (18.4 kDa).

**DNA Product Synthesized in the Purified Fraction.** *In vivo* studies have shown that $\phi$29 replication starts from either end of the DNA and proceeds by a strand displacement mechanism (6, 7). The following experiments show that the system using the purified fraction has the same properties.

The mode of replication in the purified fraction was analyzed by restriction enzyme digestion followed by agarose gel electrophoresis. After a 20- or 40-min incubation of the reaction mixture, the DNA product was treated with proteinase K, extracted, and digested with *Msp* I. Fig. 4 shows the distribution of the radioactivity incorporated into the DNA fragment, which was cut out from an agarose gel and assayed for radioactivity. Radioactivity was incorporated predominantly into both of the termini. The mode of distribution of radioactivity was basically the same after 5-min incubation (data not shown). The rate of chain elongation was quite low after the initial 15–20%, although the reaction was linear even after 40 min. This is probably due to the fact that some factors required for elongation in the purified fraction are missing.

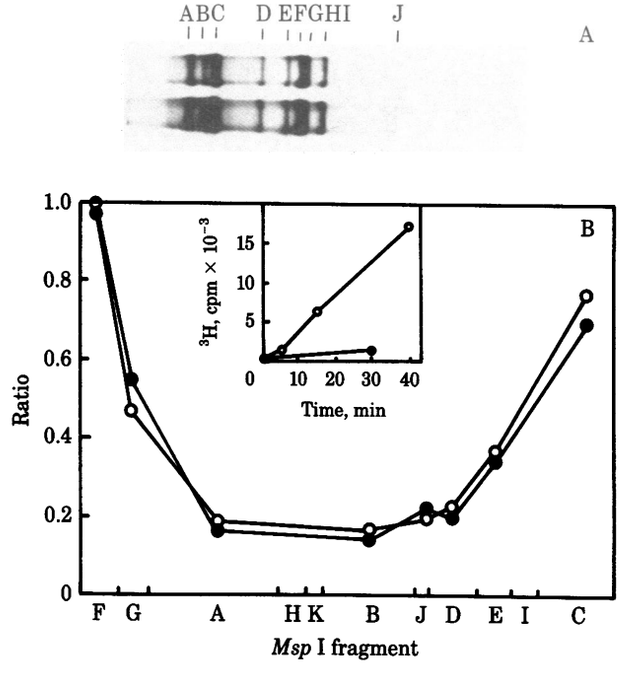


FIG. 4. Product analysis of DNA replication. After 20 min (upper lane) or 40 min (lower lane) of incubation of reaction mixture, DNA was treated with proteinase K and extracted with phenol. DNA was digested by *Msp* I and electrophoresed through a 1.4% agarose gel in Tris/borate/EDTA buffer for 5 hr at 5 V/cm. Gels were dried and autoradiographed (*A*). Each band was cut out and its radioactivity was measured in a liquid scintillation counter. The ratio of radioactivity to the DNA length of each fragment was plotted (*B*). The ratio of the fragment F was arbitrarily set as 1.0, and the other values were normalized to it. The abscissa represents the *Msp* I restriction map of $\phi$29 DNA. The actual radioactivity for fragment F was 49,333 cpm for the 20-min digestion (●) and 73,282 cpm for the 40-min digestion (○). (*Inset*) Kinetics of [$^3$H]dTTP incorporation with DNA-protein (○) or deproteinized DNA (●) as template.

## DISCUSSION

We have described the isolation of replication activity for $\phi$29 DNA from phage-infected cells. In all steps of purification, the activity of complex formation between terminal protein and dAMP is associated with the activity of DNA replication. The purified protein fraction prepared from cells infected with a temperature-sensitive mutant of gene 3 was heat labile, in contrast to a protein fraction from wild-type phage-infected cells. Moreover, immune serum against the terminal protein that was prepared from $\phi$29 DNA-protein inhibited the activity of purified fraction both in complex formation and in DNA replication (to be described elsewhere). These results indicate that the gene 3 product is included in the purified fraction and that the protein labeled with dAMP in the assay system for complex formation is indeed the terminal protein that is also necessary for DNA replication *in vitro*.

According to the protein-primer model, the first step of the replication is to make a covalent complex of terminal protein and dAMP (stage I). This complex should provide a 3′-OH for the subsequent chain elongation (stage II). Thus, one of the most direct pieces of evidence to prove the model is to show the coupling of the two reactions. Purified protein fraction catalyzed the reaction for both stages separately in each assay system. Moreover, pulse–chase experiments showed that most of the product of stage I (90%) was able to serve as a substrate for the stage II reaction. These results indicate that progeny terminal protein of $\phi$29 indeed functions as a primer for DNA replication.

Recently, Ikeda *et al.* (19) reported that when adenovirus terminal protein (Ad-pTP), prepared from adenovirus-infected HeLa cells, was incubated with $\phi$X174 single-stranded DNA, complex formation can take place between the Ad-pTP and dCMP, the terminal nucleotide of the adenovirus genome. More recently, Tamanoi *et al.* (20) reported that adenovirus DNA deproteinized by piperidine treatment and heat-denatured DNA treated with protease supported the covalent linking between the Ad-pTP and dCMP when partially purified enzyme fraction was used. Thus, they suggested the possibility that the newly synthesized terminal protein recognizes a specific sequence but not parental protein linked to the DNA. In contrast, neither $\phi$174 or M13 coliphage DNA nor heat-denatured $\phi$29 DNA treated with proteinase K was able to support the covalent linking between the $\phi$29 terminal protein and dAMP. Only the $\phi$29 DNA-protein complex isolated from the phage particle has been found to be active. Moreover, *in vivo* studies showed that mixed infection at a nonpermissive temperature with *ts2* (gene 2) and *ts3* (gene 3) mutants of $\phi$29 produce only *ts2* progeny (21). However, mixed infection with nonsense mutants of *sus2* and *sus3* in a nonpermissive host yielded both types of progeny. Thus, it is likely that the parental terminal protein bound to the template DNA is necessary for recognition by progeny terminal protein via protein–protein interaction.

Previous *in vivo* studies suggested that replication of the $\phi$29 DNA starts at both ends, not simultaneously, and occurs by a mechanism of strand displacement (6, 7). The data presented here obtained by analysis of the DNA product synthesized in the purified fraction are in good agreement with *in vivo* data. However, the rate of chain elongation is quite low. The radionucleotides incorporated into DNA are predominantly at both ends even after 40-min incubation of the reaction mixture (15–20% of the length at both ends). Possibly, one or more factors required for further elongation are missing from the purified fraction. This factor(s) appears to be missing already in the early stage of purification (fraction II), because the mode of replication was basically the same when fraction II was used for analysis of the DNA product (data not shown). A complementation assay to stimulate DNA replication should enable us to purify this unknown factor(s).

We are grateful to May-Fen Shih and Laura Weber for their invaluable assistance. We are also grateful to Drs. H. Bernstein, J. Little, and J. Duffy for critical reading of this manuscript. Excellent editorial assistance by Beth Byram and Carole Goodman are highly appreciated. This research was supported by National Institutes of Health Grant GM28013 to J.I., who is a Faculty Research Awardee of the American Cancer Society (FRA-165).

1. Geiduschek, E. P. & Ito, J. (1982) in *Molecular Biology of Bacilli*, ed. Dubnau, D. (Academic, New York), pp. 203–245.
2. Watabe, K., Shih, M., Sugino, A. & Ito, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5245–5248.
3. Shih, M., Watabe, K. & Ito, J. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1031–1036.
4. Peñalna, M. A. & Salas, M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5522–5526.
5. Mellado, R. P., Peñalna, M. A., Inciarte, M. R. & Salas, M. (1980) *Virology* **104**, 84–96.
6. Harding, N. E. & Ito, J. (1980) *Virology* **104**, 323–338.
7. Inciarte, M. R., Salas, M. & Sogo, J. M. (1980) *J. Virol.* **34**, 187–199.
8. Kornberg, A. (1980) *DNA Replication* (Freeman, San Francisco).
9. Watson, J. D. (1972) *Nature (London) New Biol.* **239**, 197–201.
10. Winnacker, E. L. (1978) *Cell* **14**, 761–773.
11. Rekosh, D. M. K., Russel, W. C., Bellett, A. J. D. & Robinson, A. J. (1977) *Cell* **11**, 283–295.
12. Mellado, R. P., Moreno, F., Vinuela, E., Salas, M., Reilly, B. E. & Anderson, D. L. (1976) *J. Virol.* **19**, 495–500.
13. Green, P. J., Betlach, M. C., Boyer, H. W. & Goodman, H. M. (1974) in *Methods in Molecular Biology*, ed. Weckner, R. (Dekker, New York), Vol. 9, pp. 87–111.
14. Hermoso, J. M. & Salas, M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6425–6428.
15. Yoshikawa, H. & Ito, J. (1982) *Gene* **17**, 323–335.
16. Wright, G. E., Baril, E. F., Brown, V. M. & Brown, N. C. (1982) *Nucleic Acids Res.* **10**, 4431–4440.
17. Yoshikawa, H., Friedman, T. & Ito, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1336–1340.
18. Escarmis, C. & Salas, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1446–1450.
19. Ikeda, J. E., Enomoto, T. & Hurwitz, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2442–2446.
20. Tamanoi, F. & Stillman, B. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2221–2225.
21. Salas, M., Mellado, R. P., Vinuela, E. & Sogo, J. M. (1978) *J. Mol. Biol.* **119**, 269–291.