JOURNAL OF VIROLOGY, Dec. 1978, p. 895-904
0022-538X/78/0028-0895$02.00/0

Vol. 28, No. 3
Printed in U.S.A.

Bacteriophage φ29 Terminal Protein: Its Association with the 5′ Termini of the φ29 Genome

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Received for publication 26 June 1978

The location of the protein bound to bacteriophage φ29 DNA has been studied with restriction endonucleases, exonucleases, and polynucleotide kinase. The protein is invariably associated with the two terminal DNA fragments generated by restriction endonucleases. The φ29 DNA prepared with or without proteinase K treatment is resistant to the action of the 5′-terminal-specific exonucleases, λ-exonuclease and T7 exonuclease. The φ29 DNA is also inaccessible to phosphorylation by polynucleotide kinase even after treatment with alkaline phosphatase. On the other hand, φ29 DNA is sensitive to exonuclease III, and the 3′ termini of the DNA can be labeled by incubating with α-[^{32}P]ATP and terminal deoxynucleotidyl transferase. The protein remains associated with the φ29 DNA after treatment with various chaotropic agents, including 8 M urea, 6 M guanidine-hydrochloride, 4 M sodium perchlorate, 2 M sodium thiocyanate, and 2 M LiCl. These results are consistent with the notion that the protein is linked covalently to the 5′ termini of the φ29 DNA.

The genomes of the small *Bacillus* phages, φ29 and φ15, are linear, nonpermuted duplex DNA of about 12×10^6 molecular weight (18,000 nucleotide pairs) (1-3, 20). A novel feature of these phage genomes isolated from either phage particles or infected cells is their tight association with protein (12, 17, 19, 30). Similar DNA-protein associations have been reported for other small *Bacillus* phages and for adenoviruses (4, 22, 35). It has been known for some time that the infectivity (transfectivity) of DNA isolated from small *Bacillus* phages is sensitive to proteolytic enzymes, suggesting that a DNA-associated protein is required for transfection (15). Treatment of the φ29 and φ15 DNA-protein complexes with the restriction endonucleases *Eco*RI and *Hpa*I has indicated that the protein may be associated with the terminal fragments (17-19).

We reported previously that a temperature-sensitive mutant in gene 3 contains a thermolabile transfecting DNA-protein complex (44). This suggested that the protein bound to the DNA may be the product or a part of the product of the φ29 gene 3. Recently it has been shown that the DNA-bound protein has a molecular weight of 27,000 to 31,000, is one of the early gene products of φ29, and is associated with both ends of the φ29 DNA (13, 37). Furthermore, Salas and colleagues have shown that tryptic peptides of the DNA-bound protein are very similar, if not identical, to those of the φ29 gene 3 protein (37). The gene 3 protein has been shown to be essential for φ29 DNA replication (11, 28, 29, 42). In this paper, further evidence is presented that the protein is linked covalently to the 5′ termini of the φ29 DNA. Salas et al. (37) have conducted similar studies and reached the same conclusions.

MATERIALS AND METHODS

Preparation of φ29 and ^{14}C-labeled φ29 phages. Bacteriophage φ29 was prepared by using *Bacillus amyloliquefaciens* H strain as host and was purified as described previously (19). ^{14}C-labeled φ29 was prepared as follows. *B. subtilis* SCR 114 (*spoA12 thyAthyB*) was grown to 5×10^7 cells per ml in 200 ml of Penassay broth. The cells were harvested by centrifugation and resuspended in 20 ml of phage adsorption medium (0.05 M Tris-hydrochloride, pH 7.4; 0.1 M NaCl; and 0.01 M $MgSO_4$). Phages were added at a multiplicity of 25, and the mixture was incubated at 37°C for 5 min without aeration. The phage-bacteria complex was diluted 10-fold with prewarmed Spizizen minimal medium containing 0.2% glucose, 1% casein acid hydrolysate, and [^{14}C]thymidine (1 μg/ml, 0.5 μCi/mmol), and shaken for 2 h at 37°C. The ^{14}C-labeled φ29 phage particles were collected by centrifugation and purified by discontinuous CsCl gradient centrifugation as described previously (20).

Preparation of φ29 DNA and DNA-protein complex. Purified phage particles (2×10^{12} PFU/ml) were treated with sodium dodecyl sulfate (SDS) (1%) and heated at 60°C for 2 min. For φ29 DNA preparation, the disrupted phages were treated with proteinase K (200 μg/ml) and extracted with phenol as de-

scribed previously (20). For the preparation of ϕ29 DNA-protein complex, the disrupted phages were shaken gently with phenol and centrifuged. The DNA-protein complex at the interface between the aqueous layer and phenol was removed, precipitated with ethanol, and then dissolved and dialyzed against 20 mM Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA.

Iodination of the ϕ29 DNA-protein complex with ^{125}I. Iodination catalyzed by insoluble lactoperoxidase was carried out at room temperature by the method of David (8). The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 50 μg of ϕ29 DNA-protein complex, 0.1% SDS, 10^{-5} M H_2O_2, 800 μCi of $Na^{125}I$, and 20 μl of the lactoperoxidase-Sepharose beads (a gift of R. McCabe) in a final volume of 200 μl. The reaction mixture was shaken for 40 min and centrifuged to remove Sepharose-bound enzyme. The supernatant was extensively dialyzed at room temperature and then at 4°C to remove free iodide. The labeled ϕ29 DNA-protein complex was purified by centrifugation through a 5 to 20% sucrose gradient. The peak fractions were pooled, dissolved, and dialyzed against 0.02 M Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA.

Enzymatic digestions. λ-Exonuclease was a gift from C. Radding and M. Rhoades. The reaction mixture (200 μl) contained 50 mM glycine-KOH (pH 9.8), 2 mM $MgCl_2$, 3 mM dithiothreitol, 0.05 to 0.1 mM ^{14}C-labeled ϕ29 DNA (expressed as nucleotide equivalent) or ^{14}C-labeled ϕ29 DNA-protein complex (74,000 cpm/μg of DNA) and λ-exonuclease (10 U) (27). Reaction mixtures (200 μl) for T7 exonuclease (a gift of P. D. Sadowski) contained 50 mM Tris-hydrochloride (pH 8.0), 5 mM $MgCl_2$, 20 mM KCl, 2 mM dithiothreitol, 0.05 to 0.1 mM ^{14}C-labeled ϕ29 DNA or ^{14}C-labeled ϕ29 DNA-protein complex, and 20 U of enzyme. The reaction mixture (200 μl) for exonuclease III (34) contained 60 mM Tris-hydrochloride (pH 8.0), 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.05 to 0.1 mM ^{14}C-labeled ϕ29 DNA or ^{14}C-labeled ϕ29 DNA-protein complex, and 10 U of enzyme (New England Biolabs, 16,000 U/mg of protein). All reactions were carried out at 37°C. Extent of hydrolysis was determined by measuring the production of acid-soluble nucleotides by the method of Guiney and Helinski (10).

Restriction endonucleases *Eco*RI and *Hpa*I were isolated as described previously (19). Other restriction endonucleases, *Hpa*II, *Hin*dIII, *Hae*II, *Hin*cII, *Xba*I, and *Pvu*I, were obtained from New England Biolabs. Enzymes *Bgl*I and *Bst*EII were purchased from Bethesda Research Laboratories. Reactions were carried out at 37°C in the following solutions: for *Eco*RI, *Xba*I, *Hpa*I, *Hin*cII, *Hha*I, *Hin*fI, *Hin*dII, *Hin*dIII, and *Pvu*II, 100 mM Tris-hydrochloride (pH 7.8), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol; for *Bgl*I, *Hpa*II, *Hae*II, and *Bst*EII, 10 mM Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol.

Dephosphorylation of ϕ29 DNA was carried out by incubation in mixtures (1 ml) containing 50 mM Tris-hydrochloride (pH 8.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 to 2.0 mM ϕ29 DNA or ϕ29 *Eco*RI fragments, and 5 U of alkaline phosphatase (Boehringer Mannheim) at 37°C for 60 min. The reaction mixture was extracted twice with buffer-saturated phenol, and the DNA was precipitated with ethanol, redissolved in 20 mM Tris-hydrochloride (pH 7.4) buffer containing 1 mM EDTA, and dialyzed against the same buffer.

In vitro 5'-end labeling. Phosphorylation of the ϕ29 DNA or ϕ29 DNA *Eco*RI fragments was performed according to the method of Richardson (33). The reaction mixture (200 μl) contained 0.1 M Tris-hydrochloride (pH 7.4), 15 mM $MgCl_2$, 20 mM dithiothreitol, 0.45 to 3.3 mM ϕ29 DNA or ϕ29 *Eco*RI fragments, 3.4 μM γ-[^{32}P]ATP (1,000 to 3,000 Ci/mmol) and 42 U of T4 polynucleotide kinase per ml (PL Biochemicals, Inc.). The reaction was carried out at 30°C for 60 min. Unreacted ^{32}P was removed from the labeled DNA by extensive dialysis at 4°C against repeated changes of buffer containing 0.02 M Tris-hydrochloride (pH 7.4), 0.1 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 1 mM EDTA.

In vitro 3'-end labeling. The 3' terminus was labeled as described by Roychoudbury et al. (36). The reaction mixture (200 μl) contained 1 mM $CoCl_2$, 48 mM cacodylate buffer (pH 6.8), 1.5 mM ϕ29 DNA, 9 μM α-[^{32}P]ATP (203 Ci/mmol), and 110 U of terminal deoxynucleotidyl transferase (PL Biochemicals, Inc.). Incubations were at 37°C for 60 min. The reaction mixtures were then dialyzed extensively against the buffer described above.

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out as described previously (19, 22).

Sucrose gradient centrifugation. Sucrose gradients were prepared at room temperature by layering successively 0.9 ml of 20, 15, 10, and 5% sucrose solutions in 50 mM Tris-hydrochloride (pH 7.4), 0.1 M NaCl, 10 mM EDTA, and 0.1% SDS approximately 15 h before use. Samples (50 to 100 μl) were layered on the gradient and centrifuged in an SW56 rotor at 25°C. After centrifugation, 10-drop fractions were collected on glass filters which were then dried, and the radioactivity was determined (19).

RESULTS

We have shown recently that a 31,000-molecular-weight protein is attached to the two terminal *Eco*RI fragments (A and C) of the ϕ29 DNA (13). However, the *Eco*RI-A and -C fragments comprise approximately 50 and 10% of the ϕ29 genome, respectively (17, 19). To identify the location of the protein more precisely on the genome, we have determined other restriction endonuclease cleavage sites on the ϕ29 DNA. The methods for mapping the DNA fragments generated with several restriction endonucleases involved the analysis of partially digested DNA fragments and the analysis of overlapping sets of DNA fragments produced by cleavage with two different restriction endonucleases. Figure 1 summarizes the cleavage maps of the ϕ29 genome. A detailed description of the mapping of the DNA fragments will be published elsewhere (J. Ito, manuscript in preparation).

It has been observed previously that DNA fragments associated with the protein either do

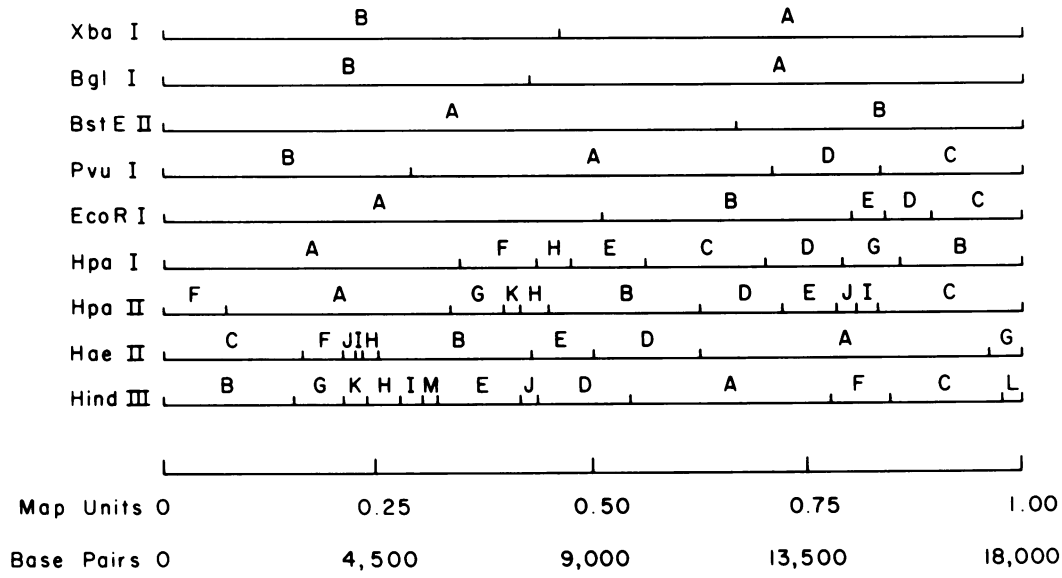

FIG. 1. *Physical maps of φ29 DNA obtained after cleavage with XbaI, BglI, Bst EII, PvuI, EcoR1, HpaI, HpaII, HaeII, and HindIII restriction endonucleases. The fragments were named alphabetically in order of decreasing size. The location of the HindIII-N fragment is not known. The EcoRI cleavage sites have been reported previously (19).*

not migrate or migrate abnormally during electrophoresis (17–19, 41). An example of this is shown in Fig. 2, which compares electrophoretic patterns of the *Hin*dIII digestion products of the φ29 DNA and the φ29 DNA-protein complex. It can be seen that the *Hin*dIII-B and -L fragments are missing from the tracks that received digests of the φ29 DNA-protein complexes. With each of the restriction endonucleases (*Hpa*II, *Hae*II, *Hinc*II, *Hin*fI, *Hha*I, and *Pvu*I), we observed that two end fragments obtained from the φ29 DNA-protein complex were either missing or altered in their mobility during electrophoresis in agarose gel (data not shown). The *Hin*dIII-L fragment contains about 200 base pairs. Thus, the 31,000-molecular-weight protein is bound within 200 nucleotides of the end of the φ29 DNA molecule.

Susceptibility of φ29 DNA to exonuclease. To study further the location of the protein on φ29 DNA, we used specific exonucleases. λ-Exonuclease is known to initiate hydrolysis of double-stranded DNA specifically from the 5′ terminus (27), whereas exonuclease III from *Escherichia coli* initiates hydrolysis at the 3′ end of the native DNA (34). It might be expected

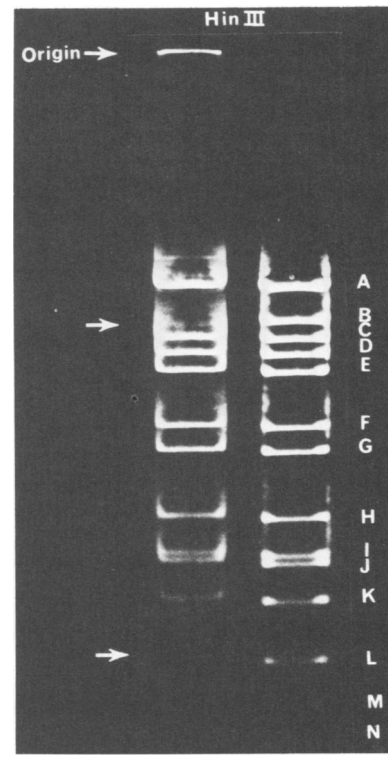

FIG. 2. *Agarose gel electrophoresis of φ29 DNA and φ29 DNA-protein complex digested with restriction endonuclease HindIII. Digested materials (1 μg of DNA) in 10% glycerol and 0.01% bromophenol blue were heated for 2 min at 60°C before application to 1% agarose slab gels. The arrows indicate terminal fragments which are missing in the digest of the φ29 DNA-protein complex.*

that protein bound at or near the 3′ or 5′ termini of the φ29 DNA interferes with hydrolysis of the DNA by one of these exonucleases. φ29 DNA prepared with or without proteinase K was incubated with either λ-exonuclease or exonuclease III, and the release of acid-soluble radioactivity was measured. The results are shown in Fig. 3. In these experiments φ29 DNA or φ29 DNA-protein complexes treated with *Eco*RI were also incubated with one or two exonucleases. The *Eco*RI digests serve as the controls. It is clear from Fig. 3A that neither φ29 DNA nor φ29 DNA-protein complex is sensitive to the λ-exonuclease, whereas *Eco*RI digests are sensitive. Figure 3B, on the other hand, shows that both φ29 DNA and DNA-protein complexes are sensitive to the exonuclease III, although somewhat less so than other DNAs such as φ3T DNA (data not shown). With increased enzyme concentration, it was observed that the digestion of both the DNA and the DNA-protein complex reached the theoretical limit of 50%. These results suggest that the 3′ termini of the φ29 DNA are free but the 5′ termini are blocked. To confirm that the 5′ termini are blocked, DNA samples treated with λ-exonuclease were analyzed by agarose gel electrophoresis. Figure 4 shows the time course for the λ-exonuclease digestion of φ29 DNA that has been treated with *Eco*RI. Fragments B, D, and E were rapidly degraded, but two distinct bands remained. Separate experiments with purified and denatured *Eco*RI-A, -B, and -C fragments have indicated that the remaining bands are derived from the *Eco*RI-A and -C fragments, respectively (data not shown). It is most likely that these bands consist of the single strands of the *Eco*RI-A and -C fragments. Similar results were obtained after treatment with T7 exonuclease which can initiate hydrolysis of double-stranded DNA specifically from either the 5′-phosphate or 5′-hydroxyl end group (23). These results clearly indicate that the 5′ termini of the φ29 DNA are blocked.

Accessibility of the φ29 DNA to phosphorylation. The study with exonucleases indicated that the protein may be attached at or near the 5′ termini of the φ29 DNA. To determine whether the protein is bound to the very ends of the φ29 DNA molecule, 5′-terminal labeling experiments were carried out with γ-[^{32}P]ATP and T4 polynucleotide kinase. Figure 5 shows the sedimentation patterns of the phos-

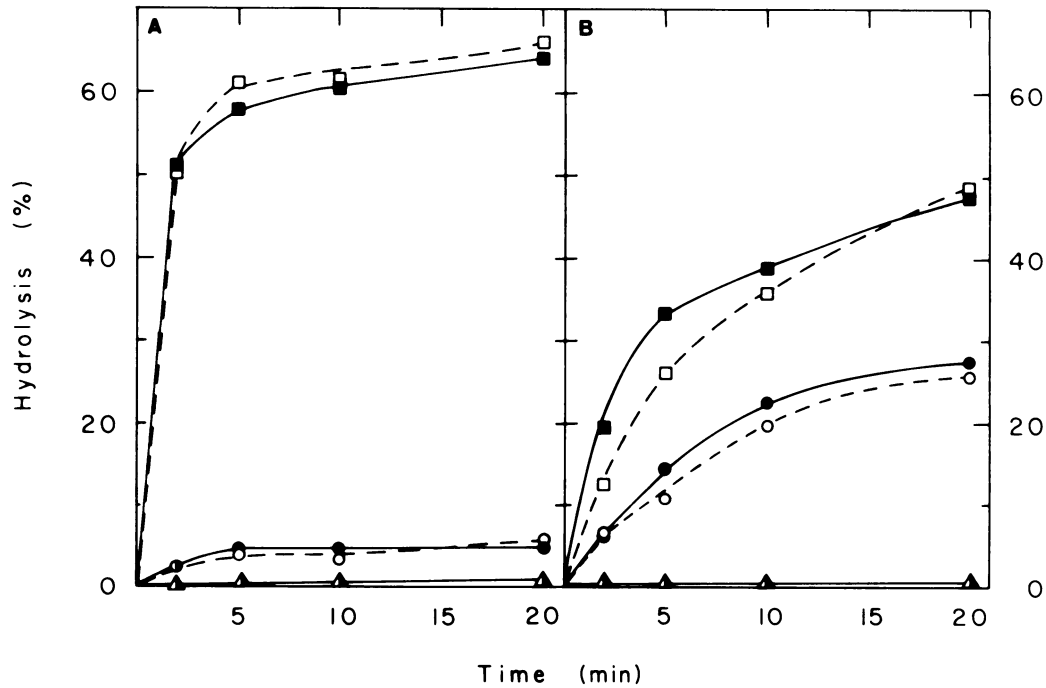

FIG. 3. *Hydrolysis of φ29 DNA and φ29 DNA-protein complex with λ-exonuclease and exonuclease III. λ-Exonuclease (A) and exonuclease III (B) digestion of φ29 DNA (●), DNA-protein complex (○), EcoRI-treated φ29 DNA (■), EcoRI-treated DNA-protein complex (□), EcoRI-treated φ29 DNA with no exonuclease (▲), EcoRI-treated φ29 DNA-protein complex with no exonuclease (△). EcoRI treatment of DNA samples was carried out in the exonuclease reaction mixture (200 μl) with 2 U of enzyme by incubating for 30 min at 37°C before the addition of the exonuclease.*

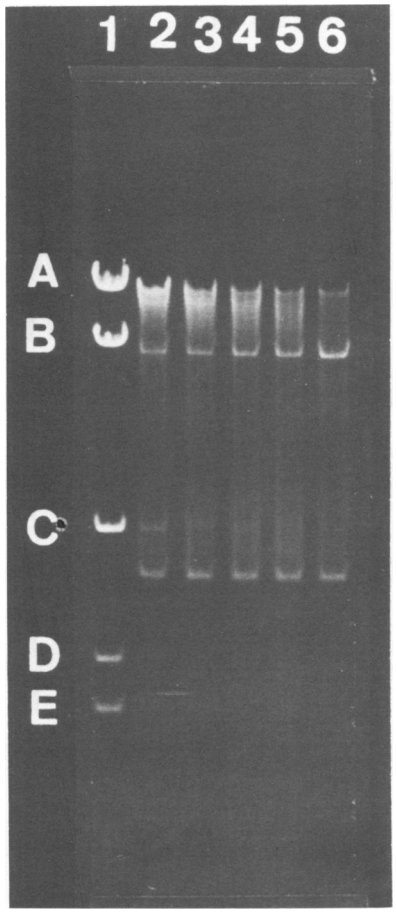

FIG. 4. *Time course of λ-exonuclease digestion of φ29 DNA EcoRI fragments. The reaction mixture consisted of 50 mM glycine-KOH (pH 9.8), 2 mM $MgCl_2$, 3 mM dithiothreitol, 5 μg of the EcoRI fragments of φ29 DNA, and 3 μl of λ-exonuclease (a gift of Rhoades) in 200 μl. Incubation was carried out at 37°C. At different times, 20 μl of the reaction mixture was withdrawn, mixed with EDTA (final 10 mM), and heated for 5 min at 65°C. The mixtures were then adjusted to 10% glycerol and 0.01% bromophenol blue and were applied to a 1% agarose slab gel. After electrophoresis, the gels were stained and photographed as described previously (19). Lane 1, zero time; lane 2, 0.5-min digest; lane 3, 1-min digest; lane 4, 2-min digest; lane 5, 5-min digest; and lane 6, 10-min digest.*

phorylated φ29 DNA and *Eco*RI-A fragment in neutral sucrose gradients. Under the conditions used, the φ29 sediments at 23S, and the *Eco*RI-A fragment sediments at 18S (19). φ29 DNA prepared with proteinase K treatment incorporated very little ^{32}P (less than 2% of the expected value). Treatment of φ29 DNA with alkaline phosphatase before incubation with γ-[^{32}P]ATP and polynucleotide kinase stimulated additional ^{32}P incorporation, although instead of the expected 100-fold stimulation, an increase of only 4-fold was observed. Under the same conditions, dephosphorylated φ29 DNA *Eco*RI-B fragments were quantitatively labeled (Table 1). It was also found that the *Eco*RI-A fragment was labeled about 50% of the expected value, indicating that one of the 5′ termini of the *Eco*RI-A fragments

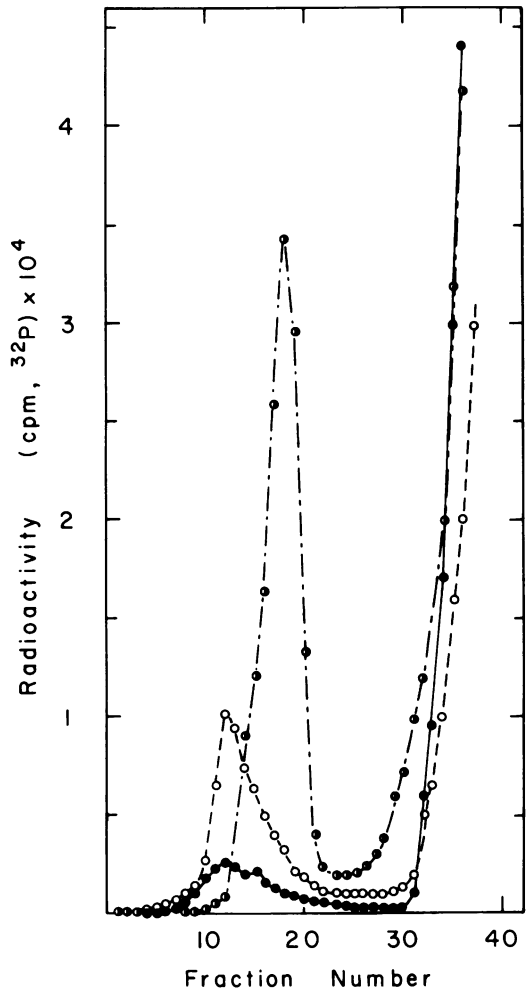

FIG. 5. *Sucrose gradient sedimentation of 5′-terminal-labeled φ29 DNAs and EcoRI-A fragment. DNA samples were phosphorylated with γ-[^{32}P]ATP and polynucleotide kinase as described in the text. Samples (50 μl) were applied to 5 to 20% sucrose gradients and centrifuged in an SW56 rotor at 45,000 rpm for 130 min at 25°C. This figure is the composite of three separate tubes. 5′-Terminal-labeled φ29 DNA (●), 5′-terminal-labeled φ29 DNA treated with alkaline phosphatase before labeling with polynucleotide kinase (○) and 5′-terminal-labeled EcoRI-A fragment treated with phosphatase before labeling with polynucleotide kinase (◑).*

may not be accessible to phosphorylation. These results suggest that most of the 5′ termini of the φ29 DNA are not accessible to phosphorylation by polynucleotide kinase even after extensive treatment with proteinase K and alkaline phosphatase.

3′-Terminal labeling of the φ29 DNA. To confirm that the φ29 DNA molecules have a free 3′-OH group, 3′-terminal labeling experiments were performed with α-[^{32}P]ATP and terminal deoxynucleotidyl transferase. It has been shown recently that the 3′-terminal ends of the double-stranded DNA molecules can be labeled by terminal transferase with Co^{2+} instead of Mg^{2+} (36). Native φ29 DNA prepared with proteinase K treatment was incubated with α-[^{32}P]ATP and terminal transferase as described above. The 3′-terminally labeled φ29 DNA was then cleaved with *Eco*RI and was subjected to sucrose gradient sedimentation to see whether the ends of the φ29 DNA were labeled specifically. It can be seen from Fig. 6 that only terminal fragments *Eco*RI-A and -C were labeled. This indicates that the φ29 DNA contains unblocked 3′-OH groups.

TABLE 1. *Phosphorylation of φ29 DNA and the EcoRI-A and -B fragments*

DNA source	Alkaline phosphatase treatment	^{32}P incorporated (mol/mol of DNA)[a]
φ29 DNA	−	0.02
	+	0.08
φ29 *Eco*RI-A fragment	+	0.95
φ29 *Eco*RI-B fragment	+	1.90

[a] Expressed as moles of ^{32}P incorporated per mole of double-stranded equivalent DNA nucleotide.

Stability of the φ29 DNA-protein association. Because the φ29 DNA-protein complex contains only 0.5% protein by weight (12), it is difficult to obtain sufficient amounts of the radioactive complexes by labeling in vivo. Accordingly, we labeled the protein-DNA complexes in the protein moiety with ^{125}I by using the solid-phase lactoperoxidase method (8).

Using the ^{125}I-labeled DNA-protein complex,

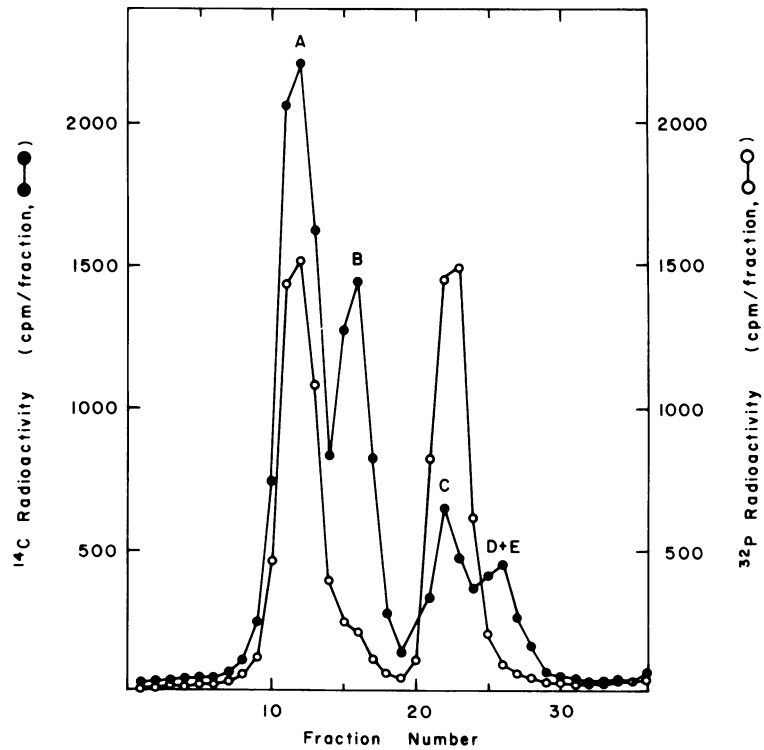

FIG. 6. *Zone sedimentation profiles of the EcoRI digestion fragments of the 3′-terminal-labeled φ29 DNA. φ29 DNA was labeled by incubation with α-[^{32}P]ATP and terminal transferase as described in the text. The labeled DNA was then cleaved with EcoRI and mixed with EcoRI fragments of ^{14}C-labeled φ29 DNA. The mixture was applied to a 5 to 20% sucrose gradient and centrifuged in an SW50 rotor at 45,000 rpm for 3.5 h at 20°C. ^{32}P-3′-terminal-labeled DNA digested with EcoRI (○). ^{14}C-labeled φ29 DNA fragments generated by EcoRI digestion (●).*

we tested the stability of the complex to various chaotropic agents and conditions which affect ionic and hydrophobic associations. These include: 8 M urea, 6 M guanidine-hydrochloride, 4 M $NaClO_4$, 2 M NaSCN, 2 M $LiCl_2$, 0.2 M KCl-HCl (pH 2.0), 0.1 M Na_3PO_4-NaOH (pH 11.5), and 0.2 M KCl-NaOH (pH 12.4), all at 30°C for 40 min. The treated complexes were analyzed by either sedimentation through neutral sucrose gradients or by CsCl band centrifugation. None of these treatments were able to dissociate the ϕ29 DNA-protein complex. Furthermore, thermal denaturation of the ϕ29 DNA-protein complex did not dissociate the protein from the DNA. Treatment of the complex with 3.75 M hydroxylamine (pH 4.5) at 32°C for 1 h also failed to release the label from the DNA. Dissociation of the protein from the DNA could be achieved, however, by treatment of the complex with 1 M NaOH (Fig. 7). When subjected to a sucrose gradient centrifugation (5 to 20%) at 45,000 rpm in an SW56 rotor for 15 h, the ^{125}I-protein component sedimented in a single peak at approximately 3S, suggesting that the dissociation was not due to degradation of the protein by the strong alkali (data not shown).

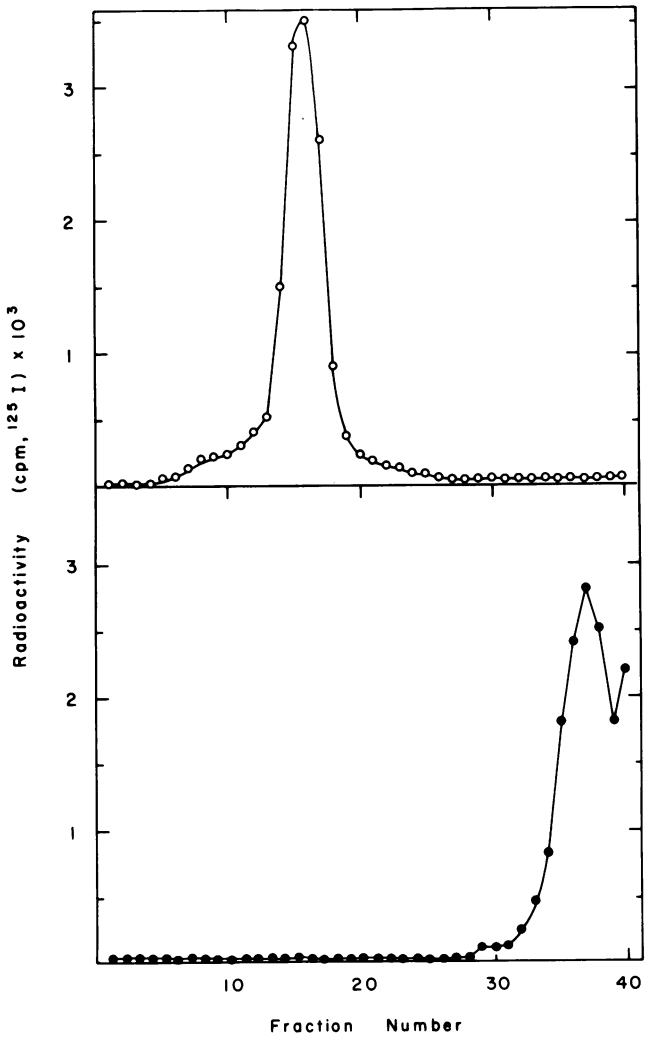

FIG. 7. *Effect of 1 M NaOH treatment on the sucrose gradient centrifugation of ^{125}I-labeled protein-DNA complex. ^{125}I-labeled ϕ29 DNA-protein complex was incubated with 1 M NaOH for 30 min at 37°C and then neutralized with HCl. The sample and an untreated control were then centrifuged at 25°C in sucrose gradients (5 to 20%) for 120 min at 45,000 rpm in an SW56 rotor. ϕ29 DNA-protein complex* (○). *ϕ29 DNA-protein complex treated with NaOH* (●).

DISCUSSION

In the present communication, evidence is presented that the 5′ termini of the φ29 DNA are structurally modified. As the result of this modification, the φ29 DNA is resistant to the 5′-terminal-specific exonucleases, λ-exonuclease and T7 exonuclease. In addition, we showed that the 5′ termini of the φ29 genome are inaccessible to phosphorylation by polynucleotide kinase. On the other hand, φ29 DNA prepared with or without proteinase K treatment is sensitive to hydrolysis by 3′-terminal-specific exonuclease III. Furthermore, the 3′ termini of the φ29 DNA can be labeled by incubating with α-[^{32}P]ATP and terminal deoxynucleotidyl transferase. Salas et al. (37) have also shown that the 5′ termini of the φ29 are not accessible to phosphorylation and that the φ29 DNA is sensitive to exonuclease III. The 5′-blocking group of φ29 DNA cannot be removed by extensive treatment with proteinase K and alkaline phosphatase. The fact that none of the chaotropic agents tested was capable of destroying the φ29 DNA-protein association suggests that the terminal protein is linked covalently to φ29 DNA. Thermal denaturation of the φ29 DNA-protein complex also failed to dissociate the protein from the DNA.

We have observed previously that φ29 DNA fragments associated with protein either do not migrate into agarose gels or migrate abnormally during electrophoresis. However, after heating in the presence of 2% SDS, the *Eco*RI-A fragment could migrate into agarose gels during electrophoresis. Therefore, we suggested that protein bound to the *Eco*RI-A fragment of φ29 may be removed by such treatment (12, 19). Subsequently, we found that DNA-protein complex treated with 2% SDS can migrate into agarose gels during electrophoresis. Larsen and Nathans (25) have made a similar observation on a mouse adenovirus DNA-protein complex. They have shown that when the agarose gel contained 0.5% SDS and the sample had been treated with 2% SDS, the DNA fragment associated with protein migrated into the agarose gel (25). Our present study with ^{125}I-labeled φ29 DNA-protein complex has clearly shown that the terminal protein is not dissociated from either end fragment by heating in the presence of 2% SDS (13).

Although the presence of some unusual 5′-terminal blocking group could not be ruled out at present, it is highly likely that an amino acid residue or a short peptide remaining from the hydrolysis of φ29 DNA-protein complex may be the 5′-terminal blocking group. Therefore, our results as well as those of Salas et al. (37) strongly argue that the terminal proteins are linked covalently to the 5′-termini of the φ29 DNA. The φ29 DNA-protein association is, however, sensitive to strong alkali treatment. This is analogous to the adenovirus 2 DNA-protein association (6). This alkali sensitivity may indicate that the terminal protein is linked to the 5′ terminus by a phosphoester bond rather than a phosphoamide bond, because the latter is generally stable in alkali (40). The fact that the φ29 DNA-protein complex is resistant to the treatment with NH_2OH at low pH tends to support this view. However, it is known that the stability of phosphoamide bond is affected by adjacent nucleotide sequences and peptide length (40). Thus, the precise linkage between the DNA and the terminal protein will require a direct chemical analysis of the complex.

It is interesting to note that all small *Bacillus* phages studied contain DNA-protein complexes, regardless of their relatedness to φ29 (4, 18, 20, 22, 31). The infectivity of the DNA-protein complexes is sensitive to proteolytic enzymes. Recently there has been an increasing number of articles describing the covalent association between proteins and nucleic acids. These include: ColE1 DNA-protein relaxation complex (14), φX174 RFI DNA-A protein complex (16), human and mouse adenovirus DNA-protein complexes (5, 25, 32, 35), simian virus 40 DNA-protein complex (21), poliovirus RNA-protein complex (9, 26), and foot-and-mouth disease RNA-protein complex (38). Most of these studies have shown that the protein is linked to the 5′ terminus of the genome and have suggested that the protein is involved somehow in nucleic acid replication.

Evidence from our laboratory and others has suggested that the protein bound to the φ29 DNA is coded by an early gene and may be the product or a part of the product of the gene 3 of φ29 (12, 44). More recently, Salas et al. (37) have shown that the major tryptic peptides of the terminal protein are very similar to those of the φ29 gene 3 protein. It is known that gene 3 is involved in DNA replication of φ29 (11, 29, 39, 42) and that normal gene 3 protein is required continuously for replication of this viral DNA (28, 42, 44). Thus, these studies suggest that the terminal proteins are directly involved in DNA replication. An essential role for DNA terminal protein in the replication of linear, nonredundant DNA molecules may be of general significance. The results obtained with φ29 resemble those reported for human adenovirus types 2 and 5 (5, 32, 35). The chromosomes of these viruses also contain linear nonredundant DNA and terminal protein. Although several models have been proposed (7, 32, 43), the replication mechanism or mechanisms of such linear nonredundant DNA molecules are completely ob-

scure at the present time (24). Our preliminary analysis of the replicating ϕ29 DNA molecules by electron microscopy has revealed two major types of intermediates (unpublished data). One class is branched, Y-shaped molecules containing one single- and one double-stranded arm, and the other class is unbranched, linear DNA molecules containing single-stranded stretches. These observations suggest that ϕ29 DNA replication may start at or near the molecular ends of the viral DNA by displacement synthesis and complementary strand synthesis. Further studies should reveal information concerning the functional role of the terminal protein in DNA replication.

ACKNOWLEDGMENTS

I thank Charles Radding and Marc Rhoades for the generous gift of λ-exonuclease, P. D. Sadowski for T7 exonuclease, and R. McCabe for lactoperoxidase. I am grateful to Nancy Harding, James Cregg, Garry Henderson, and Charles Bieger for helpful discussions. I also thank Barbara Indseth and Aida Lucero for their excellent assistance. The editorial assistance of Patricia Murphy is greatly appreciated.

This research was supported in part by Public Health Service grant GM24824 from the National Institute of General Medical Sciences and by American Cancer Society grant NP-203A. I am the recipient of a Faculty Research Award from the American Cancer Society.

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