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Genome-Linked Protein Associated with the 5′ Termini of Bacteriophage φ29 DNA

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A DNA-protein complex was isolated from *Bacillus subtilis* bacteriophage φ29 by sucrose gradient sedimentation or gel filtration in the presence of agents known to break noncovalent bonds. A 28,000-dalton protein was released from this complex by subsequent hydrolysis of the DNA. The DNA-protein complex was examined for its susceptibility to enzymes which act upon the 5′ and 3′ termini of DNA molecules. It was susceptible to exonucleolytic degradation from the 3′ termini by exonuclease III but not from the 5′ termini by λ exonuclease. Attempts to label radioactively the 5′ termini by phosphorylation with T4 polynucleotide kinase were unsuccessful despite prior treatment with alkaline phosphatase or phosphatase treatment of denatured DNA. Removal of the majority of the bound protein by proteolytic digestion did not increase susceptibility. These results suggest that the linked protein is covalently attached to the 5′ termini of φ29 DNA.

φ29 is a small *Bacillus subtilis* phage containing an infectious linear DNA molecule (1, 36). Its genome also occurs as a noncovalently closed circle due to interaction between protein molecules tightly attached to the ends of the DNA (22, 23, 33). DNA-associated protein is required for transfection (19). Transfecting DNA-protein from φ29*ts*3(132), a mutant for early protein p3 which is defective in DNA synthesis under nonpermissive conditions (50), is thermolabile (55). This indicates that protein p3 is associated with the infectious DNA isolated from phage particles. Harding and Ito (16) have shown that the DNA-bound protein is an early gene product by isolating the complex from cells blocked in the synthesis of late proteins (3, 7). Little is known about the association between the protein and the φ29 chromosome or its role in the production of mature phage particles.

Nucleic acid-protein complexes occur in a variety of biological systems. A DNA-linked protein has been observed in adenoviruses (40) and found to be covalently bound to the 5′ terminus of each DNA strand (9, 37). Covalent attachment of a protein to simian virus 40 DNA was detected by Kasamatsu and Wu (25). Relaxed colicin E1 (ColE1) plasmid DNA has a protein joined to the 5′ terminus of a single-stranded nick located at or near the origin of replication (14). Recently, Lee et al. (27) and Flanegan et al. (12) reported a similar linkage between a protein and the 5′ terminus of poliovirion RNA. Although the function of the DNA-protein complex in most systems is unknown, reduction of negative superhelical turns in DNA by *Escherichia coli* omega protein is quite well characterized. It involves the transient formation of a link between the protein and the 5′ terminus of a transient single-stranded nick (11). The product of the *nalA* gene in *E. coli* has been purified and appears responsible for a nicking-closing activity required for the introduction of supercoils by DNA gyrase (13, 48). Detergent treatment of a complex formed between *nalA* gene product and DNA results in a double-stranded break in the DNA which becomes covalently linked to the protein. Although the nucleic acid-protein associations in all of these cases have similarities, these proteins are involved in a variety of biological functions. A clearer understanding of this association may disclose that a number of nucleic acid metabolic processes have a mechanism in common.

This study focuses on the biochemistry of the association between φ29 DNA and this tightly bound protein. The protein freed from the DNA-protein complex after digestion of the DNA is similar in size to the φ29 gene 3 protein. Evidence is provided that the protein is covalently linked to the 5′ termini of the DNA molecule.

MATERIALS AND METHODS

Phage and bacteria. φ29 stocks were prepared by infection of *Bacillus amyloliquefaciens* H and titrated on *B. subtilis* 12A. Phage for the preparation of radioactively labeled DNA using [^{3}H]thymidine were prepared with *B. subtilis* SCR114 (*spoA12 thyA thyB*) obtained from B. E. Reilly.

Media. TY broth (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 0.2% glucose and 5 mM $MgCl_2$ was used for the preparation of phage stocks. Phages were titrated on TY broth containing 0.6% agar over a base layer of 1.0% tryptone–0.5% NaCl–1.5% agar. Other experiments were conducted with a minimal salts medium composed of 0.1 M Tris-hydrochloride, 0.08 M NaCl, 0.02 M KCl, 0.02 M $(NH_4)_2SO_4$, 1.0 mM KH_2PO_4, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM $MnCl_2$ (final pH adjusted to 7.3) supplemented with 0.5% glucose, 0.05% Casamino Acids (Difco), and 50 μg of tryptophan per ml.

Preparation of radioactively labeled φ29. For the preparation of phage containing labeled DNA, SCR114 was grown in supplemented minimal medium containing 5 μg of thymidine per ml. Cells at a concentration of 2×10^8/ml were infected with a multiplicity of infection of 10 to 20, and [^{3}H]thymidine (New England Nuclear Corp.; 13 Ci/mmol) was added at 1 μCi/ml. ^{32}P-labeled phage were prepared in the same medium containing $^{32}PO_4$ (New England Nuclear Corp.; carrier-free) at a concentration of 1 μCi/ml. Resulting lysates were treated with RNase (2 μg/ml) and DNase (1 μg/ml) at 37°C for 1 h, and the phage particles were precipitated with polyethylene glycol (54). After centrifugation, phage pellets were suspended in 50 mM Tris-hydrochloride (pH 7.5)–0.1 M NaCl–0.01 M $MgCl_2$ (TMS) and purified by centrifugation through CsCl step gradients consisting of 0.5 ml each of CsCl solutions of densities of 1.54, 1.46, 1.39, 1.31, 1.24, and 1.15 g/cm^3, respectively, in 50 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$. Centrifugation in a Spinco SW39 or SW50.1 rotor was at 23,000 rpm for 3 h at 20°C. Phage bands were collected by piercing the bottom of the tubes and dialyzed successively against TMS plus 1 M NaCl and then TMS.

Cells used for the preparation of amino acid-labeled phage were grown in the minimal medium supplemented with 0.5% glucose, 0.02% Casamino Acids, and 50 μg of tryptophan per ml. Cultures were infected as above and made either 100 μCi/ml in [^{35}S]methionine (New England Nuclear Corp.; 580 Ci/mmol) plus 2 μg/ml in methionine or 50 μCi/ml in ^{14}C-amino acids (Schwarz/Mann, reconstituted protein hydrolysate, 180 mCi/mmol). Incorporation of label was linear for a period of 60 min, after which cell lysis began. Lysates were treated and phage were prepared as described above.

Preparation of phage DNA-protein. Phage suspensions were made 20 mM in EDTA–1% 2-mercaptoethanol–1% sodium lauryl sarcosinate (SLS) and 6 M in guanidinium chloride. After 2 h at 45°C, solutions were subjected to fractionation by either filtration on Sepharose CL or sedimentation in sucrose-guanidinium chloride gradients. Disrupted phage were loaded on a column (0.9 by 28 cm) of Sepharose CL-4B equilibrated with 50 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl–1 mM EDTA (TES) containing 6 M guanidinium chloride and 0.1% SLS. Fractions (0.5 ml) were collected upon elution with the same buffer, and those containing DNA were pooled and dialyzed versus TES containing 2 M urea and then TES alone. DNA was precipitated with 2 volumes of cold ethanol and collected by centrifugation at 27,000 × *g* for 20 min. Precipitates were dissolved in 20 mM Tris-hydrochloride (pH 7.5)–20 mM NaCl–0.1 mM EDTA and dialyzed against the same.

Alternatively, disrupted phage were layered onto 5-ml linear 5 to 20% (wt/wt) sucrose gradients in TES plus 4 M guanidinium chloride, 1.0% SLS, and 1.0% 2-mercaptoethanol. After centrifugation at 40,000 rpm and 20°C for 3 h in an SW50.1 rotor, 200-μl fractions were collected from the bottom of the tube. Suitable portions were diluted with water to prevent subsequent guanidinium chloride precipitation and then assayed for acid-precipitable radioactivity.

Where indicated, purified phage DNA was treated with Pronase (100 μg/ml, predigested at 37°C for 30 min) or proteinase K (50 μg/ml) at 37°C for 60 min, then extracted with phenol previously saturated with 0.1 M sodium borate (pH 9.0), and precipitated with 2 volumes of cold ethanol. Precipitated DNA was collected by centrifugation at 15,000 rpm for 20 min in a Sorvall refrigerated centrifuge and dissolved as above. DNA was denatured by heating at 100°C for 5 min followed by quick cooling at 0°C. T7 [^{3}H]DNA was the gift of G. Vovis.

Endonuclease R·*Eco*RI digestion. DNA preparations in 20 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$ were treated with 1 U of endonuclease R·*Eco*RI per μg of DNA at 37°C for 2 h.

Exonuclease assays. Reaction conditions for exonuclease III were described by Richardson et al. (39). The assay for λ exonuclease was that described by Little et al. (29). Reaction mixtures (100 μl) were spotted on 2-cm squares of Whatman DE81 paper, which were then washed sequentially for 30 min in 0.35 M ammonium formate, 0.3 M ammonium bicarbonate, and ether to remove mononucleotide products from remaining polynucleotide. Dried squares were counted by liquid scintillation spectrometry.

Phosphorylation with polynucleotide kinase. DNA and DNA restriction fragments were treated with alkaline phosphatase and phosphorylated with T4 polynucleotide kinase as described by Subramanian et al. (46); 1 μg of DNA in 100 μl of 5 mM Tris-hydrochloride (pH 7.5)–5 mM $MgCl_2$ was treated with 0.2 μg of alkaline phosphatase at 37°C for 1 h. The reaction was stopped by mixing with 1 volume of phenol for 20 min. Because φ29 DNA is largely lost at the interface during conventional phenol extraction, excess phenol in this case was removed by several extractions with ether. After ether was removed under a stream of N_2, the sample was dried in vacuo. The phosphatase-treated DNA was then dissolved in 100 μl of a mixture containing 10 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μCi of [γ-^{32}P]ATP per 1 mmol, and 2 U of kinase. The reaction mixture was incubated at 37°C for 2 h. Whole genome samples were incubated with 200 U of kinase per ml at 15°C for 16 h to obtain maximum labeling of the large DNA molecules (9). [γ-^{32}P]ATP (1,000 to 3,000 Ci/mmol) was the gift of J. Ravetch or purchased from New England Nuclear Corp. The labeled DNA was separated from unreacted [γ-^{32}P]ATP by gel filtration on a Sephadex G-50 column. The kinase-treated DNA eluted in the void volume and was recovered by ethanol precipitation.

Agarose gel electrophoresis. DNA and DNA restriction endonuclease fragments were subjected to

slab gel electrophoresis in 1.0 or 1.2% agarose in 40 mM Tris-acetate-20 mM acetate-2 mM EDTA, pH 7.7 (20). Separation was accomplished at 40 V for 2 to 3 h; gels were stained with ethidium bromide (0.5 μg/ml) for 15 min and photographed under UV light. Gels containing radioactively labeled fragments were subjected to autoradiographic or fluorographic analysis (6). After separated band positions were located from the film, segments containing the labeled components were excised from the gels with a razor for quantitative estimation. Residual electrophoresis buffer was removed from each segment by washing twice with 10-ml portions of methanol for 1 h. Agarose segments were then placed in 10-ml portions of liquid scintillation fluid {4 g of 2,5-diphenyloxazole per liter and 0.1 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene in toluene per liter; New England Nuclear Corp.} and counted after fluid had permeated the gel (1 to 2 h). Counting efficiency for ^{3}H and ^{32}P was equal to that observed for samples precipitated with trichloroacetic acid and collected on Whatman GF/C glass fiber filters.

Polyacrylamide gel electrophoresis. Proteins and DNA-protein complexes obtained as described above were subjected to analysis by discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (26). A slab gel apparatus was used, and separation gels contained a 10 to 18% exponential polyacrylamide gradient. Gels were run at 15-mA constant current for 4 to 5 h, fixed in 50% trichloroacetic acid, stained for 30 min in 0.1% Coomassie brilliant blue dissolved in 30% methanol plus 7% acetic acid, and then destained overnight with 30% methanol-7% acetic acid. They were subsequently treated for autoradiographic or fluorographic analysis.

Enzymes. T4 polynucleotide kinase was purified from T4 *amN*82-infected cells by the method of Richardson (38) as modified by Panet et al. (34) through the phosphocellulose step. Specific activity was 42,000 U/mg. Bacterial alkaline phosphatase and micrococcal nuclease were purchased from Worthington Biochemicals Corp. Restriction endonuclease R·*Eco*RI was obtained from Miles Laboratories, Inc., and *E. coli* exonuclease III was obtained from New England Biolabs. Lambda exonuclease was the generous gift of J. Boeke. Pronase was purchased from Sigma Chemical Co., and proteinase K was obtained from EM Laboratories.

RESULTS

Isolation of DNA-protein complex from φ29 virion. The φ29 DNA was isolated as a DNA-protein complex from phage particles after disruption with SLS-EDTA in guanidinium chloride and separated from other structural proteins by sedimentation in sucrose gradients containing 4 M guanidinium chloride or gel filtration on Sepharose CL as described above. An example of the separation by sucrose gradient sedimentation is shown in Fig. 1. Virion proteins labeled with [^{35}S]methionine were predominantly obtained in the fractions at the top of the gradient. However, a small fraction (<0.3%) of the sulfur label sedimented with the ^{3}H label of the phage DNA.

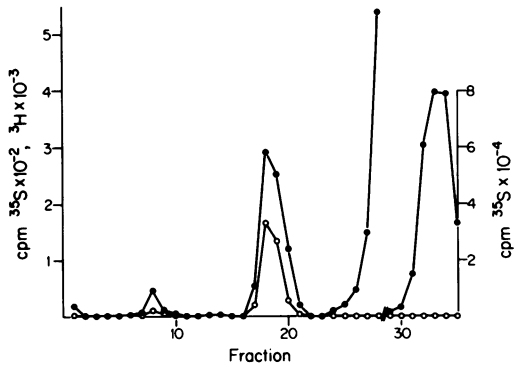

FIG. 1. *Sucrose-guanidinium chloride gradient separation of φ29 DNA-protein complex. A mixture of [^{35}S]methionine-labeled (3 × 10^{5} cpm) and [^{3}H]-thymidine-labeled (5.7 × 10^{3} cpm) phage was treated with guanidinium chloride and sedimented into sucrose-guanidinium chloride gradients as described in the text. Symbols: ●, ^{35}S radioactivity; ○, ^{3}H radioactivity. The scale on the right side refers to fractions 29 through 35.*

This label remained associated with the phage DNA upon subsequent treatment of the DNA-protein complex with high salt, 6 M urea, 4 M $NaClO_4$, or 4 M NH_2OH but was eliminated by incubation with proteinase K.

Polyacrylamide gel analysis of the protein found in the complex. As shown previously, DNA from the φ29 DNA-protein complex fails to migrate into 1% agarose gels unless the complex is treated with proteolytic enzymes (22, 23). It is presumed that the native DNA-protein complex forms large aggregates due to protein-protein interactions. The amino acid-labeled DNA-protein complex was recovered from sucrose-guanidinium chloride gradients and treated to digest the DNA component. It was then subjected to analysis by electrophoresis in discontinuous polyacrylamide gels. Figure 2 is an autoradiograph of a gel containing a sample of the DNA-protein complex which had been digested with micrococcal nuclease to free the protein component. Untreated samples (lane 1) show that when the protein remained associated with the phage DNA, it was confined to the stacking gel. After nuclease digestion (lane 2), a single polypeptide was found to migrate in a position slightly slower than that of the capsid fiber protein. Nuclease-treated (lane 4) and untreated (lane 3) virion proteins served as molecular weight standards and showed that the nuclease treatment was not accompanied by detectable proteolytic action.

Susceptibility of φ29 DNA to exonucleo-

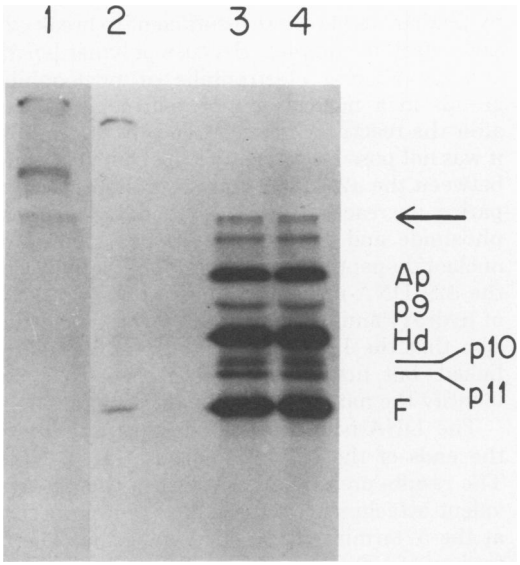

FIG. 2. *Liberation of DNA-bound protein by nuclease digestion. Sucrose gradient fractions as described in the legend to Fig. 1 containing DNA-protein complex from [^{35}S]methionine-labeled phage were pooled, dialyzed, and precipitated as described in the text. Portions dissolved in 50 mM Tris-hydrochloride (pH 8.6)–5 mM $CaCl_2$ were treated with micrococcal nuclease at 37°C for 20 min where indicated. Virion proteins were those obtained from fractions at the top of the gradient, dialyzed and concentrated by precipitation with trichloroacetic acid. (1) DNA-protein complex untreated; (2) DNA-protein complex treated with 1.5 U of micrococcal nuclease; (3) virion proteins untreated; (4) virion proteins treated with 150 U of micrococcal nuclease. The arrow indicates the position of the interface between stacking and separation gels. Virion proteins are designated by the convention of Reilly et al. (35).*

lytic degration. φ29 [^{3}H]DNA was incubated with *E. coli* exonuclease III or λ exonuclease to measure the protection of the terminal DNA sequences by the DNA-associated protein. Exonuclease III specifically degrades double-stranded DNA from 3′-phosphoryl or 3′-hydroxyl termini to a maximum of 50% acid solubility (39). Alternately, λ exonuclease hydrolyzes these same DNA preparations from the 5′ termini (28). Figure 3 shows that approximately half of the φ29 DNA was readily hydrolyzed by the action of the exonuclease III. However, under conditions which produced extensive hydrolysis of T7 [^{3}H]DNA by λ exonuclease, no appreciable degradation of φ29 [^{3}H]DNA occurred. In addition, prior treatment of the φ29 DNA with Pronase or proteinase K failed to render the molecule sensitive to the exonucleolytic activity of λ exonuclease. These results show that the terminal regions of the DNA molecule are specifically blocked at the 5′ termini. This was further examined by measuring the susceptibility of the 5′ termini of φ29 DNA to phosphorylation with T4 kinase.

Susceptibility of φ29 DNA to phosphorylation. φ29 DNA treated previously with alkaline phosphatase was incubated with T4 polynucleotide kinase and [γ-^{32}P]ATP in attempts to label its 5′ termini. Under conditions which labeled 80 to 90% of the available termini of T7 DNA, φ29 DNA incorporated less than 5% of the label expected of a molecule with reactive 5′ termini (Table 1). [^{3}H]DNA was used in these experiments to quantitate the DNA recovery. Neither prior treatment of the DNA with proteolytic enzymes nor denaturation led to an appreciable increase in the efficiency of labeling. Subsequent cleavage of this phosphorylated DNA with endonuclease R·*Eco*RI and separation of the resulting fragments on agarose gels showed that the low level of label was evenly distributed throughout the genome and, therefore, did not

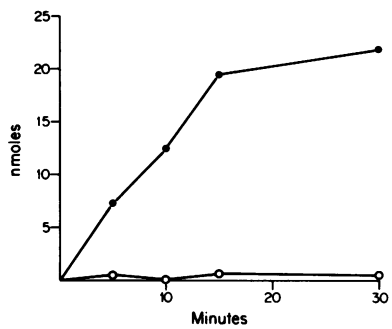

FIG. 3. *Susceptibility of φ29 DNA-protein complex to exonucleolytic digestion. Reaction mixtures (1.2 ml) under standard assay conditions contained 39 nmoles of φ29 [^{3}H]DNA (1,500 cpm/nmol) and either 27 U of exonuclease III or 3 U of λ exonuclease per ml. At indicated times, 0.1-ml portions were removed for determination of mononucleotides released. Symbols: ●, exonuclease III; ○, λ exonuclease.*

TABLE 1. *Phosphorylation of φ29 DNA*

DNA[a]	Treatment	mol of ^{32}P incorporated per mol of DNA
T7 [^{3}H]DNA		1.74
φ29 [^{3}H]DNA		0.04
φ29 [^{3}H]DNA	Proteinase K	0.06
φ29 [^{3}H]DNA	Proteinase K and denaturation	0.07

[a] Either 2.1 μg of T7 [^{3}H]DNA (3.7×10^4 cpm/μg) or 11.7 μg of φ29 [^{3}H]DNA (4.7×10^3 cpm/μg) was treated with alkaline phosphatase and T4 kinase as described in the text.

occur primarily at the ends of unit-length DNA molecules.

If the 5′ termini of ϕ29 DNA were blocked, then restriction endonuclease fragments of the ends of the molecule should incorporate only half as much label as internal restriction fragments. Endonuclease R·*Eco*RI restriction fragments of ϕ29 DNA which had been treated with proteinase K were phosphorylated with T4 kinase, and molar incorporation of ^{32}P into each fragment was measured. Table 2 shows that the internal restriction fragments B, D, and E (22, 23) incorporated approximately twice as much label per mole of fragment as did the terminal fragments A and C. Therefore, each terminal fragment has one blocked 5′ end. These results support the conclusion that the 5′ termini of the ϕ29 genome are specifically blocked from access by enzymes acting at these positions.

DISCUSSION

The DNA of ϕ29 can be isolated with an associated protein which appears to be covalently attached to it. The DNA-protein complex isolated in these studies is unusually stable; high concentrations of salt, urea, guanidinium chloride, or boiling in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, known to dissociate noncovalently bound proteins from nucleic acids, does not separate these components. Salas et al. (41) have recently isolated the ϕ29 complex by using different methods and have reached the same conclusion. These results differ from an earlier report of Harding and Ito (16) which indicates that the protein associated with the *Eco*RI-A terminal fragment of ϕ29 DNA can be removed by heating in the presence of sodium dodecyl sulfate, but agree with a recent report of Harding et al. (17) that terminal protein is firmly bound at both ends of the DNA molecule. Conditions which cleave certain synthetic nucleotide-peptide covalent complexes discussed by Shabarova (44) were insufficient to break ϕ29 DNA-protein complex. Because polymer length and neighboring electrophilic or nucleophilic groups in a nucleic acid-protein complex can alter the reactivity of such bonds to hydrolysis, it was not possible to identify the chemical bond between the ϕ29 DNA and its protein by comparing its reactivity to the reactivity of phosphoamide and phosphoester bonds studied in nucleotide-peptide complexes. The stability of the ϕ29 DNA-protein complex in the presence of hydroxylamine strongly supports the conclusion that the DNA and protein are covalently linked, but further analyses are necessary to identify the nature of the chemical bond.

TABLE 2. *Phosphorylation of ϕ29 DNA restriction fragments*[a]

*Eco*RI fragment	mol of ^{32}P incorporated per mol of DNA
A	0.87
B	1.73
C	0.83
D	1.79
E	1.68

[a] Protease-treated ϕ29 [^{3}H]DNA (11.7 μg) was digested with endonuclease R·*Eco*RI and subjected to the polynucleotide kinase labeling regimen with [γ-^{32}P]ATP at 5 μCi/mmol. The concentration of each restriction fragment was corrected for varying thymidine content by comparison with the same fragment from ϕ29 [^{32}P]DNA.

The DNA-bound protein is associated with the ends of the ϕ29 chromosome (22, 23, 33). The results presented here suggest that the covalent attachment of the linked protein occurs at the 5′ termini of the DNA molecule. The 5′ termini of ϕ29 DNA are specifically inaccessible to the degradative action of λ exonuclease or phosphorylation by sequential treatment with phosphatase and kinase (Fig. 3 and Table 1). Essentially all of the DNA molecules prepared from ϕ29 contain the same structure, as judged by the nearly total resistance of the 5′ termini to these treatments coupled with the complete susceptibility of the 3′ ends to attack by exonuclease III. Furthermore, denatured DNA remains inaccessible to labeling by polynucleotide kinase, suggesting that an unusual folded structure of the native DNA molecule is not a likely explanation for the protection of the termini. Proteolytic digestion of the complex which releases more than 95% of the bound protein does not expose the termini. It is probable that a residual oligopeptide fragment or even a single amino acid remains attached to the ends of the molecule to maintain the block. When the DNA-protein complex is treated with restriction endonuclease R·*Eco*RI, label incorporated into the terminal fragments in a subsequent kinase reaction is only half of that in the internal fragments (Table 2). Salas et al. (41) have reported a similar resistance of ϕ29 DNA to labeling with kinase and sensitivity to exonuclease III.

Until the ϕ29 DNA-linked protein is shown to be the exclusive blocking agent, alternate structures should be considered. Many chemical modifications, such as methylation of a 5′-hydroxyl group, would suffice to block the action of kinase and λ exonuclease. Most eucaryotic cellular and viral mRNA molecules and the RNA genomes of some viruses contain "capping" groups (45) which prevent labeling of their 5′ termini in polynucleotide kinase reactions.

The protein linked to ϕ29 DNA is probably the product of gene 3. Upon treatment of the

φ29 DNA-protein complex isolated by methods described here with micrococcal nuclease, a single protein was liberated which migrated as a 28,000-dalton polypeptide during polyacrylamide gel electrophoresis (Fig. 2). Harding et al. (17) have obtained from a φ29 DNA-protein complex isolated by sucrose gradient centrifugation and labeled in vitro with ^{125}I a protein with an average molecular weight of 31,000 and occasionally one of 10,000 to 13,000 daltons. The 31,000-dalton protein migrated as a diffuse band relative to the other phage structural proteins upon polyacrylamide gel electrophoresis. The broad molecular weight distribution may reflect the presence of heterogeneous oligonucleotide fragments which were not completely removed by DNase I digestion, as has been suggested in studies of the adenovirus DNA complex (37). The φ29 DNA-protein complex isolated by Salas et al. (41) contained a 27,000-molecular-weight protein. This protein compares in size to a protein(s) which is synthesized early after infection (3, 18, 32) and found to be coded by cistron 3 (3, 32). Two proteins, AF and BF, with molecular weights of 28,700 and 28,300, respectively, were found missing in cells infected with suppressor-sensitive gene 3 mutants by Anderson and Reilly (3) and their associates (18). The capsid fiber protein is the only other φ29 protein similar in size to gp3 (3, 8, 18) and which could be present in a small residual fraction in the complex. This is unlikely because the complex isolated here resisted dissociation; only treatment with a nuclease succeeded in freeing the protein. Additionally, tryptic peptide analyses indicated that the linked protein differs from the head or fiber protein and compares most closely with the gene 3 protein (41).

In accord with its proposed identity as the polypeptide component of the DNA-protein complex, gene 3 protein is required for φ29 DNA replication. Conditional lethal mutants of gene 3 are deficient in DNA synthesis under nonpermissive conditions (7, 15, 32, 43, 50). Yanofsky et al. (55) have shown that mutant *ts*3(132) and the transfectivity of its DNA are thermolabile, although McGuire et al. (31) did not observe increased thermosensitivity of *ts*3(28) virions over that of wild-type φ29. The temperature sensitivity of *ts*3(28) is apparently reversible. Protein gp3 is required continuously for DNA replication, since synthesis was found to stop immediately after shifting cultures infected with *ts*3 mutants from permissive to nonpermissive temperatures (31, 55). The gene 3 protein, therefore, seems to be a component of the phage particle and associated with the transfecting DNA-protein complex, providing a common function required for the formation of infectious centers and mature virions after infection or transfection.

Several other nucleic acid-linked proteins which appear to be involved in nucleic acid metabolism have been studied. The site-nonspecific proteins exhibit transient formation of DNA-protein complexes and are involved in nicking and closing activities which occur during replication or genetic recombination. *E. coli* ω protein, which interacts with negative supercoiled DNA to reduce the number of superhelical turns, was recently shown to form a complex with single-stranded DNA (11). When this complex is treated with alkali, the DNA is cleaved and the protein appears to be covalently linked to the DNA 5′ terminus. Another protein, the product of the *nalA* gene in *E. coli,* is associated with DNA gyrase, an enzyme which introduces negative superhelical turns into covalently closed circular DNA (13, 48). This protein subunit appears to catalyze the transient breaking and rejoining of DNA phosphodiester bonds. Treatment of the DNA-*nalA* gene product complex with detergent results in cleavage of the DNA and formation of an apparent covalent linkage between the DNA and protein.

In other cases the interaction of protein and nucleic acid is associated with a specific site on the nucleic acid molecule. The proximity of these sites to the origin of replication has suggested that these proteins may function in the initiation of replication. One or more of three proteins associated with the supercoiled DNA of the ColE1 relaxation complex acts as a strand-specific and site-specific endonuclease in which a single protein remains bound in an apparent covalent linkage to the 5′ terminus at the single-stranded break after induced conversion of the supercoiled DNA to the open circular state (5, 14, 30). The site of the nick is in close approximation to the origin of the ColE1 DNA replication, and it has been suggested that the protein components of the relaxation complex are involved in ColE1 DNA synthesis (5, 14). Similarly, the adenovirus DNA-protein complex described by Robinson et al. (40) contains protein covalently bound to the 5′ terminus of each DNA strand (9, 37) which is proposed to function in DNA synthesis as discussed below. Another instance of a DNA-protein complex has been reported in simian virus 40 (25), in which protein is covalently attached to DNA molecules at single-stranded nicks in either of the two strands but within close approximation to one another and located near the origin of replication and transcription. Lee et al. (27) and Flanegan et al. (12) recently found a protein covalently linked to the 5′ terminus of poliovirion RNA and its replicative intermediates, suggesting that it may

function in the initiation of RNA synthesis.

Constraints are placed on possible mechanisms for the replication of linear chromosomes. If DNA replication occurs by discontinuous synthesis primed by ribooligonucleotides (47), subsequent removal of the RNA primer at the ends of a linear molecule would result in a daughter duplex with a single-stranded 3′ terminus. Watson (51) has proposed that the formation of concatemers by terminally redundant linear DNA molecules during replication provides a mechanism by which the 5′-terminal nucleotide sequences can be preserved. The question of whether ϕ29 DNA is terminally redundant is unanswered; however, ϕ29 DNA is nonpermuted (2) and does not seem to form high-molecular-weight intermediates during replication (16, 24, 42). An alternate mechanism for the replication of linear DNA molecules which does not require terminal redundancy or concatemeric intermediates has been proposed by Cavalier-Smith (10). It requires the presence of terminal palindromic base sequences which could form hairpin loops at the 3′ tails to prime a gap-filling step by DNA polymerase followed by sealing of the remaining nick with ligase and suggests that the complete duplex DNA molecule is restored by the production of a single-stranded break on the opposite strand, unfolding of the hairpin loop, and filling of the resulting gap by polymerase. Such a mechanism could also function in the initiation of DNA replication as proposed for adenovirus (53). Another model for the replication of linear adenovirus DNA, presented by Rekosh et al. (37), eliminates the requirement of terminally redundant or palindromic nucleotide sequences to initiate replication or to conserve the terminal region. In this case, the terminal protein acts in the bidirectional synthesis of DNA from initiation sites near each end of the genome as a primer for the synthesis of progeny strands. A nascent terminal protein molecule is covalently linked to a deoxycytidine molecule and binds near the 3′ end of the parental DNA strand to provide the 3′-hydroxyl terminus necessary to prime DNA synthesis. Experiments (4, 21, 49, 52) showing that initiation of adenovirus DNA replication occurs near each end of the genome support this model.

Little is known about the mechanism of ϕ29 DNA replication. The association of ϕ29 DNA with the cell membrane during replication is dependent on the phage gene 2 protein (24, 42). The origin and terminus of replication have not been determined. A detailed analysis of the structures at the terminal regions of the chromosome and the nature of replicative intermediates may suggest a role for the gene 3 protein in the process of ϕ29 DNA synthesis.

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