Identification of a Protein Bound to the Termini of Bacteriophage PRD1 DNA

DENNIS BAMFORD,¹ TIMOTHY MCGRAW,² GERALD MACKENZIE,² AND LEONARD MINDICH^{2*}

Department of Microbiology, The Public Health Research Institute of The City of New York, Inc., New York, New York 10016,² and Department of Genetics, University of Helsinki, Helsinki, Finland¹

Received 28 March 1983/Accepted 10 May 1983

Lipid-containing bacteriophage PRD1 has a double-stranded DNA genome of about 14,500 nucleotide base pairs. The phage can infect *Escherichia coli* and *Salmonella typhimurium* as well as other gram-negative bacteria harboring an appropriate plasmid. [³⁵S]methionine label is incorporated into the DNA band early in infection. The label remains associated with DNA through phenol extraction and boiling with sodium dodecyl sulfate. Nuclease treatment of the genome released a protein which migrated as an early phage-specific protein (P8). This protein is also necessary for phage DNA replication. By restriction enzyme analysis it was shown that protein was associated with the terminal restriction fragments. Extracts of infected cells catalyzed the labeling of protein P8 with [α -³²P]dGTP.

Proteins have been found to be covalently linked to the 5' termini of bacteriophage ϕ 29 (11, 13, 24, 31) and adenovirus double-stranded DNA (5, 22, 23). Several single-stranded RNA viruses also have proteins covalently linked to the 5' ends of their genomes (4, 7-10, 14, 17, 25). The ϕ 29 DNA protein, an early viral protein necessary for DNA synthesis (24, 30), is linked to the 5'-terminal dAMP nucleotide (12, 29). In the adenovirus system the DNA protein is synthesized in a precursor form which is some 25,000 daltons larger than the protein found on virion DNA (6, 27). The 5'-terminal nucleotide, which is linked to this protein, is dCMP (16). These terminal proteins link to the first 5' nucleotide and are suggested to provide a free 3'-OH which can be used by DNA polymerase as a primer (6, 15, 16).

Bacteriophage PRD1 belongs to a close family of lipid-containing bacteriophages (2) which can infect a variety of gram-negative hosts, including *Escherichia coli* and *Salmonella* spp., harboring an appropriate plasmid (20). The double-stranded DNA genome of PRD1 contains about 14,500 nucleotide pairs (T. McGraw, H.-L. Yang, and L. Mindich, Mol. Gen. Genet., in press). For a more detailed description of the phage system, see Mindich et al. (18, 19) and Bamford and Mindich (1).

In this study we show that there is a strongly bound protein on PRD1 DNA termini, we identify the protein as an early phage-specific protein, P8, we show that this protein is necessary for DNA replication, and we show that extracts of

PRD1-infected cells catalyze the formation of a complex between protein P8 and [α -³²P]dGTP.

MATERIALS AND METHODS

Bacterial and phage strains. The nonsuppressor host used was *Salmonella typhimurium* LT2(pLM2), and the suppressor hosts used were *S. typhimurium* PSA(pLM2) and DB7156(pLM2) (18), the former inserting glutamine and the latter inserting tyrosine. Strain MS1550(pLM2) is a UV-sensitive strain of *S. typhimurium* that was used to follow the phage macromolecule synthesis in infected cells (32). The wild-type and nonsense mutant viruses used in this study are listed in Table 1 and were described by Mindich et al. (18, 19).

Phage production and DNA extraction. The unlabeled wild-type particles were obtained by infecting LT2(pLM2) cells at a density of 5×10^8 cells per ml in LC medium (26) with phages at a multiplicity of infection of 10. For prelabeling experiments, LT2(pLM2) cells were grown in supplemented M8 medium (26) to 3×10^8 cells per ml. [³⁵S]methionine (50 μ Ci/ml, 1,325 Ci/mmol; Amersham Corp.) was added for 5 min at 37°C. The labeling was stopped by adding unlabeled methionine to a concentration of 200 μ g/ml. The cells were harvested in an Eppendorf centrifuge at room temperature. The pellets were suspended in an equal volume of LC medium containing 200 μ g of unlabeled methionine per ml and immediately infected with wild-type PRD1 (multiplicity of infection, 10). For early labeling the cells in supplemented M8 medium were labeled at 3 to 15 min postinfection with 40 μ Ci of [³⁵S]methionine per ml. The labeling was stopped by the addition of unlabeled methionine. The cultures were incubated at 37°C with aeration until lysis occurred. The liberated phage

TABLE 1. Wild-type and nonsense mutant viruses used

Phage	Gene and protein affected	Protein characterization
Wild type		
<i>sus1</i>	IX, P9	Late (particle filling)
<i>sus2</i>	I, P1	Early (DNA synthesis)
<i>sus38</i>		
<i>sus114</i>		
<i>sus196</i>		
<i>sus233</i>	VIII, P8	Early (DNA synthesis)
<i>sus247</i>		

particles were concentrated and purified as previously described (1).

Unlabeled PRD1 DNA was extracted from purified virions. To a suspension containing 7×10^{12} PRD1 particles per ml we added 2% sodium dodecyl sulfate and 500 μ g of protease (predigested *S. griseus*) per ml and incubated this solution at 37°C for 45 min. The solution was extracted twice with phenol saturated with 0.1 M Tris (pH 8.0) buffer. The nucleic acid was precipitated from the aqueous phase by the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The DNA pellet was suspended in 10 mM Tris (pH 7.5)–0.1 mM EDTA–4 mM NaCl and stored at 4°C.

Restriction enzyme digestion. Endonuclease *HaeII* was purchased from Bethesda Research Laboratories. The restriction digests were performed at 37°C. The units of enzyme added per digestion were adjusted to give complete digests after 16-h incubations.

Nuclease treatment. Nuclease buffer (10 $\times$, 0.5 M sodium borate [pH 8.75], 5 mM CaCl₂) was added to the labeled phage preparations. The phages were boiled for 30 s, followed by chilling on ice to open the phage structure. Final concentrations of 25 and 5 U of nuclease (*Staphylococcus aureus* nuclease; Boehringer Mannheim Corp.) per ml were added, and the samples were incubated at 37°C for 2 h. The reaction was stopped by adding protein gel sample buffer containing a final sodium dodecyl sulfate concentration of 1% and mercaptoethanol (28). The samples were heated to 100°C for 1 min.

Protein and DNA analysis. The infection and labeling of UV-treated MS1550(pLM2) cells were done as previously described (18), except that 15 μ Ci of [³⁵S]methionine per ml was used. After sample buffer addition, the samples were divided into two aliquots and heated to 80 and 100°C for 1 min for DNA and protein samples, respectively.

Preparation of extracts and conditions for forming the complex between PRD1 protein P8 and [α -³²P]dGTP. An LC medium culture (200 ml) of *S. typhimurium* LT2(pLM2) was infected at a multiplicity of infection of 10 and at a cell density of about 5×10^8 cells per ml. At 15 min postinfection the cells were chilled on ice and washed once with 50 mM Tris-hydrochloride (pH 7.5) containing 2.5 mM MgCl₂. The washed cells were resuspended in 3 ml of the same buffer, disrupted in a French press, and then centrifuged at 30,000 rpm for 2

h at 5°C. The supernatant was dialyzed against 25 mM Tris-hydrochloride–2.5 mM MgCl₂–1 mM dithiothreitol at 4°C. The assay conditions for the protein nucleotide complexes were based upon those of Lichy et al. (16). The reaction mixture (0.07 ml) contained 20 mM Tris-hydrochloride (pH 7.5)–2 mM MgCl₂–0.7 mM dithiothreitol–1.5 mM ATP– 2×10^{11} phage particles (heated to 100°C for 20 s before addition)–3 μ l of the prepared extracts–10 μ Ci of [α -³²P]dATP, [α -³²P]dTTP, [α -³²P]dGTP, or [α -³²P]dCTP (~800 Ci/mmol; Amersham). The reaction mixture was incubated at 30°C for 30 min, heated to 100°C for 1 min, and chilled on ice. Micrococcal nuclease buffer and micrococcal nuclease to a final concentration of 50 U per reaction were added, and the mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of trichloroacetic acid containing 0.15% sodium PPi and 10 mM EDTA. The precipitate was collected by centrifugation, rinsed with 50 mM Tris-hydrochloride (pH 7.5), and then boiled for 2 min after the addition of protein gel sample buffer.

Gel electrophoresis. The proteins were analyzed in a 15.5% polyacrylamide gel as previously described (18). The restriction fragments of PRD1 DNA were analyzed in 5% polyacrylamide gels as described by Bolivar et al. (3). The agarose-polyacrylamide gel system used to analyze the PRD1 DNA complexes was that of Studier (28). The protein gels were dried, and autoradiograms were prepared. The DNA gels were stained with ethidium bromide, photographed, and dried, and autoradiographs were prepared.

RESULTS

The *HaeII* pattern of protease-treated PRD1 DNA showed two additional bands in the gels and less material left in the well (Fig. 1) than did that of non-protease-treated PRD1 DNA. The upper band (420 base pairs) is easily seen, but the lower band runs together with other bands (280 base pairs). It has been shown (McGraw et

FIG. 1. 5% polyacrylamide gel showing the PRD1 DNA fragments treated by *HaeII* digestion. +, Protease treatment during DNA extraction; –, no protease treatment. The calculated sizes of the fragments in base pairs are shown on the right; d, doublet.

FIG. 2. Agarose-polyacrylamide gel electrophoresis of PRD1 DNA. (A) Gel stained with ethidium bromide; (B) autoradiogram of the gel in (A). (Lane a) Protease-treated, nonradioactive standard PRD1 DNA; (lane b) heated PRD1 particles labeled 3 to 15 min postinfection with [35 S]methionine; (lanes c and d) particles shown in (b) but treated with 5 and 25 U of nuclease per ml, respectively; (lane e) heated PRD1 particles from cells prelabeled with [35 S]methionine; (lane f) nuclease-treated prelabeled PRD1 particles (25 U/ml). The arrows indicate the positions of the sample well and the PRD1 DNA band.

al., in press) that these are the end fragments of *Hae*II-digested PRD1 DNA.

When the phage was labeled with [35 S]methionine the DNA was labeled (Fig. 2b). This protein label was associated with DNA through phenol extraction and boiling with sodium dodecyl sulfate-mercaptoethanol, suggesting a protein bound to DNA. When the DNA labeling with [35 S]methionine was optimized, it was observed that the DNA was not labeled if the cells were labeled only before infection (Fig. 2e), and only a trace amount of label was associated with the DNA when late labeling (30 min and later postinfection [data not shown]) was used. The optimal conditions for DNA-protein complex labeling were achieved 3 to 15 min postinfection, the time for the synthesis of early virus-specific proteins (18). Nuclease treatment of [35 S]methionine-labeled DNA from the phage particles showed a simultaneous disappearance of the phage DNA band and the protein label from the gel (Fig. 2c and d). A new protein band appeared when the phage preparations labeled early were treated with nuclease and analyzed in protein gels (Fig. 3e and f). This band was not seen in nontreated preparations (Fig. 3d) or in treated preparations labeled before infection (Fig. 3g and h). The new band had the same migration properties as did the early phage protein P8 (Fig. 3a).

We have shown previously that mutations in complementation groups I and VIII prevent DNA synthesis in PRD1-infected cells (18). The distinction between groups I and VIII was diffi-

cult to make in ordinary labeling experiments because both classes result in poor protein synthesis. In the present study we performed labeling both early and late in infection and distinguished the classes unequivocally, showing that group VIII lacks protein P8 and group I lacks protein P1. This was especially clear in the early pattern (Fig. 4). Figure 4 illustrates the early and late protein synthesis patterns for wild-type PRD1 (Fig. 4b), *susI*-containing PRD1 (Fig. 4c), which lacks protein P9 and does not package DNA (19), several class I mutants that do not synthesize protein P1 (4d, e, f, and g), and two class VIII mutants that synthesize protein P1 but not protein P8 (4h and i). When the DNA was analyzed from these infections, it became clear that neither class I mutants nor class VIII mutants synthesize phage DNA or has protein label in the DNA position (Fig. 5). The control infections with wild-type PRD1 (Fig. 5b) and a DNA-packaging mutant (*susI*-containing PRD1) (Fig. 5c) that lacks protein P9 showed DNA synthesis as well as protein label in the DNA band. Some phage DNA was seen early in cells

FIG. 3. Polyacrylamide gel electrophoresis of [35 S]methionine-labeled PRD1 proteins. (Lane a) Early proteins (labeled 5 to 15 min postinfection); (lane b) late proteins (labeled 35 to 45 min postinfection); (lane c) purified phage particles; (lane d) phage particles labeled 3 to 15 min postinfection; (lanes e and f) phage particles labeled 3 to 15 min postinfection and treated with 5 and 25 U of nuclease per ml, respectively, before electrophoresis; (lanes g and h) nuclease-treated phage particles prepared from prelabeled cells (5 and 25 U of nuclease per ml, respectively). The arrow shows the position of the early PRD1 protein P8. The numbers at the left indicate the proteins.

FIG. 4. Polyacrylamide gel electrophoresis of [35 S]methionine-labeled proteins synthesized in UV-treated MS1550(pLM2) cells after infection with different PRD1 mutants. Early labeling was done 5 to 15 min postinfection, and late labeling was done 35 to 45 min postinfection. (Lane a) Uninfected cells; (lane b) wild-type PRD1; (lane c) *sus1*-containing mutant in gene IX lacking protein P9; (lanes d, e, f, and g) *sus2*-, *sus38*-, *sus114*-, and *sus196*-containing mutants in gene I lacking protein P1; (lanes h and i) *sus233*- and *sus247*-containing mutants in gene VIII lacking protein P8. The numbers at the left indicate, the proteins.

infected with the mutant; however, this diminished with time and appeared to result from the DNA from infecting particles and not from new synthesis.

FIG. 5. The same samples as shown in Fig. 4 electrophoresed in a polyacrylamide agarose gel to measure the synthesis of phage DNA. (A) Gel stained with ethidium bromide; (B) autoradiogram of the gel in (A). The arrows in (A) indicate the positions of the well bottoms, and the arrows in (B) indicate the position of PRD1 DNA.

FIG. 6. Polyacrylamide gel electrophoresis of the formation of a complex between protein P8 and [α - 32 P]dGTP. (Lane a) Complete reaction mixture containing 1.5 mM ATP, 10 μ Ci of [α - 32 P]dGTP, and infected-cell extract with added disrupted phage; (lane b) reaction mixture shown in (a) with added disrupted phage but with extract from uninfected cells; (lane c) reaction mixture shown in (a) but without heat-disrupted phage; (lane d) phage proteins synthesized early in infection and labeled with [35 S]methionine. The arrow shows the position of the early PRD1 protein P8. The numbers at the right indicate, the proteins.

Extracts of cells infected with adenovirus or ϕ 29 have been shown to catalyze the addition of the terminal genomic nucleotide to the DNA-associated protein (16, 21). With infected-cell extracts, disrupted phage particles, and [α - 32 P]dGTP it was possible to catalyze a reaction which resulted in a phosphorus-labeled band which migrated as the early phage-specific protein P8 (Fig. 6d). Also, another band at variable density was observed below protein P8, but it did not correspond to any phage protein. None of the other nucleotides ([α - 32 P]PdATP, [α - 32 P]dTTP, and [α - 32 P]dCTP) were able to incorporate label into the protein P8 band (data not shown). The formation of the complex was dependent upon the addition of disrupted phage particles (Fig. 6b). Uninfected-cell extracts did not catalyze this reaction (Fig. 6c).

DISCUSSION

The early virus-specific protein P8 is bound to the ends of the bacteriophage PRD1 genome. The linkage of the protein to the DNA is very strong, since neither phenol nor boiling in sodium dodecyl sulfate-mercaptoethanol released it, but protease treatment did. We believe that there is a covalent link between the protein and phage DNA, as in the case with ϕ 29 and adeno-

virus (23, 24). We do not know whether the protein is associated with the 3' or 5' ends of the DNA. Neither of these ends can be labeled by polynucleotide kinase or terminal deoxynucleotidyltransferase (unpublished data). The function suggested for terminal DNA proteins is to provide a free 3'-OH for priming the DNA polymerase activity (15), and it is possible that this model is also useful for PRD1, since protein P8 is one of the two phage proteins necessary for phage DNA replication.

It was possible to specifically catalyze an *in vitro* reaction in which dGTP and no other nucleotide became linked to protein P8 when infected-cell extracts and heat-disrupted phage particles were used. This indicates that the first nucleotide in the DNA to which protein P8 is linked is dGMP and that the formation of a protein P8-dGMP complex depends upon phage proteins. The reaction was dependent upon added disrupted phage particles, indicating that an exogenous DNA-protein P8 complex is necessary. This reaction is analogous to those which occur between the adenovirus DNA protein and dCTP (16) and between bacteriophage ϕ 29 DNA protein P3 and dATP (21).

PRD1 is the third example of a case in which there is a terminal replication protein linked to a viral DNA genome. Because this virus can be propagated in *E. coli* or *Salmonella* spp., it offers a promising system for studies of DNA replication.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-09861, awarded to L.M., from the National Institute of Allergy and Infectious Diseases and by a fellowship from the European Molecular Biology Organization awarded to D.B. for his work at The Public Health Research Institute.

LITERATURE CITED

- Bamford, D. H., and L. Mindich. 1982. Structure of the lipid-containing bacteriophage PRD1: disruption of wild-type and nonsense mutant phage particles with guanidine hydrochloride. *J. Virol.* **44**:1031-1038.
- Bamford, D. H., L. Rouhiainen, K. Takkinen, and H. Söderlund. 1981. Comparison of the lipid-containing bacteriophages PRD1, PR3, PR4, PR5, and L17. *J. Gen. Virol.* **57**:365-373.
- Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**:75-93.
- Burroughs, J. N., and F. Brown. 1978. Presence of a covalently linked protein on calcivirus RNA. *J. Gen. Virol.* **41**:443-446.
- Carusi, E. A. 1977. Evidence for blocked 5'-termini in human adenovirus DNA. *Virology* **76**:380-384.
- Challberg, M. D., S. V. Desiderio, and T. J. Kelly, Jr. 1980. Adenovirus DNA replication *in vitro*: characterization of a protein covalently linked to nascent DNA strands. *Proc. Natl. Acad. Sci. U.S.A.* **77**:5105-5109.
- Daubert, S. D., and G. Bruening. 1979. Genome-associated proteins of comoviruses. *Virology* **98**:246-250.
- Daubert, S. D., G. Bruening, and R. C. Najarian. 1978. Protein bound to the genome RNAs of cowpea mosaic virus. *Eur. J. Biochem.* **92**:45-51.
- Flanagan, J. B., R. F. Pettersson, V. Ambros, M. J. Hewlett, and D. Baltimore. 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5'-terminus of virion and replicative intermediate RNAs of poliovirus. *Proc. Natl. Acad. Sci. U.S.A.* **74**:961-965.
- Golini, F., A. Nomoto, and E. Wimmer. 1978. The genome-linked protein of picornaviruses. IV. Difference in the VPg's of encephalomyocarditis virus and poliovirus as evidence that the genome-linked proteins are virus-coded. *Virology* **89**:112-118.
- Harding, N. E., J. Ito, and G. S. David. 1978. Identification of the protein firmly bound to the ends of bacteriophage ϕ 29 DNA. *Virology* **84**:279-292.
- Hermoso, J. M., and M. Salas. 1980. Protein p3 is linked to the DNA of phage ϕ 29 through a phosphoester bond between serine and 5'-dAMP. *Proc. Natl. Acad. Sci. U.S.A.* **77**:6425-6428.
- Ito, J. 1978. Bacteriophage ϕ 29 terminal protein: its association with the 5' termini of the ϕ 29 genome. *J. Virol.* **28**:895-904.
- Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer. 1977. A protein covalently linked to poliovirus genome RNA. *Proc. Natl. Acad. Sci. U.S.A.* **74**:59-63.
- Lichy, J. H., J. Field, M. S. Horwitz, and J. Hurwitz. 1982. Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. *Proc. Natl. Acad. Sci. U.S.A.* **79**:5225-5229.
- Lichy, J. H., M. S. Horwitz, and J. Hurwitz. 1981. Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **78**:2678-2682.
- Mayo, M. A., H. Barker, and B. D. Harrison. 1979. Evidence for a protein covalently linked to tobacco ring-sport virus. *J. Gen. Virol.* **43**:735-740.
- Mindich, L., D. Bamford, C. Goldthwaite, M. Laverty, and G. Mackenzie. 1982. Isolation of nonsense mutants of lipid-containing bacteriophage PRD1. *J. Virol.* **44**:1013-1020.
- Mindich, L., D. Bamford, T. McGraw, and G. Mackenzie. 1982. Assembly of bacteriophage PRD1: particle formation with wild-type and mutant viruses. *J. Virol.* **44**:1021-1030.
- Olsen, R. H., J.-S. Siak, and R. H. Gray. 1974. Characteristics of PRD1, a plasmid-dependent broad host range DNA bacteriophage. *J. Virol.* **14**:689-699.
- Peñalva, M. A., and M. Salas. 1982. Initiation of phage ϕ 29 DNA replication *in vitro*: formation of a covalent complex between the terminal protein p3 and 5'-dAMP. *Proc. Natl. Acad. Sci. U.S.A.* **79**:5522-5526.
- Robinson, A. J., and A. J. D. Bellett. 1974. A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. *Cold Spring Harbor Symp. Quant. Biol.* **39**:523-531.
- Robinson, A. J., H. B. Younghusband, and A. J. D. Bellett. 1973. A circular DNA-protein complex from adenoviruses. *Virology* **56**:54-69.
- Salas, M., R. P. Mellado, E. Viñuela, and J. M. Sogo. 1978. Characterization of a protein covalently linked to the 5' termini of the DNA of *Bacillus subtilis* phage ϕ 29. *J. Mol. Biol.* **119**:269-291.
- Sangar, D. V., D. J. Rowlands, T. J. R. Harris, and F. Brown. 1977. Protein covalently linked to foot-and-mouth disease virus RNA. *Nature (London)* **268**:648-650.
- Sinclair, J. F., A. Tzagoloff, D. Levine, and L. Mindich. 1975. Proteins of bacteriophage ϕ 6. *J. Virol.* **16**:685-695.
- Stillman, B. W., J. B. Lewis, L. T. Chow, M. B. Mathews, and J. E. Smart. 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. *Cell* **23**:497-508.
- Studier, F. W. 1973. Analysis of bacteriophage T7 early RNA's and proteins on slab gels. *J. Mol. Biol.* **79**:237-248.
- Watabe, K., M.-F. Shih, A. Sugino, and J. Ito. 1982. *In vitro* replication of bacteriophage ϕ 29 DNA. *Proc. Natl.*

- Acad. Sci. U.S.A. **79**:5245-5248.
30. **Yanofsky, S., F. Kawamura, and J. Ito.** 1976. Thermolabile transfecting DNA from a temperature-sensitive mutant of phage ϕ 29. *Nature (London)* **259**:60-63.
 31. **Yehle, C. O.** 1978. Genome-linked protein associated with the 5' termini of bacteriophage ϕ 29 DNA. *J. Virol.* **27**:776-783.
 32. **Youderian, P., and M. M. Susskind.** 1980. Identification of the products of bacteriophage P22 genes, including a new late gene. *Virology* **107**:258-269.