JOURNAL OF VIROLOGY, Oct. 1992, p. 6220–6222
0022-538X/92/106220-03$02.00/0

Vol. 66, No. 10

Protein P4 of the Bacteriophage φ6 Procapsid Has a Nucleoside Triphosphate-Binding Site with Associated Nucleoside Triphosphate Phosphohydrolase Activity

PAUL GOTTLIEB, JEFFREY STRASSMAN, AND LEONARD MINDICH*

Department of Microbiology, The Public Health Research Institute, New York, New York 10016

Received 1 June 1992/Accepted 8 July 1992

Bacteriophage φ6 contains three segments of double-stranded RNA. The procapsid consists of proteins P1, P2, P4, and P7, which are encoded by the viral L segment. cDNA copies of this segment have been cloned into plasmids that direct the production of these proteins, which assemble into polyhedral procapsids. These procapsids are capable of packaging plus-sense φ6 RNA in the presence of nucleoside triphosphate and synthesizing the complementary minus strand to form double-stranded RNA. In this article, we report the presence of a nucleotide-binding site in protein P4. The viral procapsid and nucleocapsid exhibit a nucleoside triphosphate phosphohydrolase activity that converts nucleoside triphosphates into nucleoside diphosphates.

Bacteriophage φ6 infects the plant pathogen *Pseudomonas phaseolicola* (*Pseudomonas syringae* pv. phaseolicola) HB10Y (24). The viral genome consists of three separate pieces of double-stranded RNA encapsidated within a polyhedral nucleocapsid (19). Virus particles contain one copy of each genome segment (5). The nucleocapsid has RNA polymerase activity and functions as both a replicase and a transcriptase (11, 18). RNA polymerization proceeds via a semiconservative mechanism with transcripts released by strand displacement (6, 23).

The first intermediate detected in the φ6 assembly pathway is a polyhedral procapsid which becomes filled with one copy each of the three pieces of double-stranded RNA (2, 5, 16, 17). The filled procapsids are then covered with a shell of protein P8. The resulting nucleocapsid is subsequently enveloped within a lipid-protein membrane (14).

Cloned cDNA copies of the genomic L segment direct the synthesis of proteins P1, P2, P4, and P7, which assemble in both *Escherichia coli* and *P. phaseolicola* into structures resembling the φ6 procapsid. These particles have both replicase and transcriptase activities (9, 11) and can package message-sense φ6 RNA in the presence of ATP or dATP. They subsequently synthesize the complementary minus-sense strands if all four ribonucleoside triphosphates (rNTPs) are present (10).

Analysis of the amino acid sequence of protein P4 reveals a sequence characteristic of nucleotide-binding sites (25). The amino acid sequence of P4 from amino acid 111 to 138 is RWPSEGIYSGVTALMGATGSGKSITLNE; the boldface residues are those of the nucleotide-binding motif. To confirm that this protein has a nucleoside triphosphate (NTP)-binding capacity, we derivatized it with a radiolabeled photoaffinity analog of ATP, 8-azido-[α-^{32}P]ATP (8-N_3ATP) (specific activity, 7.0 Ci/mmol; ICN Radiochemicals). UV irradiation of this compound converts the azido group into a reactive nitrene which forms a covalent bond with amino acids (4). Ten micrograms of nucleocapsid was mixed with about 150 pmol (6 μM) of 8-N_3ATP. The reaction volume was brought to 25 μl in buffer A (9), and irradiation took place in a droplet on Parafilm for 5 to 30 min at a distance of 8 cm from the UV source (model R51 mineral light; Ultraviolet Products Inc.). After the irradiation was complete, the reaction mix was diluted with concentrated electrophoresis running buffer (15). Radiolabeled proteins were visualized by autoradiography after denaturation and electrophoresis through a 15.5% polyacrylamide–sodium dodecyl sulfate (SDS) gel (21). Gels were soaked for 1 hour in 1 M sodium salicylate before being dried. φ6 proteins used for standard molecular size markers were labeled with [^{3}H]leucine (20).

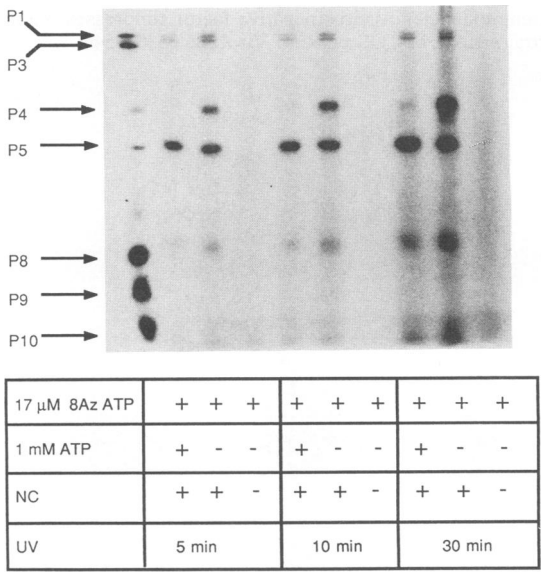

FIG. 1. The derivatization of φ6 proteins with the photoaffinity analog of ATP, 8-N_3ATP (8Az ATP). Isolated nucleocapsids (NC) were mixed with 8-N_3ATP with or without unlabeled competitor ATP. UV irradiation was for 5, 10, or 30 min. The derivatized proteins were separated by electrophoresis through a 15.5% polyacrylamide–SDS gel after denaturation and were visualized by autoradiography of the dried gel. The leftmost lane contains φ6 protein standards labeled with ^{3}H-labeled leucine.

* Corresponding author.

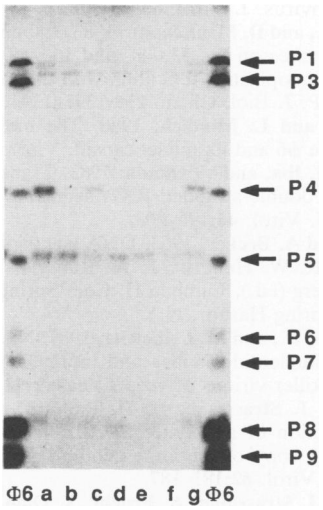

FIG. 2. 8-N_3ATP-binding competition with unlabeled nucleotides. Isolated nucleocapsids were mixed with 8-N_3ATP in the presence of unlabeled competitor nucleotide. UV irradiation was for 10 min. The derivatized proteins were separated by electrophoresis through a 15.5% polyacrylamide–SDS gel after denaturation and were visualized by autoradiography of the dried gel. Reaction mixtures: lane a, no competing nucleotide; lane b, unlabeled ATP competitor; lane c, unlabeled CTP competitor; lane d, unlabeled GTP competitor; lane e, unlabeled UTP competitor; lane f, unlabeled ADP competitor; lane g, unlabeled AMP competitor. ϕ6 protein standards labeled with [^{3}H]leucine are displayed in lanes ϕ6.

In the absence of competing nucleotide, all of the nucleocapsid proteins became labeled (Fig. 1). However when unlabeled ATP was present at 1 mM, the reaction of the photoaffinity analog with protein P4 was substantially reduced (Fig. 1).

This competition also occurred in the presence of 1 mM concentrations of each of the three other NTPs or ADP. However, AMP did not prevent the binding of the affinity analog to P4 (Fig. 2). The inhibition of labeling by CTP appears weaker than the inhibition by the other NTPs in Fig. 2, but in repeated experiments the difference was not apparent. dATP was an effective competitor of 8-N_3ATP binding to P4 (data not shown). This inhibition of covalent binding of the photoaffinity analog to protein P4 by all four NTPs and dATP suggests that the NTPs all bind to the same site.

The procapsid structure produced in *E. coli* from the cDNA copy of segment L is identical to that produced by ϕ6 virus infection (9). This structure is simpler than the nucleocapsid in that it lacks RNA and protein P8. We have found that the packaging by the procapsid of plus-sense RNA occurs in the presence of ATP or dATP (10). We therefore examined the binding of 8-N_3ATP to this structure. Plasmid pLM378 carries large (L)-segment cDNA under control of the *tac* promoter. It encodes the four proteins that constitute the procapsid, P1, P2, P4, and P7 (9). Plasmid pLM406 carries L-segment cDNA with a deletion so that it encodes only proteins P1 and P4. It was constructed from pLM369 by producing an *Sph*I deletion from within the L-segment sequence to the *Eco*RI site of the vector pUC8 multiple-cloning site sequence (9). *E. coli* JM109 [Δ(*lac-proAB*) *thi relA1* λ$^-$ *recA gyrA96 endA supE44 hsdR17* (F′ *traD36 proAB lacI*^{q}ZΔM15)] carrying these plasmids was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and cell lysates were centrifuged on sucrose gradients. The

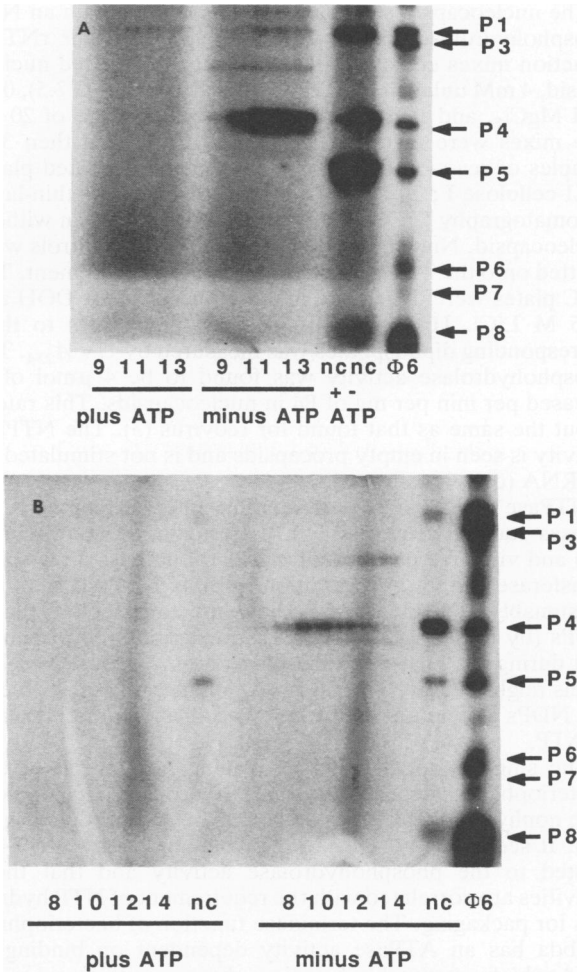

FIG. 3. *E. coli* JM109 cultures containing either plasmid pLM378 or pLM406 were induced with IPTG. Cell lysates were centrifuged in sucrose gradients. Twenty-microliter aliquots of the gradient fractions which contained the procapsid particle peaks were used in the 8-N_3ATP photoaffinity assay. Each fraction was assayed in the presence or absence of unlabeled competing ATP. Derivatized proteins from each fraction were separated by electrophoresis through a 15.5% polyacrylamide–SDS gel after denaturation and were visualized by autoradiography of the dried gel. (A) Sucrose gradient fractions of lysates containing the complete particle produced by pLM378. Fraction numbers are shown below the gel. The reaction mixture in lane nC + ATP contains ϕ6 nucleocapsid with unlabeled competing ATP. [^{3}H]leucine-labeled ϕ6 protein standards are in lane ϕ6. (B) Sucrose gradient fractions of lysates containing particles composed of proteins P1 and P4, produced by pLM406.

gradient fractions which contained the structure were identified on SDS-polyacrylamide gels (9). Twenty-microliter aliquots of the sucrose gradient fractions containing the particle peaks were used in the photoaffinity labeling assay (Fig. 3A). It can be seen in this figure (fractions 9 to 13) that the presence of unlabeled competing ATP prevents 8-N_3ATP binding to protein P4 in the peak fractions. The P4 bands are clearly derivatized when no unlabeled ATP is present in the photoaffinity assay (Fig. 3A, fractions 9 to 13). The peak fractions of the partial particle encoded by pLM406 were photoaffinity labeled also, showing that covalent binding to P4 is specifically blocked in the presence of 1 mM ATP (Fig. 3B).

The nucleocapsid structure was found to contain an NTP phosphohydrolase activity that acts upon all four rNTPs. Reaction mixes consisted of about 1 μg of isolated nucleocapsid, 4 mM unlabeled NTP, 55 mM Tris-HCl (pH 7.5), 0.25 mM $MgCl_2$, and 1 mM sodium azide in a volume of 20 μl. The mixes were incubated for 1 h at 25°C, and then 3-μl samples of reaction mix was spotted onto precoated plates (PEI-cellulose F; 20 by 20 cm; E.M. Science) for thin-layer chromatography (TLC). Control reactions were run without nucleocapsid. Nucleoside diphosphate (NDP) controls were spotted onto the TLC plates at the time of development. The TLC plates were developed in a mixture of N HCOOH and 0.25 M LiCl. Hydrolysis of all four nucleotides to their corresponding diphosphates was measured by UV A_{254}. The phosphohydrolase activity was found to be 4 μmol of P_i released per min per mg of P4 in nucleocapsids. This rate is about the same as that found for reovirus (3). The NTPase activity is seen in empty procapsids and is not stimulated by ϕ6 RNA (data not shown).

NTPase activity has been found in other segmented RNA viruses such as reovirus (3, 13). Vesicular stomatitis virus (22) and viruslike particles of yeasts (8) possess a phosphotransferase activity in addition to the NTPase activity. Presumably, these systems allow utilization of available NDPs (by NTP regeneration) for transcription and replication during the host cell stationary phase, when some NTP levels might be low. The ϕ6 RNA polymerase is not able to use NDPs as precursors for RNA synthesis in the presence of ATP.

The single-stranded RNA packaging mechanism of this bacteriophage is dependent on NTP hydrolysis (10). Analogs with nonhydrolyzable γ phosphate do not support packaging (10). It seems likely that the NTP-binding activity of P4 is related to the phosphohydrolase activity and that these activities are correlated with the requirement of NTP hydrolysis for packaging. The terminase function of bacteriophage lambda has an ATPase activity dependent on binding to DNA which presumably functions during nucleic acid packaging (7). Many double-stranded DNA bacteriophages use ATP hydrolysis for the packaging of DNA (12). The complexing of protein C in bacteriophage ϕX174 to the phage DNA replication complex and procapsid structure is also dependent on ATP or dATP binding (1).

In addition to its role in RNA packaging, the NTPase activity could also be involved in the assembly of the procapsid itself. Particles that are missing P4 are poorly formed and are unstable (9). Preliminary experiments done in our laboratory indicate that defective P4 results in defective procapsid assembly. It is not clear whether this is due to defects in the NTPase activity, but mutations in the NTP-binding motif lead to assembly defects.

This work was supported by Public Health Service grant GM-34352 awarded to L.M.

REFERENCES

1. **Aoyama, A., M. Hamatake, and M. Hayashi.** 1981. Morphogenesis of ϕX174: in vitro synthesis of infectious phage from purified viral components. Proc. Natl. Acad. Sci. USA **78:**7285–7289.
2. **Bamford, D. H., and L. Mindich.** 1980. Electron microscopy of cells infected with nonsense mutants of bacteriophage ϕ6. Virology **107:**222–228.
3. **Borsa, J., J. Grover, and J. D. Chapman.** 1970. Presence of nucleoside triphosphate phosphohydrolase activity in purified virions of reovirus. J. Virol. **6:**295–302.
4. **Campbell, K., and D. MacLennan.** 1983. Labeling of high affinity ATP binding sites on the 53,000- and 160,000-dalton glycoproteins of the sarcoplasmic reticulum with the photoaffinity probe 8-N3-^{32}P-ATP. J. Biol. Chem. **258:**1391–1394.
5. **Day, L. A., and L. Mindich.** 1980. The molecular weight of bacteriophage ϕ6 and its nucleocapsid. Virology **103:**376–385.
6. **Emori, Y., H. Iba, and Y. Okada.** 1983. Transcriptional regulation of three double-stranded RNA segments of bacteriophage ϕ6 in vitro. J. Virol. **46:**196–203.
7. **Feiss, M., and A. Becker.** 1983. DNA packaging and cutting, p. 305–330. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Georgopoulos, D., and M. Leibowitz.** 1987. Nucleotide phosphotransferase, nucleotide kinase and inorganic pyrophosphatase activities of killer virions of yeast. Yeast **3:**117–129.
9. **Gottlieb, P., J. Strassman, D. H. Bamford, and L. Mindich.** 1988. Production of a polyhedral particle in *Escherichia coli* from a cDNA copy of the large genomic segment of bacteriophage ϕ6. J. Virol. **62:**181–187.
10. **Gottlieb, P., J. Strassman, A. Frucht, X. Qiao, and L. Mindich.** 1991. In vitro packaging of the bacteriophage ϕ6 ssRNA genomic precursors. Virology **181:**589–594.
11. **Gottlieb, P., J. Strassman, X. Qiao, A. Frucht, and L. Mindich.** 1990. In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage ϕ6: studies with procapsids assembled from plasmid-encoded proteins. J. Bacteriol. **172:**5774–5782.
12. **Guo, P., C. Peterson, and D. Anderson.** 1987. Prohead and DNA-gp3-dependent ATPase activity of the DNA packaging protein gp16 of bacteriophage ϕ29. J. Mol. Biol. **197:**229–236.
13. **Kapuler, A. M., N. Mendelsohn, H. Klett, and G. Acs.** 1970. Four base-specific nucleoside 5′-triphosphatases in the subviral core of reovirus. Nature (London) **225:**1209–1213.
14. **Mindich, L.** 1988. Bacteriophage ϕ6: a unique virus having a lipid-containing membrane and a genome composed of three dsRNA segments. Adv. Virus Res. **35:**137–176.
15. **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.
16. **Mindich, L., and R. Davidoff Abelson.** 1980. The characterization of a 120 S particle formed during ϕ6 infection. Virology **103:**386–391.
17. **Mindich, L., J. F. Sinclair, and J. Cohen.** 1976. The morphogenesis of bacteriophage ϕ6: particles formed by nonsense mutants. Virology **75:**224–231.
18. **Partridge, J. E., J. L. Van Etten, D. E. Burbank, and A. K. Vidaver.** 1979. RNA polymerase activity associated with bacteriophage ϕ6 nucleocapsid. J. Gen. Virol. **43:**299–307.
19. **Semancik, J. S., A. K. Vidaver, and J. L. Van Etten.** 1973. Characterization of a segmented double-helical RNA from bacteriophage ϕ6. J. Mol. Biol. **78:**617–625.
20. **Sinclair, J. F., J. Cohen, and L. Mindich.** 1976. The isolation of suppressible nonsense mutants of bacteriophage ϕ6. Virology **75:**198–208.
21. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNA's and proteins on slab gels. J. Mol. Biol. **79:**237–248.
22. **Testa, D., and A. Banerjee.** 1979. Nucleoside diphosphate kinase activity in purified cores of vesicular stomatitis virus. J. Biol. Chem. **254:**9075–9079.
23. **Usala, S. J., B. H. Brownstein, and R. Haselkorn.** 1980. Displacement of parental RNA strands during in vitro transcription by bacteriophage ϕ6 nucleocapsids. Cell **19:**855–862.
24. **Vidaver, A. K., R. K. Koski, and J. L. Van Etten.** 1973. Bacteriophage ϕ6: a lipid-containing virus of *Pseudomonas phaseolicola*. J. Virol. **11:**799–805.
25. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α and β subunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.