Biochemical Characterization of an ATPase Activity Associated with the Large Packaging Subunit gp17 from Bacteriophage T4*

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Double-stranded DNA-packaging in icosahedral bacteriophages is believed to be driven by a packaging “machine” constituted by the portal protein and the two packaging/terminase proteins assembled at the unique portal vertex of the empty prohead shell. Although ATP hydrolysis is evidently the principal driving force, which component of the packaging machinery functions as the translocating ATPase has not been elucidated. Evidence suggests that the large packaging subunit is a strong candidate for the translocating ATPase. We have constructed new phage T4 terminase recombinants under the control of phage T7 promoter and overexpressed the packaging/terminase proteins gp16 and gp17 in various configurations. The hexahistidine-tagged-packaging proteins were purified to near homogeneity by Ni²⁺-agarose chromatography and were shown to be highly active for packaging DNA in vitro. The large packaging subunit gp17 but not the small subunit gp16 exhibited an ATPase activity. Although gp16 lacked ATPase activity, it enhanced the gp17-associated ATPase activity by >50-fold. The gp16 enhancement was specific and was due to an increased catalytic rate for ATP hydrolysis. A phosphorylated gp17 was demonstrated under conditions of low catalytic rates but not under high catalytic rates in the presence of gp16. The data are consistent with the hypothesis that a weak ATPase is transformed into a translocating ATPase of high catalytic capacity after assembly of the packaging machine.

Recent biochemical studies with a number of double-stranded DNA phage packaging systems (λ, T3, T4, P1, and SPP1) revealed that the nonstructural terminase complex, constituted by one small subunit and one large subunit, is a key component of the DNA-packaging machine (3). Indeed, among the multiple biochemical functions and interactions that are required to catalyze the DNA-packaging process, the two key functions, (i) DNA cutting, which generates the termini of the packed DNA (hence, the name terminase), and (ii) ATP hydrolysis, which is evidently the driving force for translocation of DNA into the capsid shell, have been shown to be associated with the large terminase subunit (λ, gpA (4, 6); T3, gp19 (7)). Genetic evidence also supports this implication. In phage T3, a mutation in one of the consensus ATP binding sites of the large terminase subunit gp19 resulted in partial uncoupling of the ATPase from DNA translocation (7). In phage T4, ts mutations in g17 accumulated partially filled heads, suggesting a defective DNA translocation pathway (8). Recently, certain mutants in phage λ large terminase subunit gpA were reported, which showed apparent defects in DNA translocation.1 However, despite much circumstantial evidence, a direct linkage between a biochemically defined ATPase and DNA translocation is yet to be established for any phage system.

The phage T4 terminase, analogous to the other phage terminases, is constituted by a 70-kDa large subunit, gp17, and an 18-kDa small subunit, gp16 (9, 10). Although these proteins have been purified and an in vitro DNA-packaging system has been constructed (9), not much is known about the biochemical functions associated with these proteins and their role in the DNA-packaging pathway. Of particular interest to us is the putative translocating ATPase, which has not yet been identified in any phage system. In this study we asked, do either (or both) of the terminase subunits exhibit an ATPase activity? If so, what are the biochemical characteristics of the ATPase? And, do these characteristics implicate a mechanistic relationship to DNA translocation?

We report here the first biochemical characterization of the T4-packaging/terminase subunits with respect to the ATPase function. The data show that the large packaging subunit gp17 is the catalytic subunit exhibiting an ATPase activity, whereas the small subunit gp16 acts as an essential accessory subunit. We also discovered a novel feature associated with the small subunit gp16, namely its substantial stimulation of gp17 ATPase activity. Implications of these findings to the mechanism of DNA packaging in bacteriophage T4 and other double-stranded DNA viruses are discussed.

**EXPERIMENTAL PROCEDURES**

*Bacteria and Phage—Escherichia coli B40 (su−) was used as the amber suppressor for preparation of phage stocks. E. coli F301 (sup-) was used as the non-suppressor strain for preparation of packaging

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1 M. Feiss, personal communication.
extracts. *E. coli* VR34 (N59259 alpha lambda phage) was used for plating the reaction packaging mixture (11). *E. coli* CR63 (su-9) and *E. coli* CR63 (lambda su-9) were used for determining the yield of phage with appropriate genetic markers. *E. coli* strains BL1, BL1(DE3), BL2(DE3)pLys- S, and BL2(DE3)pLys-E (Novagen, Inc. (20)) were used to construct the plasmid strains. The recombinant plasmids were either transformed directly into competent cells or grown in EX200 (Novagen, Inc.) or BL21(DE3)pLys-S (Novagen, Inc.) cells. The *E. coli* strain B834 (DE3) was used for amplification of the overlapping region. This was constructed by cloning the sequences 5'-GCCGATCGGATGGAAGGTCTTGATATAAACAAAC-3' and 5'-GATGGAAGGTCTTGATATAAACAAAC-3' and 5'-GCCGATCGGATGGAAGGTCTTGATATAAACAAAC-3' (forward nucleotides 1–25) and 5'-GCCGATCGCTTTACATCGTGTTCCATCTATCC-3' (reverse nucleotides 497–472). The corresponding primers for amplification of *g17* coding sequence were 5'-GCCGATCGGATGGAAGGTCTTGATATAAACAAAC-3' (forward primer) and 5'-GCCGATCGGATGGAAGGTCTTGATATAAACAAAC-3' (reverse primer). The nucleotide numbers in parentheses correspond to the coding sequence of the corresponding gene. Each primer had a tag sequence at the 5'-end of the oligonucleotide (shown in italics), which allowed efficient cutting at the adjacent BamHI sequence (shown in bold). Polymerase chain reaction amplifications were performed as described earlier using about 100 ng of purified mature phage T4 DNA as a template (20). The amplified DNA was digested with BamHI at 37 °C for 2 h, and the DNA was ligated with the linearized and dephosphorylated plasmid vector DNA. The ligated DNA was transformed into competent *E. coli* BL21 cells, and the amp' transformants were scored for gene-positive transformants by marker rescue using the 16amN87amN66 and 17amNG178 mutants (21). The presence of appropriate size insert and orientation was confirmed by restriction mapping. Miniprep DNAs were prepared from the transformants and were re-transformed into the expression strain *E. coli* BL21(DE3)pLys-S. We found that the BL21(DE3)pLys-S strain was weaker than the BL21(DE3)pLys-E strain for overexpression of gp17, and hence, all the expression studies were performed under the BL21(DE3)pLys-S strain. The plasmid pRL could not be maintained in BL21(DE3), presumably due to the toxicity of a high basal level expression of gp17-associated nucleases (21, 22).

The clone pR16–17 was constructed by cloning the contiguous *g16* and *g17* coding sequences in its native configuration in which the RBS and the ATG initiation codon of *g17* are nested within a 30-base pair-overlapping sequence in the 3'-end of *g16* (10). The clone pR16,17 was constructed by deleting the 3'-end-overlapping sequence in *g16* and incorporating a second T7 RBS in the upstream of the *g17* ATG initiation codon (12). The clone R16–17 was constructed by a similar strategy except for the deletion of the 3'-end-overlapping sequence in *g16* and the incorporation of an additional T7 RBS upstream of the *g17* ATG initiation codon (12). This was done to minimize potential translational regulation in the overlapping sequence, which might limit the overexpression of both gp16 and gp17 (23). All the expression strains used in this project contained the ampicillin marker, and the amp' kan' resistant colonies were selected. A number of such combinations were constructed and analyzed since a variety of constructs with different antibiotic markers for *E. coli* were used in this study.

**Construction of Terminase Recombinants.—** The recombinant terminase genes were constructed in *E. coli* BL21(DE3)pLys-S carrying the clone pRL-H16. The procedure was essentially the same as described for gp17 except for the following differences. In the DEAE-Sepacel column chromatography, the non-specific proteins were eluted with 0.1 M NaCl and washed with 0.1 M NaCl, 60 mM imidazole. gp17 was then eluted with a 50-ml linear gradient of 60 mM–1 M imidazole. The fractions (0.5–1 ml) were analyzed by SDS-PAGE and in vitro DNA-packaging. Fractions containing gp17, which eluted at about 350 mM imidazole, were pooled, dialyzed against 20 mM Tris-HCl, pH 7.9, 50 mM MgCl2, 3 mM β-mercaptoethanol, and 0.5 mM EDTA and resuspended in SDS sample buffer. The supernatants (soluble fraction) and pellets (insoluble fraction) were analyzed by SDS-PAGE. Equivalent to about 10 μl of the original culture volume was loaded into each well.

**Purification of gp17.—** A fresh culture of *E. coli* BL21(DE3)pLys-S containing the clone pH17 was grown at 30 °C in Moore's tryptone-phosphate medium (24) containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml) to a density of around 2 × 108 cells/ml. The cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellet was resuspended in 0.1–0.2 μl of the Buffer P. The resuspended cells were lysed by two passages through a French press cell at 20,000 lb/inch², and the cell lysates were centrifuged at 12,000 rpm for 15 min to remove cell debris. The supernatant was directly loaded onto a DEAE-Sepacel ion-exchange column (20-ml packed column per 200–400 ml of original culture). The column was thoroughly washed by passing the buffer through 3–5 column volumes of Buffer P. The column was then washed with 0.13 M NaCl in Buffer P to remove nonspecific proteins. Bound gp17 was eluted with 0.2 μl NaCl in Buffer P, and the fractions were analyzed by SDS-PAGE and in vitro DNA-packaging. Fractions containing the bulk of gp17 were pooled and dialyzed against two changes of buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole), 1 liter each. The sample was then loaded onto a Ni2+-agarose column that was pre-equilibrated with the binding buffer (3-ml packed column per 200–400 ml of original culture). After washing the column with 10–15 column volumes of Buffer P, loosely adhered nonspecific proteins were removed by further washing with 10–15 column volumes of Wash buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 60 mM imidazole). gp17 was then eluted with a 50-ml linear gradient of 60 mM–1 M imidazole. The fractions (0.5–1 ml) were analyzed by SDS-PAGE and in vitro DNA-packaging. Fractions containing gp17, which eluted at about 350 mM imidazole, were pooled, dialyzed against two changes of 500 μl of Buffer P containing 200 mM NaCl, and stored at 70 °C.

**Purification of gp16–gp17.**—gp16 was purified from the E. coli BL21(DE3)pLys-S carrying the clone pRL-H16. The procedure was essentially the same as described for gp17 except for the following differences. In the DEAE-Sepacel column chromatography, the non-specific proteins were eluted with 0.17 M NaCl and washed, and gp16 was eluted with 0.2 M NaCl. The final purified gp16 was dialyzed against Buffer F (no NaCl) and was stored at −70 °C. **ATPase.—** The purified gp17, gp16, or the control proteins were incubated.
bated in a reaction mixture (10–30 μl) containing 1–10 μCi of [γ-32P]ATP or [α-32P]ATP (specific activity 3000 Ci/mmol) in Buffer P at 37 °C for 10–30 min. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM. A 1-μl reaction mixture was spotted on a 10-cm-long polyethyleneimine-cellulose thin layer chromatography strip at about 1 cm from the bottom. The strips were developed in a solvent containing 0.5 M LiCl and 1 M formic acid until the solvent reached about 0.5 cm from the top of the strip (~45 min (25)). The chromatograms were air-dried and autoradiographed. The NTase activity was determined under the same conditions using 10 μCi of α-32P-labeled ATP, dATP, CTP, GTP, or TTP (as the substrate specific activity). Whenever nonradioactive nucleotides were used as standards, the spots were visualized by exposing the chromatogram to UV light and marking the position of the nucleotide spot. In the quantitative experiments, the purified gp16 and/or gp17 were incubated in duplicates or triplicates with either [32P]ATP or a mixture of [32P]ATP and cold ATP, and the 32Pi or [32P]ADP formed was quantitated. Quantitation was performed by cutting the spots from the TLC strips and counting in a Beckman LS-133 scintillation counter at a pre-set efficiency of 0.5–1.5%. For some experiments, quantitation was performed using a Molecular Dynamics PhosphorImager. From the 32P value, the total P, produced, which includes both the cold and radioactive P, was calculated. In the single turnover experiments, the reaction mixture contained, on a molar basis, 3–10-fold more protein when compared with the ATP substrate. The data represent an average of duplicate or triplicate values for each variable.

### RESULTS

#### Purification of the Terminase Proteins and Composition of the Purified Proteins—

| Terminase Construct | gp16 | gp17 |
|---------------------|------|------|
| pr16                | 20   |      |
| pr17                | 5    |      |
| pr16 × 2             |      |      |
| pr15-17             | 1    | 1    |
| pr1617-17            |      |      |
| pr16-17              | 1    | 1    |
| pr16 + pr1L17        |      |      |

| **TABLE I** Recombinant constructs for overexpression of the packaging proteins gp16 and gp17 |
|----------------------------------|------|------|
| pr16 × 2                         | 20   |      |
| pr17 × 5                         | 5    |      |
| pr16 × 2 + pr1L17                |      |      |
| pr16-17 × 10                      |      |      |

**Notes:** T7 promoter; g17 ribosome binding site; hexahistidine tag; ompT tag; g16 coding sequence; g17 coding sequence; overlapping sequence at the 3' end of g16; g17 ribosome binding site. See "Experimental Procedures" for details. The numbers in the gp16 and gp17 columns represent estimated levels of gp16 or gp17 expression as a percentage of the total cell protein. + represents low but detectable levels of the expressed protein. The data in parentheses indicate the levels of expression in other pR16 + pR17-type constructs.

#### Immunoadsorption of gp17—

gp17 was partitioned out of solution by passing through an anti-gp17 IgG-protein A-Sepharose column. Antibodies against gp17 were raised in a rabbit using the most highly purified gp17 preparation (Jossan Laboratories). The specificity of gp17 antibodies was confirmed by Western blotting using crude cell lysates of uninduced and induced pRL-H17 extracts and control induced lysates. The purified anti-gp17 IgG and the pre-immune IgG were purified by 50% ammonium sulfate precipitation to remove any nonspecific IgG. The anti-gp17 IgG and pre-immune IgG were immobilized by SDS/PAGE and, more importantly, lacked any nonspecific nuclease or ATPase activities. The IgGs were immobilized by passing about 180 μg of purified IgG through mini-protein A-Sepharose columns (~200-μl beads) (25). Each column was thoroughly washed with the buffer, and about 3 μg of purified gp17 was passed through the column. The adsorbed samples, which were recovered in the flow-through from the pre-immune and the anti-gp17 IgG columns, were analyzed for ATPase activity.

**Phosphorylation of gp17**—The purified gp16, gp16, or the control proteins were incubated with either [γ-32P]ATP or [α-32P]ATP in Buffer P (10–30 μl) at 37 °C for 10–45 min. The reaction was terminated by adding EDTA to a final concentration of 10 mM. After taking a small aliquot of the sample (1 μl) for TLC to determine the ATPase activity, the proteins in the reaction mixture were precipitated by adding 10 volumes of 10% trichloroacetic acid. The samples were incubated on ice for 30 min. The precipitated proteins were recovered by centrifugation at 12,000 rpm for 20 min at room temperature. The pellets were washed twice with 1 ml of trichloroacetic acid followed by two additional washings with 1 ml of acetone to remove trichloroacetic acid. The pellets were dissolved in 50 μl of 1× SDS sample buffer, and the proteins were resolved by SDS-PAGE. The proteins were stained with Coomassie Blue, destained, dried, and autoradiographed. The 32P in the gp17 position was quantitated using the PhosphorImager.

#### Overexpression of the Phage T4 Terminase Proteins gp16 and gp17—

Using the phage T7 pET system, the terminase proteins gp16 and gp17 were expressed either independently or together in a number of configurations (Table I). When expressed independently, gp17 was overexpressed up to about 1% of the total cell protein, and gp16 was expressed up to about 20% of the total cell protein. However, when expressed together, regardless of the configuration, the level of expression was much lower, in most cases only up to about 1% of the total cell protein. Although most of the overexpressed gp16 (except OmpT) was in the soluble fraction, most of the gp17 partitioned into the insoluble fraction (compare lanes 4 for the insoluble fraction with lanes 3 for the soluble fraction in Fig. 1, panels A and C). Although large quantities of near homogeneous gp17 could be purified from the insoluble fraction, it could not be renatured into an active form having in vitro DNA-packaging activity. Therefore, we experimented with the growth conditions to maximize the expression of gp17 as a soluble protein. We found that more of the expressed gp17 was recovered in the soluble form when tryptone-phosphate medium was used and by lowering the growth temperature to 30 °C (compare lane 4 for the insoluble fraction with lane 3 for the soluble fraction in panel B). This is in agreement with a previous report showing the expression of foreign proteins in soluble form when the E. coli was grown in the enriched tryptone-phosphate medium (24).

Qualitative in vitro DNA-packaging and in vivo terminase assays showed that the recombinant terminase proteins were active. After considering various factors, we chose to focus our efforts on the His-tagged constructs pRL-H17 and pRL-H16. A careful analysis showed that the hexahistidine tag did not appear to interfere with the functionality of gp17. For instance, the His-gp17 was highly active for in vitro packaging of externally added phage T4 DNA (~106 plaque-forming units/10-μl lysate). Second, induction of His-gp17 expression resulted in the degradation of plasmid DNA, yielding a characteristic DNA smear extending throughout the lane, as has been observed with the native gp17 (21, 22); however, the extent of degradation with His-gp17 was lower than that with the native gp17. Finally, sequencing of the entire g17 from the pRL-H17 clone showed no mutations introduced by polymerase chain reaction.

**Purification of the Terminase Proteins and Composition of the Purified Proteins**—Attempts to purify gp16 and gp17 in a single step by directly passing the crude extract through the Ni2+-agarose column were not successful since some nonspecific E. coli proteins apparently competed with gp17 for binding to the column, decreasing the efficiency of gp17 binding and recovery. Therefore, gp17 was first partially purified by DEAE-Sepharcel column chromatography, and the eluate within a narrow window of 130–200 mM NaCl (Fig. 1B, lane 6), which contained most of the gp17, was further purified by Ni2+-
agarose affinity chromatography. Virtually all the proteins in the DEAE fraction except His-gp17 were removed either in the flow-through fraction (lane 9) or in the 60 mM imidazole fraction (lane 10). The bound gp17 was then eluted as a single peak at about 350 mM imidazole when the column was developed with a linear 60 mM–1 M imidazole gradient. The purified gp17 was estimated to be about 95% homogeneous (lane 11); a typical yield from the two-step protocol was about 2 mg of gp17/liter of induced culture.

gp16 was purified by the same two-step protocol (Fig. 1C). In the case of gp16, the eluate from even a narrower window between 170 and 200 mM NaCl from the DEAE-Sephaloc column was loaded onto the Ni2+-agarose affinity column (lanes 8–10). After washing off nonspecific proteins with the Bind buffer followed by 60 mM imidazole, gp16 was eluted as a single peak at about 375 mM imidazole. The purified gp16 was estimated to be about 95% homogeneous (lanes 16 and 17), and the yield was about 20 mg of gp16/liter of induced culture.

The composition of purified gp16 and gp17 was analyzed by SDS-PAGE. In the case of gp17, in addition to the predominant band, another band which is about 1 kDa shorter was seen (Fig. 1C, lane 11, marked with a small arrow). In some preparations, we also observed at least one additional band, which is about 3 kDa shorter than the major band (see Fig. 3A, panel D). Using the molecular mass standards, we confirmed that the predominant band corresponded to the full-length His-gp17 (27). The shorter form(s) was gp17, which apparently arose by a C-terminal truncation, as inferred from the following observations: (i) The lower band reacted strongly with the gp17 antibodies upon Western blotting and immunostaining; (ii) although the full length form is the predominant band in the crude extract, the ratio of full-length versus truncated forms varied in different purified preparations (in some, the truncated forms were in fact predominant, suggesting varying extents of proteolytic cleavage during purification); and (iii) the truncated forms quantitatively bound to the Ni2+-agarose column, which would be possible only if the N-terminal His-tag was intact.

In the case of gp16, the predominant band had a C-terminal truncation (Fig. 1C, marked with a small arrow), whereas the full-length protein appeared as a faint band immediately above the truncated band (large arrow). A C-terminal truncation was inferred based on the fact that the truncated gp16 quantitatively bound to the Ni2+-agarose column. This agrees with the recent data by Lin et al., who showed by mass spectrometry and amino acid sequencing that the predominant band in their purified preparation was truncated by nine amino acids at the C terminus (23). They suggested that, most likely, a -1 translational frameshift occurs at the 30-base pair-overlapping junction between the 3′-end of g16 and the 5′-end of g17, resulting in translation termination immediately after the Arg-155 codon (also see “Experimental Procedures”). In addition to the predominant truncation, we also observed additional shorter forms of gp16. These minor forms, visualized below the predominant truncated form, were seen upon overloading the gel (e.g. see Fig. 7, lanes 6–8). These may represent alternative translational terminations near the overlap region, although proteolytic cleavage cannot be ruled out.

Oligomeric State of the Packaging Proteins—The oligomeric state of the purified gp16 and gp17 was analyzed by native PAGE. In the case of gp17, a ladder of bands was observed that presumably corresponded to the monomer, dimer, trimer, etc. of gp17. However, the intensity of the monomeric gp17 versus the multimeric ladder varied in different experiments (e.g. compare lanes 1 and 2 in Fig. 1D). To confirm that the ladder bands were indeed gp17, individual bands were sliced out, the protein was extracted from gel slices, and the eluate was subjected to SDS-PAGE and Western blotting with gp17 antibodies. Indeed, the eluted protein strongly reacted with the gp17 antibodies (data not shown). The native PAGE results are also consistent with the preliminary gel exclusion chromatography and scanning transmission electron microscope data, which showed that a predominant fraction of gp17 existed as a monomer.3

3 In the case of gp16, a distinctly different pattern was re-

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Footnote:

3 M. Simon and V. Rao, unpublished data.
in contrast to gp17, a vast majority of gp16 molecules existed as microscopically revealed double rings, clusters (aggregates) of putative electron microscopy and scanning transmission electron microscopy with the earlier gel exclusion chromatography data (9). Negative band 2 and gp17 to package DNA was determined by the previous preparations obtained using the preparations was 1–2 orders of magnitude greater than that of preparations showed that the packaging activity of the gp17 gp16 exhibited none. Analysis of a number of purified gp17 (9), gp17 alone exhibited DNA-packaging activity, whereas vice versa. coli (sup’), as the plating bacteria since this strain allows plaque formation of all possible phage recombinants present in the reaction mixture. The wild type yield (5b) and the am or rII’ recombinant phage yield (5c) were determined by first plating the packaging reaction mixture on E. coli F901 and testing the plaque-forming units (p.f.u.) on CR 63 (λ) and vice versa.

| Terminase protein | Phage yield |
|-------------------|-------------|
| 1 None            | <50         |
| 2 gp16            | <50         |
| 3 gp17            | 2.8 × 10^4  |
| 4 a gp17          | 3.4 × 10^4  |
| b gp17 + gp16     | 4.8 × 10^4  |
| c                 | 8.8 × 10^4  |
| d                 | 3.4 × 10^4  |
| e                 | 2.4 × 10^4  |
| 5 a gp17          | 6.2 × 10^4 (total) |
| b                 | 5.1 × 10^4 (wild type) |
| c                 | 2.8 × 10^4 (am or rII’ recombinants) |

Native PAGE of gp16 revealed three bands; the most intense band was closest to the well (band 1), another intense band just below (band 2), and a faint band near the bottom of the gel (band 3) (Fig. 1D, lane 3). This pattern suggested that, in contrast to gp17, a vast majority of gp16 molecules existed as large multimeric complexes (bands 1 and 2) and is consistent with the earlier gel exclusion chromatography data (9). Negative electron microscopy and scanning transmission electron microscopy revealed double rings, clusters (aggregates) of double rings, and single rings. Although our preparations predominantly consisted of double rings and clusters of double rings, the mass and disposition of the rings are the same as the single and double gp16 rings observed by Lin et al., each single ring consisting of about eight gp16 monomers (23).

**DNA-packaging in Vitro**—The ability of the purified gp16 and gp17 to package DNA was determined by in vitro DNA-packaging assays (Table II). In agreement with our earlier data (9), gp17 alone exhibited DNA-packaging activity, whereas gp16 exhibited none. Analysis of a number of purified gp17 preparations showed that the packaging activity of the gp17 preparations was 1–2 orders of magnitude greater than that of the previous preparations obtained using the λ-P2 expression system. Although gp16 exhibited no DNA-packaging activity, it enhanced the gp17-dependent packaging activity by about 100-fold (Table II, lines 4, a–e). The enhancement was seen only when the gp17 concentration in the reaction mixture was limiting, suggesting that at low concentrations DNA-packaging requires both gp16 and gp17, but gp17 alone is sufficient at high concentrations. This may explain partly why 16am mutations are lethal, since the levels of gp17 in the infected cell are estimated to be very low.

A very interesting observation was that when we analyzed the total DNA-packaging efficiency, i.e. packaging efficiency of the externally added mature wild type DNA plus the endogenous am-rII’ concatameric DNA, it was about 100-fold greater than the packaging efficiency of the externally added DNA alone (lines 5a and b). This analysis has not thus far been possible because of low packaging efficiencies of our earlier preparations (10^4–10^5 plaque-forming units/μg of DNA), which were no greater than the background unadsorbed/revertant phage in the packaging extracts. From these data, it is clear that the T4 in vitro DNA-packaging system is not as inefficient as was once believed. In fact, these data would argue that the presumed inefficiency of the T4 packaging system was because the assay measures the packaging efficiency of externally added DNA, which is poor. Indeed, the packaging efficiency of the externally added wild type DNA was comparable with that of the recombined DNAs (am or rII’-DNAs), which clearly arose by in vitro recombination between the endogenous am-rII’-DNA and the externally added wild type DNA (line 5c).

The above data are potentially very interesting with regard to the mechanism of substrate recognition and packaging initiation by phage T4 terminase and also question the notion that the exogenously added mature DNA is packaged end to end. It means that it is unlikely that the preferred mode of T4 in vitro system is to package the unit-length DNA molecule from one end to the other using the already cut termini for packaging initiation and termination. Most of the measured packaging of wild type DNA may indeed represent the packaging of endogenous concatameric DNA after a recombinational exchange of its am and rII’ markers with the exogenous mature wild type DNA (28). Presumably, structural features of the endogenous concatameric DNA and/or proteins associated with it may favor assembly of the terminase complex on the endogenous DNA, facilitating preferential packaging initiation. This should not, however, be confused with the fact that the T4 system (using purified gp17) is capable of performing end-to-end packaging of the externally added foreign DNA such as the P1 plasmid-based pNS88, pNS-Ada10, etc. (Ref. 29 and data not shown). Our argument however, is that when the externally added packaging substrate is the mature T4 DNA, the highly recombinogenic T4 system and the 100-fold higher packaging efficiency for the endogenous DNA would bias the system against the end-to-end packaging of individual externally added T4 DNA molecules. Further experiments with defined polymerase chain reaction-amplified DNA fragments are currently under way to further investigate this issue.

**ATPase Activity**—Since a translocating ATPase is believed to be the driving force for DNA-packaging, the ability of the purified packaging proteins to hydrolyze ATP was analyzed. In numerous experiments, gp16 did not show significant hydrolysis of ATP regardless of the concentration of ATP used (Fig. 2, lane 2 (23)). On the other hand, gp17 showed an ATPase activity; incubation of gp17 with [γ-32P]ATP resulted in the generation of inorganic 32P (lane 3), suggesting that gp17 hydrolyzed the β-γ phosphodiester bond, generating ADP and 32P (the position of P was verified using the 32P product from alkaline phosphatase and Na+K+-ATPase reactions as a standard). To establish this point definitively, gp17 was incubated with [α-32P]ATP, and the reaction products were resolved by TLC (Fig. 2, panel B). Nonradioactive standards of AMP, ADP, and ATP were resolved on the same strip, and their positions were visualized under the 254-nm UV light (Fig. 2, lane 6). Comparison of the reaction products in Fig. 2, lane 5, to the migration pattern of the standards (lane 6) clearly showed that the 32P product corresponded to ADP, which is the product expected upon the hydrolysis of the β-γ phosphodiester bond.

**Evidence That the ATPase Activity Is Inherently Associated with gp17**—A number of approaches were employed to test whether the ATPase activity exhibited by purified gp17 is inherently associated with gp17 as opposed to a minor contaminant in the preparation.

The gradient elution profile of gp17 and the in vitro DNA-packaging and ATPase activities from the Ni2+-agarose column

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4 M. Simon, A. Zlotnick, and V. Rao, unpublished data.
5 M. Simon, personal communication.
were compared (Fig. 3A). In numerous purifications, a close overlap of gp17 elution profile and both these activities was evident. The relative concentration of gp17 in the peak gradient fractions 22 to 27 (panel I) mirrored the plaque-forming units produced in an in vitro DNA-packaging assay (panel III) and the \(^{32}P\) liberated in the ATPase assay (panel II).

An immunoadsorption approach was used to deplete gp17 from solution and see whether it resulted in a corresponding depletion of the ATPase activity. Purified gp17 was passed through an anti-gp17 IgG/protein A-Sepharose column as well as a control pre-immune IgG/protein A-Sepharose column, and the flow-through fractions were analyzed (Fig. 3B). The data showed that passage of gp17 through the anti-gp17 IgG column resulted in depletion of gp17 (panel I) and a much reduced ATPase activity (panel II).

**Characteristics of the ATPase**—The nucleotide specificity of gp17 ATPase was assessed by quantitating the inorganic \(^{32}P\) released using ATP, dATP, GTP, UTP, TTP, or CTP as a substrate. As shown in Table III, the gp17 ATPase is highly specific to ATP; dATP was also cleaved but at a reduced efficiency. None of the other NTPs were hydrolyzed to a significant extent.

The catalytic parameters \(K_m\) and \(K_{cat}\) of gp17 ATPase were quantitated (Table IV). The \(K_m\) for ATP hydrolysis was about 110 \(\mu\)M, and the \(K_{cat}\) was about 2 molecules of ATP hydrolyzed/gp17 subunit/min. These data, in particular the low \(K_{cat}\), are consistent with the notion that the basal ATPase activity of the putative translocating ATPase must be low when it is not coupled to DNA translocation.

We have also analyzed and quantitated the effect of various reaction components on the gp17 ATPase activity. Variables such as salt concentration and the presence of non-ionic detergents such as Triton X-100 did not significantly affect the ATPase activity. Interestingly, the gp17 ATPase activity did not show a significant DNA dependence when the substrate used was either the mature T4 DNA or various plasmid DNAs. Finally, the ATPase activity also was not significantly affected by the presence of gp20 portal protein rings or the purified empty proheads (data not shown; proheads themselves showed no ATPase activity).

**gp16 Enhanced the gp17 ATPase Activity**—We reported earlier that, under limiting concentrations of gp17, the gp17-associated in vitro DNA-packaging activity was enhanced by about 100-fold in the presence of gp16 (Ref. 9, Table II). This was recently confirmed by Lin et al. (23). However, as evident from Fig. 2, gp16 itself did not exhibit a significant ATPase activity. One hypothesis is that, although gp16 does not possess an ATPase activity, it may stimulate the gp17-associated ATPase, which in turn may be responsible for the enhancement of in vitro DNA-packaging.

To test this hypothesis, gp16 was added to the ATPase reaction mixture, and its effect on the ATPase activity was determined. A striking result was that, in numerous experiments, the gp17 ATPase activity was dramatically stimulated in the presence of gp16 (Fig. 4, panel A) with lanes 2 (both gp16 and gp17) and, panel B, compare lane 3 (gp17 alone) with lane 4 (both gp16 and gp17).

A number of parameters of gp16 enhancement were characterized, and gp16 specificity was rigorously tested. The enhancement of gp17 ATPase was specific to gp16, since no such enhancement was observed in the presence of a nonspecific protein such as acetylated BSA (Fig. 4, panel A, lanes 3). Neither did gp16 significantly enhance the ATPase activity of
The NTPase assays were performed as described under “Experimental Procedures” using purified gp17 (1 μM) and [α-32P]NTP (150 μM). [32P]NTP was quantitated by cutting the TLC strips and scintillation-counting. The activity with ATP (37.5 pmol hydrolyzed/assay) was taken as 100% and used as a reference to determine the relative activity toward the other NTP substrates. Each value represents an average of triplicate assays.

| Substrate | NTPase activity |
|-----------|-----------------|
| ATP       | 100%            |
| dATP      | 60%             |
| CTP       | 1.2             |
| GTP       | 0.4             |
| TTP       | <0.1            |
| UTP       | <0.1            |

### Catalytic properties of gp17 ATPase

The purified gp17 was incubated with varying concentrations of ATP (10 μM–5 mM) in duplicate or triplicate assays. The concentration of the cold ATP was varied while keeping a fixed amount of [γ-32P]ATP. The 32P formed was quantitated either by scintillation-counting or phosphorimaging. From the 32P value, the total P formed, which includes both the cold and radioactive P, was calculated. Km values were determined from Lineweaver-Burk plots. The Kcat is defined as the number of ATPs hydrolyzed/gp17 subunit/min under saturating concentrations of ATP (5 mM).

| Terminase protein(s) | K_m (μM) | K_cat (min⁻¹) |
|----------------------|----------|--------------|
| Gp17                 | 110       | 2            |
| Gp17 + gp16          | 256       | 107          |

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The data clearly ruled out the first hypothesis and are in support of the second hypothesis. Although the third hypothesis is still a viable one, other evidence shown below would argue against it.

### gp16 Is Phosphorylated in the Presence of ATP

To further investigate the biochemical pathway of the gp17 ATPase function, we cross-linked ATP to gp17 in the presence of UV light to analyze the binding parameters. gp17 was incubated with [γ-32P]ATP, UV-cross-linked at 310 nm, and analyzed by SDS-PAGE and autoradiography. Surprisingly, a radioactive gp17 complex was stable to precipitation by trichloroacetic acid and SDS-PAGE.

The specificity of gp17 phosphorylation was evident in a number of experiments. In the control experiments, no incorporation of 32P into nonpivious proteins such as acetylated BSA was evident (Fig. 5, panel C, lanes 5 and 13). and the radioactive incorporation was completely quenched in the presence of 100 μM cold ATP (data not shown). Consistent with the fact that gp16 possesses no ATPase activity, no significant linkage was evident with gp16 at twice the molar concentration of gp17 (e.g. lanes 3 and 4).

The data clearly ruled out the first hypothesis and are in support of the second hypothesis. Although the third hypothesis is still a viable one, other evidence shown below would argue against it.

The above data implicated a specific gp16-gp17 interaction underlying the ATPase enhancement. This was also supported by the fact that the extent of enhancement was dependent on the gp16-gp17 ratio. As shown in Fig. 4D, a proportional increase in the ATPase activity was evident as the ratio of gp16 to gp17 increased. Maximum enhancement was obtained at 6–8 molecules of gp16 to 1 molecule of gp17.

**gp16 Enhancement Was Due to an Increase in Catalytic Rate of ATP Hydrolysis**—Three hypotheses were put forward to explain the gp16 enhancement of gp17 ATPase activity. First, the enhancement was due to an increase in the affinity of the gp16 ATPase toward the ATP substrate. Since the enhancement was proportional in the presence of gp16, the Km for ATP hydrolysis in the presence of gp16 was 256 μM and 107 ATPs hydrolyzed/gp17 subunit/min, respectively. In comparison with the corresponding values with gp17 alone, these data clearly demonstrated that the Km had not changed significantly in the presence of gp16 (Table IV); in fact, an increase in Km was observed, suggesting that the affinity toward ATP has decreased somewhat in the presence of gp16. On the other hand, the Kcat for ATP hydrolysis had increased by 53-fold in the presence of gp16.

These data clearly ruled out the first hypothesis and are in support of the second hypothesis. Although the third hypothesis is still a viable one, other evidence shown below would argue against it.

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A phosphorylated gp17-P is formed upon incubation of gp17 with \([\gamma^{32}\text{P}]\text{ATP}\). A, ATPase assay. Lanes 1, 6, and 11, buffer controls. Lanes 2, 7, and 12, gp17 (2.3 \(\mu\)M). Lanes 3, 8, and 13, gp17 (2.3 \(\mu\)M) + gp16 (4.1 \(\mu\)M). Lanes 4, 9, and 14, gp16 (4.1 \(\mu\)M). Lanes 5, 10, and 15, acetylated BSA (2 \(\mu\)M). B, the samples from A were analyzed for protein by SDS-PAGE and Coomassie Blue staining (see “Experimental Procedures”). Lane S, purified gp16 and gp17 standards. C, autoradiogram of B showing the \([\alpha^{32}\text{P}]\)labeled gp17 band. Only the lanes showing \([\alpha^{32}\text{P}]\)gp17 band are marked.

Fig. 5. A phosphorylated gp17-P is formed upon incubation of gp17 with \([\gamma^{32}\text{P}]\text{ATP}\). A, ATPase assay. Lanes 1, 6, and 11, buffer controls. Lanes 2, 7, and 12, gp17 (2.3 \(\mu\)M). Lanes 3, 8, and 13, gp17 (2.3 \(\mu\)M) + gp16 (4.1 \(\mu\)M). Lanes 4, 9, and 14, gp16 (4.1 \(\mu\)M). Lanes 5, 10, and 15, acetylated BSA (2 \(\mu\)M). B, the samples from A were analyzed for protein by SDS-PAGE and Coomassie Blue staining (see “Experimental Procedures”). Lane S, purified gp16 and gp17 standards. C, autoradiogram of B showing the \([\alpha^{32}\text{P}]\)labeled gp17 band. Only the lanes showing \([\alpha^{32}\text{P}]\)gp17 band are marked.

Both with \([\gamma^{32}\text{P}]\)ATP and \([\alpha^{32}\text{P}]\)ATP. The data showed that gp17 was linked to \(32\text{P}\), since labeling was seen only with \([\gamma^{32}\text{P}]\)ATP (Fig. 5, panel C, lanes 2, 3, and 7) but not with \([\alpha^{32}\text{P}]\)ATP (panel C, lanes 12 and 13).

Formation of Phosphorylated gp17 Follows the Course of ATP Hydrolysis—A consistent result in many experiments was that the phosphorylated gp17 can be trapped only under conditions of low catalytic rates for ATP hydrolysis but not under high catalytic rates. For instance, it was visualized either in the 4 °C samples (Fig. 5, panel C, lanes 2 and 3) or in the 37 °C samples only when gp17 alone was present (lane 7) but not (or at a much reduced extent) in the samples containing both gp16 and gp17 at 37 °C (lane 8), wherein the catalytic rates are known to be the highest. When the time course of the reaction was followed under conditions of low catalytic rates, the progression of ATP hydrolysis precisely mirrored that of \(32\text{P}\)-gp17 formation (Fig. 6). The fraction of \(32\text{P}\)-gp17 at any given time point (panel A) was approximately one-tenth of the total \(32\text{P}\) released (panel B).

gp16 Enhanced the Release of \(P_1\) from the gp17-P Complex—Is the gp16 enhancement of the gp17 ATPase due to an enhanced rate of \(P_1\) release from the gp17-P complex? To address this question, gp17 was first phosphorylated in the absence of gp16 at 37 °C (low catalytic rates). The reaction mixture was then diluted with gp16, and the release of \(32\text{P}\) from the gp17-P was quantitated. The data showed that, indeed, gp16 stimulated a partial release of \(P_1\) from the gp17-P complex. As shown in Fig. 7, in the control experiments neither the buffer (lanes 2 and 3) nor ATP (lanes 4 and 5) could stimulate the release of \(P_1\) from the complex. In additional controls, no further labeling of gp17 occurred during the incubation conditions (lane 8) nor was any labeling evident with gp16 or acetylated BSA (lanes 8 and 9). But incubation of the \(32\text{P}\)-gp17 complex with gp16 stimulated the release of about 40% of the \(32\text{P}\) from the complex (lanes 6 and 7). Interestingly, the released \(32\text{P}\) was transferred to gp16.

**DISCUSSION**

Although a number of interesting models have been proposed for DNA translocation, viz. portal protein as a translocating rotor (30, 31), topoisomerase nicking and resealing, (32), osmotic pump (33), tracking along the DNA (1, 2), and conformational switching (34), there is as yet no direct evidence for any of the models, although the rotating portal vertex model (30) has gained much interest in recent years. A consensus has emerged, however, with regard to the energy source. It appears that a translocating ATPase “motor” provides the driving force for the thermodynamically uphill DNA translocation. In fact, two defined in vitro DNA-packaging systems estimate that hydrolysis of one ATP is coupled to translocation of two base pairs of DNA (35, 36). Although the translocating ATPase has not yet been identified for any phage system, genetic and biochemical evidence point to the large terminase subunit as a strong candidate (6–8). With the ultimate goal of generating a molecular picture of
the translocating ATPase in phage T4, we asked in this study, do either or both of the packaging/terminase proteins exhibit an ATPase activity? If so, do either or both exhibit characteristics consistent with a translocating ATPase? To address these questions, we have constructed and tested a number of new recombinants for overexpression of the packaging proteins and developed simple, rapid procedures for purification of milligram recombinants for overexpression of the packaging proteins and experimental conditions used.

An ATPase activity is associated with the purified gp17 but not with gp16. This activity hydrolyzed ATP into ADP and P_i and specifically cleaved ATP and dATP but not the other NTPs. These data are consistent with the fact that gp17, but not gp16, possesses two consensus ATP binding sites (10, 35). This is also analogous to other large terminase proteins such as the gpA from phage λ and gp19 from phage T3, which consist of 1–3 consensus ATP binding site(s) and exhibit an ATPase activity (7, 39). Our evidence from a number of approaches clearly demonstrated that the observed ATPase activity is indeed inherently associated with gp17. The catalytic properties of the ATPase, a weak \( K_{\text{cat}} \) in the absence of DNA-packaging, its specific enhancement by gp16, and concurrent stimulation of both DNA-packaging and ATPase by gp16, provide compelling functional linkage between the ATPase and gp17 and its modulation by gp16-gp17 interactions.

The appearance of an acid-stable phosphorylated gp17 is an interesting observation and one that has not been reported in any phage terminase system. Its formation was observed with \( [\gamma^{32}\text{P}]\text{ATP} \) but not with \( [\alpha^{32}\text{P}]\text{ATP} \) under conditions of low catalytic rates but not under high catalytic rates and in the presence of gp16. These data are consistent with the hypothesis that gp17-P is an intermediate in the gp17 ATPase catalytic cycle and its hydrolysis to release P\(_i\) is a rate-limiting step. Consequently, gp17 by itself would be a weak ATPase, but in the presence of gp16, the kinetic limitation is overcome by gp16-gp17 interactions, thus enhancing the catalytic turnover. This behavior in some respects is similar to that of the P-type ATPases such as the Na\(^+\)/K\(^-\)-ATPase, in which the formation of an acid-stable enzyme-P intermediate in the catalytic pathway has been well documented (40). More in-depth biochemical analyses of the mechanistic relevance of gp17-P in ATPase catalysis and gp16 enhancement, including an alternative possibility that the gp17-P formation may be due to a gp17-associated protein kinase activity, are currently under investigation.

The most important discovery in this study, which has also not been reported in any other phage terminase system, is the enhancement of gp17 ATPase activity by >50-fold by the small terminase subunit gp16. The enhancement is specific and is dependent on the ratio of gp16 to gp17 in the reaction mixture. The data also show that the enhancement was due to an increase in the catalytic rate of the ATPase but not due to an increase in its affinity toward the ATP substrate. Previously, we reported that gp16 enhanced the gp17-associated in vitro DNA-packaging activity by about 100-fold (9). Although these could be coincidental observations, it is tempting to link the enhanced ATPase catalysis to enhanced DNA translocation. Recent evidence also supports such a linkage. We have recently constructed mutants in the critical N terminus proximal ATP binding site of gp17. Any substitution at the conserved Lys-166 residue within the putative Walker-A motif SQR\(_{166}\) of the consensus ATP binding site I was lethal (21), and preliminary data indicate that the gp17-K166G mutant lost both the in vitro DNA-packaging activity and the gp16-stimulated ATPase activity.\(^6\)

The established role of the small terminase subunit thus far has been in DNA substrate recognition. There is abundant evidence to show that the small subunit recognizes the cos (R1, R2, and R3 sites in cosB) or pac sequence on the viral chromosome, leading to the assembly of a holo-terminase-DNA complex (4), following which, the large terminase subunit makes a cut at a nearby site. Further events in the packaging pathway, for instance linkage of DNA to the empty prohead, interactions with the portal protein, and DNA translocation, were attributed solely to the large packaging subunit (3). Such a functional separation was also implicated even in the circularly permuted phage T4, which does not use a unique cutting site (41). However, the discovery in this study of gp17 ATPase enhancement by gp16 would implicate the small terminase subunit in a broader and more complex role in the DNA-packaging mechanism per se. Clearly, the large terminase subunit by itself is a weak ATPase. The observed level of its catalytic capacity \( K_{\text{cat}} \approx 2 \) is too low to account for the required \( K_{\text{cat}} \) of about 10^5/min/packaging complex for DNA-packaging. The latter value was inferred from the defined phage T3 and d29 in vitro DNA-packaging systems and is based on the translocation of approximately 2 base pairs of DNA per molecule of ATP hydrolyzed at a rate of about 30 kilobases/min (6, 35, 36).

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\(^6\) V. Rao and P. Lin, unpublished data.
Although these values may need correction, the important point is that, to account for the projected ATP consumption, either some component of the packaging machine must serve as the translocating ATPase, and with a high catalytic capacity, or the gp17 ATPase activity has to be dramatically stimulated upon the assembly of the packaging machine. There is yet no evidence for the first hypothesis. As reported here, no other component of the packaging machinery, the gp16, the gp20 portals, or the preheads, exhibited significant ATPase activity nor are there any consensus ATP binding sites evident in any of these components. On the other hand, the ability of gp16 to stimulate gp17 ATPase and the data on the putative gp17-P intermediate are consistent with the second hypothesis in that a pre-exiting translocating ATPase, but with a dramatically increased catalytic rate, may emerge consequent to the assembly of the packaging machine. Although such a hypothesis was implied earlier (42), this report presents the first biochemical evidence in that direction. Although the observed >50-fold stimulation in the catalytic rate still cannot account for the estimated catalytic rate required for DNA translocation, the formation of a holo-terminase complex should be viewed only as a partially assembled packaging machine. Further stimulation may occur after docking the holo-terminase-DNA complex onto the portal vertex of the prehead receptor. Furthermore, as believed, if the terminase complex functions as a multimeric unit, the $K_{\text{cat}}$ value of the packaging unit could be numerically increased by 6–12-fold (assuming that one gp17 subunit interacts with either two or one gp20 portal subunits).

Specific stimulation of a weak basal ATPase activity by a functionally relevant second component has been documented numerically increased by 6–12-fold (assuming that one gp17 Walker-A and Walker-B motifs, many of which have conservational properties discussed above provide a plausible connectivity between the gp17 ATPase and DNA translocation in phage T4. We have recently reported that the consensus ATP binding site I in gp17 is critical for function (21). Using a powerful combinatorial approach, we have isolated numerous mutants in the putative Walker-A and Walker-B motifs, many of which have conservative substitutions, yet exhibited a null phenotype.7 The biochemical analyses of these mutants to elucidate the relationship between the gp16-stimulated ATPase, ATP binding site I, and DNA translocation are in progress.

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7 V. Rao, M. Mitchell, and K. Goetzinger, unpublished data.