The terminase enzyme from bacteriophage λ is composed of two viral proteins (gpA, 73.2 kDa; gpNu1, 20.4 kDa) and is responsible for packaging viral DNA into the confines of an empty procapsid. We are interested in the genetic, biochemical, and biophysical properties of DNA packaging in phage λ and, in particular, the nucleoprotein complexes involved in these processes. These studies require the routine purification of large quantities of wild-type and mutant proteins in order to probe the molecular mechanism of DNA packaging. Toward this end, we have constructed a hexahistidine (hexa-His)-tagged terminase holoenzyme as well as hexa-His-tagged gpNu1 and gpA subunits. We present a simple, one-step purification scheme for the purification of large quantities of the holoenzyme and the individual subunits directly from the crude cell lysate. Importantly, we have developed a method to purify the highly insoluble gpNu1 subunit from inclusion bodies in a single step. Hexa-His terminase holoenzyme is functional in vivo and possesses steady-state and single-turnover ATPase activity that is indistinguishable from wild-type enzyme. The nuclease activity of the modified holoenzyme is near wild type, but the reaction exhibits a greater dependence on Escherichia coli integration host factor, a result that is mirrored in vivo. These results suggest that the hexa-His-tagged holoenzyme possesses a mild DNA-binding defect that is masked, at least in part, by integration host factor. The mild defect in hexa-His terminase holoenzyme is more significant in the isolated gpA-hexa-His subunit that does not appear to bind DNA. Moreover, whereas the hexa-His-tagged gpNu1 subunit may be reconstituted into a holoenzyme complex with wild-type catalytic activities, gpA-hexa-His is impaired in its interactions with the gpNu1 subunit of the enzyme. The results reported here underscore that a complete biochemical characterization of the effects of purification tags on enzyme function must be performed prior to their use in mechanistic studies.

Terminase enzymes are found in all of the large, tailed double-stranded DNA bacteriophages and are responsible, at least in part, for the insertion of a viral genome into an empty, pre-formed shell or procapsid (1–3). In bacteriophage λ, the enzyme is composed of large (gpA, \(^{1} 73.2 \text{ kDa}\)) and small (gpNu1, 20.4 kDa) subunits that are isolated as a gpA\(_{1}\)gpNu1\(_{2}\) holoenzyme complex (4–6). These proteins are an integral part of a series of nucleoprotein intermediates involved in DNA packaging, however, and the subunit stoichiometry in each of these intermediates likely differs (7–9).

The preferred packaging substrate in phage λ consists of a linear concatemer of viral genomes, linked head-to-tail and up to 10 genomes in length (10). The cohesive end site (cos) of the viral genome represents the junction between successive genomes in the concatemer and is the site where the terminase subunits assemble to initiate DNA packaging (11–14). A model for genome packaging has been proposed as follows (7–9) (see Fig. 1): 1) the terminase gpA subunit assembles as a symmetric dimer onto the cos\(_{N}\) subsite of cos; 2) cooperative binding of gpNu1 to three repeating R-elements found within the cos\(_{B}\) subsite is required for efficient gpA assembly at cos\(_{N}\) and the stability of the resulting pre-nicking complex; 3) the endonuclease activity of the gpA subunit nicks the duplex at cos\(_{N}\) and, after terminase-mediated strand separation, yields the mature 12-base single-stranded left end of a DNA bound and protected by the terminase subunits; 4) this stable nucleoprotein intermediate, known as complex I, next binds to an empty procapsid that triggers an ATP-dependent translocation across the duplex and initiates active DNA packaging; 5) upon encountering the next downstream cos in the concatemer (the end of the viral genome), terminase again nicks the duplex at cos\(_{N}\) and strand separation simultaneously releases the DNA-filled capsid and regenerates complex I, which again captures an empty procapsid; and 6) attachment of the tail to the DNA-filled capsid completes the assembly process and yields a fully infectious virus.

Genetic experiments have identified functional domains within the terminase subunits. An N-terminal domain of gpNu1 contains a putative helix-turn-helix DNA binding motif identified by sequence homology (15). A. Becker, cited in Ref. 7), and site-specific DNA binding has been localized to this terminase; gpA-hexa-His, a gpA subunit containing a hexahistidine purification tag at the C terminus of the protein; bp, base pair; β-ME, 2-mercaptoethanol; cos, cohesive end site, the junction between individual genomes in immature concatemeric λ DNA; gpNu1, the small subunit of phage λ terminase; GdmHCl, guanidinium hydrochloride; hexa-His-gpNu1, a gpNu1 subunit containing a hexahistidine purification tag at the N terminus of the protein; hexa-His terminase, phage λ terminase enzyme containing a hexahistidine purification tag at the C terminus of the gpA subunit; IHF, E. coli integration host factor; PAGE, polyacrylamide gel electrophoresis; phage λP1 A\(_{\text{wild-type}}\), bacteriophage lambda that carries a wild-type terminase holoenzyme; phage λP1 A\(_{\text{hexa-His}}\), bacteriophage lambda virus that carries a hexa-His terminase holoenzyme construct; reconstituted terminase holoenzyme, enzyme that was prepared by mixing the individually purified subunits; terminase holoenzyme, enzyme directly purified as a gpA\(_{1}\)gpNu1\(_{2}\) holoenzyme complex from cells simultaneously expressing both enzyme subunits; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; NI-NTA, nickel-nitrilotriacetic acid; pfu, plaque-forming units.

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This paper is available online at http://www.jbc.org
IHF binding element (indicated, as are the three gpNu1-binding elements (R1–R3) for the purification of phage protein subunits and have developed simple, one-step protocols for the purification of phage protein, even with "cleavable" purification tags where one or two amino acids remain after removal of the tag. It is thus imperative that the functional significance of these "minor" changes in protein structure be clearly defined prior to utilization of the constructs for detailed structural and mechanistic studies. We have therefore examined the effect of these hexahistidine tags on the catalytic competence of terminase holoenzyme and the isolated enzyme subunits.

EXPERIMENTAL PROCEDURES

Materials and Methods—Tryptone, yeast extract, and agar were purchased from Difco. Restriction enzymes were purchased from Promega. Mung bean nuclease was purchased from New England Biolabs. Guanidinium hydrochloride was purchased from Mallinckrodt. [γ-32P]ATP was purchased from ICN. Unlabeled nucleoside triphosphates and ampicillin were purchased from Sigma. Ni-NTA-agarose was purchased from Qiagen. All other materials were of the highest quality commercially available.

Bacterial cultures were grown in shaker flasks utilizing a New Brunswick Scientific series 25 incubator-shaker. Bacterial growth media and agar were prepared as described by Sambrook and co-workers (24). When required, kanamycin and ampicillin were added at 50 and 100 μg/ml, respectively. UV-VIS absorbance spectra were recorded on a Hewlett-Packard HP8452A spectrophotometer. Automated DNA sequence analysis was performed by the University of Colorado Cancer Center Macromolecular Resources Core facility or by the DNA sequencing facility at the University of Iowa. Both strands of the duplex were examined to verify the expected DNA sequence.

Bacterial Strains, DNA Preparation, and Protein Purification—The viral and bacterial strains and the plasmids used in these studies are shown in Table I. Plasmids pSF1 and pAFP1 were purified from the Escherichia coli strains C600[pSF1] (25) and JM107[pAFP1] (26), respectively, using Qiagen DNA Prep columns. Synthetic oligonucleotides used in this study were purchased from either Integrated DNA Technologies, Inc., or Life Technologies, Inc., and were used without further purification. Purification of wild-type terminase holoenzyme and the isolated wild-type gpA and gpNu1 subunits was performed as described previously (6, 27). E. coli integration host factor was purified from HN880 (generously provided by H. Nash, National Institutes of Health, Bethesda) by the method of Nash et al. (28). All of our purified proteins were homogenous as determined by SDS-PAGE and densitometric analysis using a Molecular Dynamics laser densitometer and the ImageQuant® data analysis package. Unless otherwise indicated, protein concentrations were determined spectrophotometrically using millimolar extinction coefficients (6, 27).

Construction of pQH101, a Hexa-His Terminase Holoenzyme Overexpression Plasmid—A vector that overexpresses terminase holoenzyme with six histidines fused to the natural C-terminal glutamic acid of the gpA subunit (see Fig. 2) was constructed by PCR methods using pASY20 as a PCR template (Table I). This plasmid contains a λ DNA extending from bp 2216 (SpIHI site) to bp 3522 (BsHII site) cloned into a pIBI30 (International Biotechnologies) background (29). pASY20 thus contains the wild-type λ sequence for the A gene (3’end), except for an XbaI site introduced at bp 2628 (Fig. 2) (29). The forward PCR primer was complementary to bp 2206–2229 in the A gene sequence and contained the SpHII site. The reverse PCR primer was complementary to bp 2623–2229 in the A gene sequence and contained the SpHII site. The reverse PCR primer was complementary to bp 2615–2638 in the A gene sequence, except that the TCC Ser codon at bp 2619–2621 (Ser-637, Fig. 2) was changed to an AGC. This introduced an A/II site at bp 2615 in the A sequence but maintained a wild-type serine codon. PCR amplification yielded the expected 415-bp fragment that was isolated, digested with SpHII and XbaI, and ligated into similarly digested pASY20. This afforded the plasmid pASY30 which was identical to pASY20 except for the A/II site introduced at bp 2615. pASY30 was cut with A/III, and the cohesive ends were removed by digestion with mung bean nuclease. The linearized plasmid was next digested with BsrEI (bp 3329) which deleted a 714-bp A/II-BsrEI fragment (Fig. 2). The following synthetic duplex 1 was ligated into doubly digested pASY30.

**DNA sequences are listed as described by Daniels et al. (41).**

![Image](https://example.com/image.png)

**FIG. 1. Model for terminase assembly at cos.** The cos region of the λ genome is shown at top. The three subsites, cosQ, cosN, and cosB, are indicated, as are the three gpNu1-binding elements (R1–R3) and the IHF binding element (I) found within cosB. The terminase subunits and IHF assemble at cos forming a pre-nicking complex which, in the presence of Mg2++, nicks the duplex ultimately yielding the stable packaging intermediate complex I. Complex I binds an empty procapsid which initiates an ATP-dependent insertion of viral DNA into the capsid (Active DNA Packaging). We note that the stoichiometry of the terminase subunits in each of the nucleoprotein intermediates remains speculative. Details are presented in the text.
where the BspEI restriction site is indicated in italics. The resulting plasmid, pQH70, contains the 3’ end of the A gene (bp 2633) with six histidine codons appended but is deleted for the BspEI segment between the sites at bp 2637 and 3229 of pASY20 (Fig. 2). To replace this missing plasmid DNA, pASY20 was digested with BspEI, and the 692-bp fragment was cloned into BspEI-linearized pQH70. The resulting plasmid was digested with Sse83871 (Panvera), and the 263-bp fragment containing the modified segment of the A gene (see Fig. 2) was ligated into the corresponding Sse83871 site of the terminase expression plasmid pQH101. The sequence from the SpaI site at 2216 through the end of the A gene was found to be as predicted from the manipulations. We note that protein expression in this cell line is heat-inducible.

Construction of Phage λ-P1 A<sup>hexa-His</sup>—A lysogen that expresses hexa-His terminase (λ-P1 A<sup>hexa-His</sup>) in place of the wild-type holoenzyme expression plasmid pQH101. The sequence from the SpaI site at 2216 through the end of the A gene was found to be as predicted from the manipulations. We note that protein expression in this cell line is heat-inducible.

**TABLE I**

<table>
<thead>
<tr>
<th>Strains and plasmids used in this study</th>
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<tbody>
<tr>
<td><strong>Strain/plasmid</strong></td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
</tr>
<tr>
<td>HN880</td>
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<tr>
<td>MF1427</td>
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<tr>
<td>MF1972</td>
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<td>MF2517</td>
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</tbody>
</table>

**Phage λ strains**

- λ-P1 (λ-P1<sup>wild-type</sup>)
- λ-P1 A<sup>hexa-HIS</sup>
- λ-P1 A<sup>hexa-HIS</sup>

<table>
<thead>
<tr>
<th><strong>Plasmids</strong></th>
<th><strong>pSF1</strong></th>
<th>Derivatives of pBR322 carrying λ DNA extending from bp 44,141 (HindIII) to 5,505 (BamHI). This fragment contains the late promoter p&lt;sub&gt;λ&lt;/sub&gt;, the lysis genes S, R, and cos, the terminase genes Nu1 and A, and genes W and B</th>
<th>This work</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJM1</td>
<td>Derivative of pSF1 containing two additional restriction sites, XbaI (bp 48,442) and EcoRI (bp 194)</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pJM1-&lt;sup&gt;hexa-HIS&lt;/sup&gt;</td>
<td>Derivative of pJM1 that contains a hexa-His-modified A gene</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pASY20</td>
<td>Derivative of pIB130 (International Biotechnologies, Inc.) carrying λ DNA segment from bp 2,216 (SphI) to 3,522 (BstEI)</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>pAF1</td>
<td>Derivative of pUC19 carrying λ DNA segment extending from bp 48,425 to 194</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>pCM101</td>
<td>Wild-type terminase expression vector pCM101 (30) yielding the hexa-His terminase holoenzyme expression plasmid pQH101</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pH6-A</td>
<td>Derivative of pKK77(-H) expressing gpa-hexa-His</td>
<td>This work</td>
<td></td>
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<tr>
<td>pH6-Nu1</td>
<td>Derivative of pKK77(-H) expressing hexa-His-gpNu1</td>
<td>This work</td>
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</table>

**Expression and Purification of gpA-Hexa-His—** One liter of 2× YT media containing 25 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 1% glucose, and 50 μg/ml ampicillin was inoculated with a 10-ml overnight culture of *E. coli* OR1265[pQH101] and maintained at 30°C until an optical density of 0.6 (600 nm) was obtained. The cells were then heat-induced and harvested as described previously for wild-type holoenzyme (6). The cell pellet was resuspended in 100 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl, 1 mM PMSF, 0.4 mg/ml lysozyme, and 10 μg/ml aprotinin, and placed on ice for 20 min. The cells were then lysed by sonication, and the insoluble cellular debris was removed by centrifugation (12,000 × g for 15 min, followed by 12,000 × g for 30 min). The clarified supernatant was mixed with 5 ml of Ni-NTA agarose followed by gentle shaking on ice for 1 h. The mixture was loaded into an empty column and bound protein was eluted with 2× 4-ml aliquots of 20 mM Tris-HCl buffer, pH 8, containing 500 mM NaCl. Bound protein was eluted in a stepwise fashion with 20 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl and increasing concentrations of imidazole (4 × 0.5 ml each, 100, 150, and 250 mM imidazole). Hexa-His terminase holoenzyme eluted in the 250 mM imidazole fractions that were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA, 7 mM β-ME, and 50% glycerol. The purified protein sample was stored at −70°C.

**Expression and Purification of gpA-Hexa-His—** One liter of 2× YT
media containing 25 mM NaH₂PO₄, pH 7.2, 1% glucose, and 50 µg/ml ampicillin was inoculated with a 10-ml overnight culture of E. coli BL21(DE3)pLH6-A cells, and the culture was maintained at 37 °C until an optical density of 0.6 (600 nm) was obtained. The cells were then induced with the addition of IPTG to 1 mM, and the culture was maintained at 30 °C for 3 h while the optical density increased. The cells were harvested by centrifugation, and the cell pellet was resuspended in 100 ml of 25 mM Tris-HCl, pH 8.0, buffer containing 100 mM NaCl, 2 mM EDTA, and 7 mM β-ME. Cell lysis was affected as described above for hexa-His-terminase holoenzyme, and the clarified supernatant was dialyzed against 25 mM NaH₂PO₄ buffer, pH 8.0, containing 500 mM NaCl and 25 mM imidazole. Five milliliters of Ni-NTA-agarose was added to the clarified supernatant which was gently shaken on ice for 1 h. The mixture was then loaded into an empty column, and the unbound protein was eluted with 2 × 4 ml aliquots of 50 mM NaH₂PO₄ buffer, pH 8.0, containing 500 mM NaCl and 50 mM imidazole. gpA-hexa-His was finally eluted with 7 × 0.5-ml aliquots of 50 mM NaH₂PO₄ buffer, pH 8.0, containing 500 mM NaCl and 250 mM imidazole. The elution fractions were examined by SDS-PAGE, and the appropriate fractions were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 2 mM EDTA, 7 mM β-ME, and 50% glycerol. The purified protein sample was stored at −70 °C.

Expression and Purification of Hexa-His-gpNu1—Growth and induction of E. coli BL21(DE3)pLH6-Nu1 cells was performed as described above for gpA, except with kanamycin; however, similar to the wild-type gpA (27, 31, 32), all of the expressed hexa-His-gpNu1 protein was found in the crude cell lysate (data not shown). The protein pellet was resuspended in 50 ml of 25 mM Tris-HCl, pH 8.0, buffer containing 100 mM NaCl, 2 mM EDTA, and 7 mM β-ME and re-pelleted by centrifugation (11,500 × g for 30 min). The washed pellet was next solubilized with 20 ml of 6 M guanidinium HCl (GdmHCl), pH 8.0, gently shaken on ice for 1 h, and insoluble material was removed by centrifugation (10,000 × g for 30 min). Five milliliters of Ni-NTA-agarose was added to the clarified supernatant which was gently shaken on ice for 1 h. The mixture was then loaded into an empty column, and the unbound protein was eluted with 2 × 4-ml aliquots of wash buffer (10 mM Tris-HCl, 100 mM NaH₂PO₄ buffer containing 6 M GdmHCl) at pH 5.9. Nonspecifically bound protein was eluted with 2 × 4-ml aliquots of wash buffer at pH 5.5, followed by 1 ml of wash buffer at pH 5.0. Hexa-His-gpNu1 protein was finally eluted with 6 × 0.5-ml aliquots of the same buffer at pH 4.5. The fractions were examined by SDS-PAGE, and the appropriate fractions were pooled, dialyzed against 25 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA, 7 mM β-ME, 2.5 mM GdmHCl, and 20% glycerol. The concentrated protein samples showed no signs of aggregation upon prolonged storage at −70 °C in the presence of 2.5 M GdmHCl.

Reconstitution of Terminase Holoenzyme—Reconstitution of terminase holoenzyme from the individually purified subunits was accomplished by mixing gpA and gpNu1 (wild-type or hexa-His-tagged) in a 1:2 molar ratio and incubating on ice for 5 min. We note that gpNu1 is stored as a concentrated stock in 2.5 M GdmHCl. Dilution of the protein sample during reconstitution simultaneously diluted the GdmHCl thus allowing the folding of gpNu1 into an active enzyme complex. Control experiments confirmed that GdmHCl concentrations as high as 50 mM had no effect on the catalytic activities of the enzyme (not shown).

In Vitro DNA Packaging Assay—The in vitro packaging assay was performed as described by Chow et al. (30). The reaction mixture (20 µl) contained 10 µl of a sonic extract of an induced culture of MF2517 (Table I) in 30 mM Tris-HCl buffer, pH 9.0, containing 10 mM MgCl₂, 3 mM spermidine, 6 mM putrescine, 7 mM β-mercaptoethanol, 1.5 mM ATP, 1.5 mM of mature λ c5875 Sam7 DNA, and the indicated concentration of either wild-type or hexa-His-tagged terminase holoenzyme. The sonicate extract was prepared as described previously (33) and provides proheads, tails, and assembly proteins required for virus assembly. The reaction samples were incubated at room temperature for 30 min to allow the assembly of infectious virus in vitro, and appropriate dilutions were plated on the supF strain MF1968 to determine virus yield.

In Vivo Virus Development (Virus Yield Assay)—MF1427 (IHF*) or MF1972 (IHF⁻), lysogenized with λ-P1 carrying either a wild-type A gene (λ-P1 A⁺) or with λ-P1 carrying the hexa-His-A gene (λ-P1 Ahexa-His), were grown overnight with aeration in L broth plus kanamycin at 31 °C. To determine the lysogen number of viable lysogens in each culture, the cultures were diluted into L broth (1:100 dilution) and grown to approximately 2 × 10⁶ cells/ml. Then portions of each culture were removed, diluted, and spread on L plates plus kanamycin; the plates were incubated overnight at 31 °C. Lysogens were induced by thermal induction at 42 °C for 20 min and then incubated at 37 °C for 60 min. The lysates were treated with chloroform, clarified, and plated for phage yield on the IHF⁺ strain MF1427.

Terminase Activity Assays—The cos cleavage assay was performed as described previously using pAFF1 as a nuclease substrate (6, 34). ATPase catalytic activity was examined as described previously (22). Where indicated, DNA (Scal-linearized pAFF1) was added to the ATPase assay mixtures at a concentration of 25–50 nM. The concentration of protein used in these assays is indicated in each individual experiment.

Kinetic Analysis—Steady-state ATPase activity was analyzed using linear regression techniques as described previously (6, 34). Only data within the linear portion of the reaction time course were used in the analysis. Diter for turnover experiments were analyzed according to both Equations 1 and 2, which describe monophasic and biphasic reaction time courses, respectively.

\[
\text{Products} = A - B \cdot \exp(-k_1 \cdot t) - C \cdot \exp(-k_2 \cdot t) \quad (\text{Eq. 1})
\]

\[
\text{Products} = A - B \cdot \exp(-k_1 \cdot t) - C \cdot \exp(-k_2 \cdot t) - \exp(-k_3 \cdot t) \quad (\text{Eq. 2})
\]

Where Products refer to the ADP formed at time t, and A is the extent of the reaction at t = 0. B and C describe the fraction of the observed rate associated with the slow and fast phases, respectively, and k₁ and k₂ represent the observed rate constants for the slow phase (k₆₋₇) and fast phase (k₇₋₈) of the reaction, respectively. The indicated constants were determined by nonlinear regression analysis of the experimental data using the Igor® data analysis program (Wave Metrics, Lake Oswego, OR) as described previously (6). A mono-exponential curve function describes appropriate data as the data if the values of the rate constants, k₁ and k₂, obtained by nonlinear regression analysis of the data to Equation 2 differed by less than 10-fold and 2) the χ² value obtained from fitting to Equation 1 was within an order of magnitude that obtained from fitting to Equation 2.

**RESULTS**

Construction, Expression, and Purification of Hexa-His-gpNu1, gpA-Hexa-His, and Hexa-His Terminase Holoenzyme—Previous studies have demonstrated that the C-terminal 38 amino acids of gpA define a functional domain that is required for interaction of complex I with the procapsid (18, 19). Interestingly, whereas the penultimate 5 amino acids are strictly required for procapsid binding (19), addition of up to 4 random amino acids to the C terminus of the gpA subunit did not appear to significantly affect the phage yield in E. coli (29). Based upon these data, we reasoned that the addition of six histidines to the C terminus of the gpA subunit might provide a convenient and efficient purification tag with little effect on enzyme function. Vectors that express the hexa-His-tagged gpA subunit, alone and co-expressed with the wild-type gpNu1 subunit, were thus constructed as described under “Experimental Procedures.” We further constructed a vector for the expression of an isolated gpNu1 subunit that contains a hexa-His tag at the N terminus. The C-terminal amino acid sequence of gpA-hexa-His and the N-terminal amino acid sequence of hexa-His-gpA-Nu1 are shown in Fig. 2. For the purpose of clarity, we use the term terminase holoenzyme to describe the enzyme that was directly purified as a gpA-gpNu1 holoenzyme complex from cells simultaneously expressing both enzyme subunits and the term reconstituted terminase holoenzyme to describe enzyme that was prepared by mixing the individually purified subunits.

Both hexa-His terminase holoenzyme and the isolated gpA-hexa-His subunit were efficiently expressed in E. coli (Fig. 3) and were found in the soluble fraction of the crude cell lysate (not shown). The proteins were purified from the clarified cell lysate in a single step as described under “Experimental Procedures” and yielded hexa-His-terminase holoenzyme (10 mg/liter cells) and gpA-hexa-His (12 mg/liter cells) preparations.

Given that gpA-gpNu1 interactions occur at the C terminus of the gpNu1 subunit, we did not attempt to construct a C-terminally hexa-His-tagged gpNu1 protein as we felt that reconstitution of terminase holoenzyme would be adversely affected.
that were >95% homogenous as determined by SDS-PAGE. The isolated hexa-His-gpNu1 subunit was similarly efficiently expressed in E. coli (Fig. 3). However, as is observed with the wild-type protein (31, 32, 35), hexa-His-gpNu1 was found exclusively in the insoluble cell lysis pellet (data not shown). The protein was solubilized from these inclusion bodies using 6 M guanidinium hydrochloride and purified to homogeneity in a single step using the nickel-chelate column as described under “Experimental Procedures” (29 mg/liter cells, 95% pure).

Hexa-His-gpNu1 has been stored as a concentrated protein solution in 2.5 M GdmHCl for up to 12 months with no evidence of aggregation and with no loss of catalytic activity.

**In Vitro Packaging Activity of Wild-type and Hexa-His Terminase Holoenzyme**—An in vitro packaging assay was used to examine the biological activity of the purified hexa-His-tagged terminase holoenzyme. This assay utilizes extracts of induced cultures of E. coli MP2517 (Table I) as a source of viral procapsids, tails, and all the necessary assembly proteins, except for a functional terminase enzyme (30). Addition of viral DNA and terminase holoenzyme allows virus assembly in vitro and yields fully infectious phage that are quantitated by their ability to form plaques (pfu). Fig. 4 shows that both wild-type and hexa-His terminase holoenzymes are biologically active and may be used to assemble infectious virus in vitro. We note, however, that the concentration dependence of pfu formation is slightly greater for hexa-His terminase than for the wild-type enzyme, with ~10-fold more enzyme required for similar phage yields. This difference disappears at enzyme concentrations greater than 10 nM, however, presumably because terminase is no longer limiting in the assay mixture.

**ATPase Activity of Wild-type and Hexa-His Terminase Holoenzyme**—We have previously identified two ATPase catalytic sites in terminase holoenzyme, a high affinity site in gpA (K_m ~5 μM) and a low affinity, DNA-stimulated site in gpNu1 (K_m...
1,300 and 500 μM, minus and plus DNA, respectively) (22, 23). Fig. 5A shows the steady-state rate of ATP hydrolysis using an ATP concentration of 1 mM and thus examines ATPase activity of both catalytic sites in the holoenzyme. The figure shows that the ATPase activity of both wild-type and hexa-His-tagged terminase holoenzymes are essentially identical. Moreover, the ATPase activity of both the wild-type and mutant holoenzymes is significantly stimulated by DNA, and the degree of stimulation is virtually identical for each (Fig. 5A, Table II). Furthermore, the steady-state rate of ATP hydrolysis by both enzymes using an ATP concentration of 20 μM, a concentration where catalytic activity is predominantly localized within the gpA subunit, is similarly identical (Fig. 5B and Table II).

In order to characterize more fully the effect of the hexa-His purification tag on ATP hydrolysis activity, we next examined the rate of ATP hydrolysis in a single-turnover experiment. In this experiment, the concentration of enzyme and ATP were both 25 nM, and a single catalytic turnover by the enzyme is observed. Fig. 5C shows that under these experimental conditions, both wild-type and hexa-His terminase holoenzymes hydrolyze ATP with a similar time course and that both proteins are similarly stimulated by DNA. These data were analyzed as described under “Experimental Procedures” yielding the observed rate constants presented in Table III.

**Endonuclease Activity of Wild-type and Hexa-His Terminase Holoenzyme**—Terminase holoenzyme possesses a site-specific

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**Fig. 3. Expression and purification of hexa-His terminase holoenzyme, gpA-hexa-His, and hexa-His-gpNu1.** Denaturing polyacrylamide gel showing the uninduced cell lysate (U), the 2-h post-induced cell lysate (I), and the final purified protein preparations (P) for terminase holoenzyme and the individual subunits as indicated. Lane M contains molecular mass standards as follows: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; glutamate dehydrogenase, 55 kDa; ovalbumin, 42 kDa; aldolase, 40 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

**Fig. 4. In vitro DNA packaging activity of wild-type and hexa-His-tagged terminase holoenzyme.** The packaging assay was performed as described under “Experimental Procedures” using MF1427 (IHF+) as the plating bacteria. Phage λ-P1 A<sup>wild-type</sup> (●) possesses a wild-type terminase holoenzyme, and phage λ-P1 A<sup>hexa-His</sup> (○) possesses a gpA-hexa-His-tagged terminase enzyme.

**Fig. 5. ATPase activity of wild-type and hexa-His-tagged terminase holoenzymes.** Circles represent data obtained with wild-type holoenzyme in the absence (○) and presence (●) of DNA. Triangles represent data obtained with hexa-His holoenzyme in the absence (▲) and presence (●) of DNA. A, steady-state ATP hydrolysis by wild-type (solid line) and hexa-His (dashed line) terminase using an ATP concentration of 1 mM. The concentration of enzyme used in these experiments was 100 nM. B, steady-state ATP hydrolysis by wild-type (solid line) and hexa-His (dashed line) terminase using an ATP concentration of 20 μM. Steady-state ATPase assays were conducted as described under “Experimental Procedures” using an enzyme concentration of 100 nM. C, single-turnover hydrolysis of ATP by wild-type (solid line) and hexa-His (dashed line) terminase. ATPase assays were conducted as described under “Experimental Procedures” using enzyme and ATP concentrations of 25 nM.
nuclease activity that is required for excision of a single genome from a concatemeric precursor. The site at which terminase assembles and cleaves the duplex is known as cos, an abbreviation for the cohesive end site of the viral genome (see Fig. 1). Fig. 6A shows the results of a cos-cleavage activity assay for wild-type and hexa-His terminase holoenzymes. Whereas both enzymes exhibit significant activity in this assay, the time course for the hexa-His-tagged enzyme lags slightly behind that of wild-type terminase. We have suggested that assembly of the terminase subunits onto DNA is the rate-limiting step in the cos-cleavage reaction (6, 34), and these data suggested that the hexa-His-tagged holoenzyme might have impaired DNA binding interactions. In order to explore more fully this possibility, we examined the cos-cleavage activity of these enzymes in the absence of E. coli integration host factor (IHF). IHF, although not strictly required for cos-cleavage activity in vitro or virus assembly in vivo, stimulates these reactions (6, 36, 37); however, IHF becomes essential for plaque formation when terminase assembly at cos is impaired by mutations in the cos sequence of the λ genome (26, 38). Fig. 6B demonstrates that the cos-cleavage activity of hexa-His terminase is significantly reduced in the absence of IHF, whereas that of wild-type enzyme is only modestly affected. These data support the postulate that the hexa-His-tagged holoenzyme is slightly impaired in its interactions with cos-containing DNA.

**Catalytic Activity of the Isolated Wild-Type and Hexa-His-Tagged Terminase Subunits**—We (17) and others (39) have previously demonstrated that whereas gpNu1 is devoid of cos-cleavage activity, the isolated gpA subunit possesses a weak nuclease activity that is strongly stimulated in the holoenzyme complex. Table IV demonstrates that the hexa-His-tagged subunits behave similarly to the wild-type subunits. Moreover, the mutant proteins may be reconstituted into catalytically competent holoenzyme complexes that possess cos-cleavage activity. This is particularly true for the hexa-His-tagged gpNu1 subunit that may be reconstituted into an enzyme complex that is fully active compared with wild-type reconstituted enzyme (Table IV). Consistent with the cos-cleavage activity of wild-type and hexa-His terminase holoenzymes, terminase reconstituted with a hexa-His-tagged gpA subunit possesses nuclease activity that is slightly impaired when compared with wild-type reconstituted enzyme (see Table IV and Fig. 6A).

Studies on the ATPase activity of the isolated terminase subunits have similarly revealed that the isolated gpNu1 subunit possesses weak ATPase activity while gpA efficiently hydrolyzes ATP (17, 34, 40). Consistently, the isolated hexa-His gpNu1 subunit possesses modest ATP hydrolysis activity, whereas hexa-His gpA hydrolyzes ATP at a rate similar to that of the wild-type subunit (Table V). Reconstitution of terminase with wild-type gpA and hexa-His gpNu1 subunits affords a holoenzyme with ATPase activity that is virtually identical to that obtained with a fully wild-type enzyme (Table V). Interestingly, however, reconstitution of the isolated gpA-hexa-His subunit with either wild-type or hexa-His-gpNu1 yields an enzyme complex that does not possess ATP hydrolysis activity beyond that of the isolated gpA-hexa-His subunit alone (Table V).

In order to characterize more fully the ATPase activity of the isolated gpA-hexa-His subunit, a single-turnover kinetic analysis of ATP hydrolysis was performed, and the results are presented in Fig. 7 and Table VI. The wild-type gpA subunit hydrolyzes ATP with a time course that is well described by a single exponential curve function (Fig. 7A) and with an observed rate that is similar to that observed for terminase holoenzyme in the absence of DNA (compare Tables III and VI). This confirms that at this concentration of ATP, hydrolysis in the holoenzyme is limited to the gpA subunit of the enzyme.

![Fig. 6. Endonuclease activity of wild-type and hexa-His-tagged terminase holoenzyme. Cos-Cleavage activity of wild-type (●) and hexa-His terminase (▲) in the presence (A) and absence (B) of IHF. The assay was conducted as described under “Experimental Procedures” using an enzyme and DNA concentration of 400 and 100 nm, respectively. IHF was included at a concentration of 100 nm as indicated.](http://www.jbc.org/)

**TABLE II**

<table>
<thead>
<tr>
<th>DNA</th>
<th>k&lt;sub&gt;obs&lt;/sub&gt; (1 mM ATP)</th>
<th>k&lt;sub&gt;obs&lt;/sub&gt; (20 μM ATP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type holoenzyme Minus</td>
<td>27 ± 3.7</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Hexa-His holoenzyme Minus</td>
<td>23 ± 2.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Wild-type holoenzyme Plus</td>
<td>138 ± 7.9</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Hexa-His holoenzyme Plus</td>
<td>156 ± 2.0</td>
<td>7.1 ± 0.2</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>DNA</th>
<th>k&lt;sub&gt;obs&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type holoenzyme Minus</td>
<td>0.097 ± 0.01</td>
</tr>
<tr>
<td>Hexa-His holoenzyme Minus</td>
<td>0.042 ± 0.003</td>
</tr>
<tr>
<td>Wild-type holoenzyme Plus</td>
<td>0.525 ± 0.019</td>
</tr>
<tr>
<td>Hexa-His holoenzyme Plus</td>
<td>0.445 ± 0.011</td>
</tr>
</tbody>
</table>
The terminase enzyme from bacteriophage lambda is responsible, at least in part, for the insertion of viral DNA into a procapsid (7–9). Our laboratories are interested in the genetic, biochemical, and biophysical aspects of phage λ assembly and, specifically, the nucleoprotein complexes required for genome packaging. These studies require a simple, rapid, and efficient purification scheme for the isolation of large quantities of wild-type and hexa-His-tagged holoenzymes, respectively (Table I). Initial studies demonstrated that although λ-P1 A hexa-His formed normal plaques an IHF + host, the mutant phage formed minute pinpoint plaques on an IHF-deficient host (data not shown). To quantify these effects, virus burst studies were performed using λ-P1 A wild-type and λ-P1 A hexa-His viruses that express wild-type and hexa-His-tagged terminase holoenzymes, respectively (Table I). Initial studies showed that the yield of λ-P1 A hexa-His was, within experimental error, identical to that of λ-P1 A wild-type when an IHF + strain was used. This result is similar to that observed in the in vitro DNA packaging (Fig. 4) and cos-cleavage (Fig. 6) assays, where only modest effects are observed between the wild-type and hexa-His-tagged holoenzymes in the presence of IHF. Whereas both phages showed significantly reduced yields in the IHF host, the deficit was much more pronounced for λ-P1 A hexa-His (Table VII). In fact, the observed decrease in burst size λ-P1 A hexa-His was sufficient to lower virus yield to a level just above that required for plaque formation on an IHF + host, thus yielding minute pinpoint plaques.

**Discussion**

Addition of DNA to the reaction mixture strongly stimulates the ATPase activity of the wild-type gpA subunit and the rate of ATP hydrolysis is more appropriately described by a double-exponential curve function. Analysis of these data yield the fast and slow rate constants presented in Table VI. Interestingly, the rate constant for the fast phase of the reaction is similar to that observed for terminase holoenzyme in the presence of DNA (compare Tables III and VI), suggesting that at this concentration of ATP, the observed stimulation of ATP hydrolysis in holoenzyme is mediated by DNA interactions with the gpA subunit.

Unlike wild-type protein, single-turnover ATP hydrolysis by the isolated gpA-hexa-His subunit exhibits biphasic behavior, even in the absence of DNA (Fig. 7B). Moreover, the rate constants obtained from analysis of these data are quite similar to the $k_{\text{slow}}$ and $k_{\text{fast}}$ rate constants obtained for wild-type gpA in the presence of DNA (Table VI). Only a small proportion of the time course (14–22%) is attributable to $k_{\text{fast}}$ for the hexa-His-tagged protein, however, and unlike the wild-type protein, gpA-hexa-His is unresponsive to DNA (Fig. 7B and Table VI).

The effect of hexa-His terminase in vivo virus development—λ-P1 A hexa-His was constructed by crossing phage λ-P1 Aam42 (Table I) and plasmid pJM1-hexa-His as described under “Experimental Procedures.” The Aam42 mutation is a lethal amber mutation located in the 5th-to-last codon of the A gene. Since there are only 4 codons between the Aam42 mutation in the phage and the six His codons of the modified A gene in the plasmid, it was expected that virtually all plaque-forming λ-P1 A hexa-His recombinants would contain the hexa-His modification. This was confirmed by sequencing studies on the λ-P1 A hexa-His recombinants, which directly demonstrated the presence of the hexa-His modification. The yield of plaque-forming (λ +) recombinants was 1.43 × 10^4 and 1.62 × 10^7 pfu/ml in crosses with pJM1-A wild-type and pJM1-A hexa-His plasmids, respectively. A control cross with no plasmid yielded less than 1 × 10^4 pfu/ml, indicating that revertants of the λ-P1 Aam42 phage were not contributing significantly to the titers of the cross-lysates. Since the frequencies of plaque-forming recombinants were essentially the same for each plasmid, we conclude that terminase with the hexa-His modification is functional in virus development.

**Effect of IHF on λ-P1 A wild-type and λ-P1 A hexa-His in Vivo Virus Development**—The distinct requirement for IHF in cos-cleavage by hexa-His terminase holoenzyme (Fig. 6) suggested that phage development in vivo might exhibit a similar requirement for IHF. This is especially important as the hexa-His purification tag might be expected to weaken interactions with the procapsid and thus require increased stability of complex I to ensure that progression toward active DNA packaging would occur. We thus examined the requirement for IHF on in vivo phage development by λ-P1 A wild-type and λ-P1 A hexa-His viruses that express wild-type and hexa-His-tagged terminase holoenzymes, respectively (Table I). Initial studies demonstrated that although λ-P1 A hexa-His formed normal plaques an IHF + host, the mutant phage formed minute pinpoint plaques on an IHF-deficient host (data not shown). To quantify these effects, virus burst studies were performed using λ-P1 A wild-type and λ-P1 A hexa-His lysogens in IHF + and IHF – hosts. Table VII shows that the yield of λ-P1 A hexa-His was, within experimental error, identical to that of λ-P1 A wild-type when an IHF + strain was used. This result is similar to that observed in the in vitro DNA packaging (Fig. 4) and cos-cleavage (Fig. 6) assays, where only modest effects are observed between the wild-type and hexa-His-tagged holoenzymes in the presence of IHF. Whereas both phages showed significantly reduced yields in the IHF + host, the deficit was much more pronounced for λ-P1 A hexa-His (Table VII). In fact, the observed decrease in burst size λ-P1 A hexa-His was sufficient to lower virus yield to a level just above that required for plaque formation on an IHF + host, thus yielding minute pinpoint plaques.

**Discussion**

The terminase enzyme from bacteriophage lambda is responsible, at least in part, for the insertion of viral DNA into a procapsid (7–9). Our laboratories are interested in the genetic, biochemical, and biophysical aspects of phage λ assembly and, specifically, the nucleoprotein complexes required for genome packaging. These studies require a simple, rapid, and efficient purification scheme for the isolation of large quantities of wild-type and mutant terminase holoenzymes, as well as the isolated enzyme subunits. The use of λ terminase in the biotechnology industry further underscores the need for simple and efficient purification protocols. Several vectors have been developed for the expression of terminase holoenzyme and the individual subunits in E. coli (27, 30), and several purification schemes have been published over the years (4, 6, 23, 31, 32). Even the most efficient protocols are relatively laborious and time consuming, however, for the routine purification of large quantities of enzyme. Purification of hexa-His-tagged proteins
Hexa-His Lambda Terminase

The concept provides a simple method to purify the proteins and avoids a proteolysis step that would add time and significant expense to the purification procedure.

Although the addition of 4 random amino acids to the C terminus of the gpA subunit did not affect terminase function in vivo, it was necessary to confirm that addition of the hexa-His purification tag similarly did not affect the catalytic activities of the enzyme. Initial experiments demonstrated that the tag only modestly affected phage development in vivo and phage assembly in vitro. A more detailed investigation is required, however, if these proteins are to be used for mechanistic studies on the enzyme, and we next examined the ATPase activity of the modified enzyme. Both steady-state and single-turnover kinetic experiments demonstrated that ATP hydrolysis by terminase holoenzyme is little affected by introduction of a hexa-His purification tag into the gpA subunit. Importantly, both enzymes hydrolyze ATP with identical rates, and importantly both enzymes are similarly responsive to DNA. Similarly, only modest differences were observed between the enzymes in our in vitro and in vivo terminases, whereas phage λ-P1 A<sup>hexaHis</sup> possesses a gpA-hexa-His-tagged terminase.

**Table VI**

Single turnover kinetic analysis of ATP hydrolysis by the wild-type and hexa-His-tagged gpA

<table>
<thead>
<tr>
<th>DNA</th>
<th>Fast</th>
<th>Slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wild-type gpA Minus</td>
<td>0.082 ± 0.007</td>
<td><em>a</em></td>
</tr>
<tr>
<td>Hexa-His gpA Minus</td>
<td>0.014 ± 0.033</td>
<td>0.610 ± 0.146</td>
</tr>
<tr>
<td>Wild-type gpA Plus</td>
<td>0.035 ± 0.040</td>
<td>0.895 ± 0.096</td>
</tr>
<tr>
<td>Hexa-His gpA Plus</td>
<td>0.017 ± 0.025</td>
<td>0.706 ± 0.089</td>
</tr>
</tbody>
</table>

* The data were equally well described by a single-exponential curve function, and the single rate constant is presented.

**Table VII**

Effect of IHF on phage development in vivo

<table>
<thead>
<tr>
<th>Phage A-P1 A&lt;sup&gt;wild-type&lt;/sup&gt;</th>
<th>Host IHF&lt;sup&gt;-&lt;/sup&gt;</th>
<th>Host IHF&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>% relative</td>
<td>pfu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>% relative</td>
</tr>
<tr>
<td>Wild-type gpA Minus</td>
<td>120.7 ± 16.5 pfu</td>
<td>(100%)</td>
</tr>
<tr>
<td>Hexa-His gpA Minus</td>
<td>107.5 ± 7.7 pfu</td>
<td>(89%)</td>
</tr>
</tbody>
</table>

* pfu, plaque-forming units. Data represent the average of at least three experiments.

This result was also apparent in experiments that similarly showed a significant requirement for IHF in the in vivo development of a hexa-His terminase-containing phage.

The mild defect in hexa-His terminase holoenzyme was significantly magnified in the isolated gpA-hexa-His subunit. Unlike wild-type protein, gpA-hexa-His exhibited biphasic ATPase kinetics in the absence of DNA and was completely unresponsive to the addition of polynucleotides. These data suggest that while gpA-hexa-His in a holoenzyme complex is relatively “normal,” the isolated subunit is more severely impaired, particularly in its interactions with DNA. Moreover, the isolated gpA-hexa-His subunit does not appear to interact appropriately with gpN1 to form a “natural” holoenzyme complex. Although the nuclease activity of terminase reconstituted from the gpA-hexa-His subunit is near wild type, this reconsit-
Hexa-His Lambda Terminase

tuted holoenzyme does not possess ATPase activity beyond that observed with the isolated enzyme subunits. Conversely, however, terminase reconstituted from wild-type gpA and a hexa-His-tagged gpNu1 subunit yields a catalytically competent holoenzyme complex with wild-type nuclease and ATPase activities.

During the course of this investigation, we have uncovered an interesting aspect of ATP hydrolysis by terminase holoenzyme. Previous studies have suggested that the steady-state rate of ATP hydrolysis by the gpA subunit in terminase holoenzyme was unaffected by DNA and that DNA-mediated stimulation of ATPase activity occurred primarily at the low affinity gpNu1 ATP-binding site of the enzyme (22). Contrary to these earlier results, however, the data presented here demonstrate that DNA directly stimulates ATP hydrolysis at the high affinity gpNu1 subunit under these conditions is essentially zero. ATPase activity at the low affinity gpNu1 subunit were thus performed to provide additional mechanistic insight. The single-turnover rate constant for ATP hydrolysis by the isolated gpA subunit was virtually identical to that of the holoenzyme, confirming that ATP hydrolysis at the gpNu1 subunit under these conditions is essentially zero. Addition of DNA to gpA resulted in biphasic kinetics. The mechanistic implications of the biphasic kinetics is, at first glance, indistinguishable from the wild-type enzyme. Moreover, phages that express these mutant proteins efficiently replicate in vivo, perhaps the most stringent test of biological activity. Upon closer examination, however, the catalytic properties of these proteins reveal subtle defects. The primary defect appears to be in the interaction of gpA-hexa-His with DNA. Although most apparent with the isolated subunit, effects are also observed with the hexa-His-tagged holoenzyme. The deficiency can be overcome with the addition of IHF to the reaction mixture, and these proteins will find utility in a number of experimental systems; however, the results reported here underscore that a complete biochemical characterization of the effects of purification tags on enzyme function must be performed prior to their use in mechanistic studies.

REFERENCES
Cloning, Expression, and Biochemical Characterization of Hexahistidine-tagged Terminase Proteins
Qi Hang, Liping Woods, Michael Feiss and Carlos Enrique Catalano

doi: 10.1074/jbc.274.22.15305

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