Packaging of double-stranded DNA into bacteriophage capsids is driven by one of the most powerful force-generating motors reported to date. The phage T4 motor is constituted by gene product 16 (gp16) (18 kDa; small terminase), gp17 (70 kDa; large terminase), and gp20 (61 kDa; dodecameric portal). Extensive sequence alignments revealed that numerous phage and viral large terminases encode a common Walker-B motif in the N-terminal ATPase domain. The gp17 motif consists of a highly conserved aspartate (Asp255) preceded by four hydrophobic residues (251MIYID255), which are predicted to form a β-strand. Combinatorial mutagenesis demonstrated that mutations that compromised hydrophobicity, or integrity of the β-strand, resulted in a null phenotype, whereas certain changes in hydrophobicity resulted in cs/ts phenotypes. No substitutions, including a highly conservative glutamate, are tolerated at the conserved aspartate. Biochemical analyses revealed that the Asp255 mutants showed no detectable in vitro DNA packaging activity. The purified D255E, D255N, D255T, D255V, and D255E/E256D mutant proteins exhibited defective ATP binding and very low or no gp16-stimulated ATPase activity. The nuclease activity of gp17 is, however, retained, albeit at a greatly reduced level. These data define the N-terminal ATPase center in terminases and show for the first time that subtle defects in the ATP-Mg complex formation at this center lead to a profound loss of phase DNA packaging.

In double-stranded DNA bacteriophages and herpes viruses, genome packaging is the fulfillment of viral DNA metabolism and an indispensable step in the assembly of infectious virions. Packaging is initiated by the endonucleolytic cleavage of the newly synthesized concatemeric DNA, which in T4 is a highly branched, head-to-tail polymeric network with very few, if any, ends (1, 2). The cleavage is catalyzed by hol terminase, a nonstructural, multisubunit complex, composed of the small subunit gp16 (18 kDa) and the large subunit gp17 (70 kDa) (3). The cleaved end is linked to the unique dodecameric portal vertex of the empty prohead through specific interactions between the terminase and the portal protein (gp20, 61 kDa). A packaging motor is thus assembled, which drives directional translocation of DNA into the prohead by an ATP-dependent mechanism (4). Following head filling, the terminase makes a second cut, and the terminase-DNA complex dissociates from the packaged head and reassociates with another empty prohead to continue head filling in a processive fashion.

Numerous DNA packaging models have been proposed, yet the basic mechanism is still a mystery (5–7). Single molecule packaging studies determined that the phage DNA-packaging motor is the strongest molecular motor measured to date (4). Cryo-electron microscopy imaging and atomic structure of phage Phi29 portal are consistent with the symmetry mismatch model, which postulates that the mismatch between the 5-fold viral capsid and 12-fold portal allows an ATP-driven portal rotation that is coupled to DNA translocation (8, 9). Simpson et al. (9) suggested that the subunits of a pentameric ATPase “fire” sequentially, promoting compression and relaxation of the portal, which is coupled to DNA movement. Unfortunately, it has been difficult to experimentally test the portal rotation model and no direct evidence is yet available.

Our ultimate goal is to elucidate the biochemical mechanism by dissecting the catalytic transitions of the packaging ATPase and linking them to specific steps in the pathway. Sequence analyses by Mitchell et al. (10) showed that numerous phages encode a common ATPase domain in the N-terminal half of the large terminase protein. Despite a lack of overall sequence similarity, the functional signatures of this ATPase are strictly conserved. Molecular genetics and biochemical evidence implicate this ATPase in DNA packaging. The evidence includes the following: (i) the T4 gp17 alone exhibits ATPase and in vitro DNA packaging activities (11, 12); (ii) both the activities are stimulated 50–100-fold by the small terminase protein gp16 (12, 13); (iii) the N-terminal Walker A motif, 165SRLGKT167, is critical for function and any substitution in the conserved residues results in a loss of stimulated ATPase and in vitro DNA packaging activities (14); and (iv) a critical catalytic carboxylate, Glu256, which is involved in the cleavage of the β,γ-phosphoanhydride bond of ATP (15), has been identified.

The catalytic center of ATPase motors consists of a conserved Walker B signature, which is required to position the ATP-Mg complex in a precise configuration for harnessing the ATP energy (16). It encodes a critical aspartate preceded by four hydrophobic residues that form a β-strand. Atomic structures and biochemical data show that the carboxylate coordinates with Mg²⁺ through a water bridge, which is chelated to the β,γ-phosphates of ATP (17, 18). Sequence analyses suggest that the gp17 sequence 252MIYID255 is a potential Walker B motif (10, 19). Its disposition with an adjacent catalytic carboxylate (Glu256) and other similarities in the region suggested an interesting connection between terminases and superfamilly 2 DEAD box helicases (10, 20). Additional Walker-B motifs have also been predicted in T4 and other phage terminases (21–23), but the functional relevance of any of these motifs is unknown. In this study, using a combination of bioinformatics, molecular genetics, and biochemical approaches, we have performed a functional analysis of the bacteriophage T4 DNA-packaging ATPase motor.
thorough and rigorous analysis of the functional significance of the 251-MIVD255 sequence in the context of its potential involvement in phage DNA packaging. Combinatorial mutagenesis clearly demonstrated that the Asp255 residue is extremely critical for function. Not even a highly conserved glutamate substitution was tolerated. Biochemical analyses using a series of purified mutant proteins revealed that the mutants exhibited defects in ATP binding and lost the gp16-stimulated ATPase and in vitro DNA packaging activities.

These data define the N-terminal ATPase catalytic center in gp17. It is remarkable that subtle perturbations in the catalytic pocket, such as an increase of the aspartyl side chain by a single C–C bond resulted in a profound loss of stimulated ATPase and DNA packaging activities. The evidence is compelling to propose that this ATPase is a core component of the phage DNA packaging machine and potentially involved in the ATP energy-coupled DNA translocation mechanism. Additionally, this study also reports the most thorough molecular genetic analysis of the Walker B motif from any ATPase motor, generating the first set of conditionally lethal mutants. Implications of these results for the structure and function of ATPase motors in general are discussed.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids—The Escherichia coli P301 (sup7) strain was used for marker rescue and preparation of packaging extracts. The E. coli suppressor strains were kindly provided by Dr. Jeffrey Miller (UCLA) (24). In addition, the tRNA suppressor plasmids for Gly, Ala, Cys, Lys, Glu/Gln,5 Arg, Pro, His, and Phe, were transferred into the genetic background of E. coli BL21(DE3). These strains, for reasons unknown, gave a stronger and more uniform suppression (14, 25). These and the original suppressor strains were used to rule out any ambiguities in the suppression efficiencies. Phage yields in the packaging reaction mixtures were determined by plating the samples on E. coli NS3529 (26).

The following E. coli strains (Novagen) (27) were used to transform pET plasmids containing the g17 inserts: (i) BL21, lacks T7 RNA polymerase, nonexpression strain, used for initial transformation of ligation mixtures and long term maintenance of recombinant plasmids; (ii) BL21 (DE3), expression strain, produces significant basal levels of T7 RNA polymerase, used for in vivo terminase lethality assays; (iii) BL21 (DE3) pLys-S, expression strain, produces very low basal levels of T7 RNA polymerase, used for overexpression and purification of gp17, in vivo terminase toxicity, and DNA cleavage assays.

Wild-type phage T4, 16am-17Q425am-RII(del), 17K166am, 17Q425am, and 17Y253am were prepared in this laboratory. The T7 expression vectors, pET-15b (His tag), and pET-9d (non-His tag) (Novagen) were used for recombinant constructions.

Mutagenesis and Clone Construction—A site-specific, PCR-directed, splicing by overlap extension strategy (14, 28, 29) was used to construct (i) Y253am mutant, (ii) combinatorial mutant libraries at residues Met251, 251Y1251, and Asp255, (iii) D255N,Q,K,H library, and (iv) D255E/E256D double mutant. The four primers and two successive PCRs required to engineer the respective mutation(s) were designed using the basic principles described earlier (14, 28, 29). The combinatorial libraries consisted of all possible nucleotide combinations at the mutant site. The g17 end primers consisted of the BamHI restriction site.

Amplifications were carried out using the purified wild-type phage T4 DNA as a template and the high fidelity “TaqPlus Precision” DNA polymerase (Stratagene). The amplified mutant g17 DNA having BamHI ends was digested with BamHI and purified by agarose gel electrophoresis using a Qiagen spin column protocol (Qiagen). The mutant DNA was ligated with the BamHI-linearized and dephosphorylated pET-15b vector. The ligated DNA was transformed into E. coli BL21, and amp6 colonies were selected.

The Y253am mutation was transferred into phage by recombinational marker rescue (14, 15). The suppression pattern was determined using the two sets of suppressor strains mentioned above. In addition, two control amber phages (17-K166am and 17-Q425am), whose suppressor patterns were well established by earlier studies (14), were used for comparison.

Combinatorial mutant libraries were screened using the amber mutant phages, Y253am and K166am (control; for some experiments, Q425am was used), and the phenotypes were scored according to the procedures described earlier (14, 15). More than one independent phage mutant/plasmid clone were sequenced to confirm the mutant phenotype.6

For overexpression and purification of mutant proteins, the inserts in the right orientation with respect to the T7 promoter were selected. The g17 end primers were designed so that cloning in the right orientation would fuse the N-terminal hexahistidine tag sequence (25 aa) in frame with gp17. Following marker rescue, the orientation of the g17 insert was tested, and the clones with the right orientation were directly used for overexpression and purification of the His-tagged gp17 by nickel affinity chromatography. Incorrectly oriented inserts were excised with BamHI and religated into pET-15b, and clones with the right orientation were selected.

Non-His-tagged recombinants were constructed by subcloning the mutant g17 into the NcoI site of PET-9d vector, which does not have a hexahistidine sequence. The mutant DNA was amplified using g17 end primers containing the NcoI tags. The clones having the insert in the right orientation were isolated and induced with isopropyl 1-thio-β-D-galactopyranoside to overexpress the mutant proteins in the non-His-tagged format.

Standard Procedures—Transformations were performed either by the CaCl2 method or the electroporation method. The orientation of g17 insert DNA was determined by restriction enzyme analysis, using BglII or XbaI. DNA sequencing was done using the Fentamole cycle sequencing kit (Promega) or the Thermo Sequenase Cycle Sequencing Kit (U.S. Biochemical Corp.). Overexpression of g17 mutants and SDS-PAGE was performed according to Studier et al. (27). Wild-type gp17 and the D255 mutants were purified according to Leffers and Rao (12). In vitro DNA packaging, ATPase, in vivo nucleosome toxicity, and DNA cleavage assays were performed according to the procedures described earlier (12, 28). Azido-ATP cross-linking was done according to Suana et al. (30).

RESULTS

Sequence Analyses—The Walker B aspartate in ATPases is commonly located 50–130 residues downstream of the Walker A lysine (31), although in rare cases it can be as close as 27 residues (superfamily 3 helicases) (32) or as far away as 750 residues (Rad50) (33) downstream of the Walker A lysine. Two Walker B motifs have been proposed in gp17: 251-MIVD255 (10, 19) and 6-GSVVAKSL1MD73 (21, 22). Sequence alignments show that only the G and D residues of the latter are conserved among the T4 family terminases; more importantly, the Walker

5 It was reported that this mutant glutamate tRNA is charged with glutamine 20% of the time (24).

6 All of the mutants constructed in this study were sequenced to confirm/identify the DNA sequence at the mutant site. In many cases, more than one independent phage/clone was sequenced, and the phenotypes were confirmed to eliminate the possibility of any second site mutations.
Phage T4 DNA Packaging ATPase

B Asp^{273} is 307 residues downstream of the Walker A lysine (Lys^{166}) and preceded by a predicted α-helix, not a β-strand. These do not satisfy the basic features of the classic Walker B motif (20, 31). On the other hand, the Asp^{255} residue is 89 aa downstream of the Walker A lysine; it is preceded by four hydrophobic residues that are predicted to form a requisite β-strand (Fig. 1). Furthermore, it is followed by catalytic glutamate (Glu^{156}) (15), a key feature of Walker B from DEAD box helicases (20).

Alignments of more than 200 phage and viral large terminase sequences from the data base support the above analyses (Table 1; data not shown). The terminases show a striking conservation of the putative Walker A and Walker B motifs. Of the 59 mutants analyzed, 44 resulted in a loss of function. These results are consistent with the data from combinatorial mutagenesis experiments (see below).

Suppressor-based Mutagenesis of Residue Tyr^{252}—In order to perform combinatorial mutagenesis, it is necessary to first construct an amber mutation within (or very close to) the putative Walker B motif (14). The Tyr^{252} residue, which is flanked by sequences that are favorable for efficient amber suppression (24), was chosen to introduce the amber mutation.

As summarized in Fig. 2A, the 17Y253am mutation was suppressed by the native Tyr as well as Phe, Gln, Glu/Gln, His, Leu, Cys, and Ser suppressors, but not by the Gly, Pro, Ala, Lys, or Arg suppressors. These suppressors, but not by the Gly, Pro, Ala, Lys, or Arg suppressors. These suppressors, but not by the Gly, Pro, Ala, Lys, or Arg suppressors. These data and secondary structure predictions (Fig. 2B) suggest that three of the five features of the Walker B motif are important for function ((i) integrity of the β-strand, (ii) hydrophobicity, and (iii) electronic environment), because a break in the predicted β-strand in mutants Y253G and Y253P, reduced hydrophobicity in the mutant Y253A, or introduction of a charged/polar side chain in mutants Y253K and Y253R, respectively, resulted in a loss of function. These results are consistent with the data from combinatorial mutagenesis experiments (see below).

Combinatorial Mutagenesis of Tyr^{252}/Phe^{254} Sequence—A mutant library was constructed, wherein the Tyr^{252}/Phe^{254} sequence was replaced by all possible codon combinations. Of several hundred random mutants screened by marker rescue using the Y253am mutant, only six functional phenotypes were recovered (Fig. 3A). It is striking that all functional mutants retained hydrophobicity, β-strand structure, and no charged aa. On the other hand, all of the null mutations disrupted one or more of these features, most of them interfering with the integrity of the predicted β-strand (Fig. 3B).

Further phenotypic analysis revealed that the LAL mutant produces small to minute plaques at 37 °C, while a contribution due, which is situated at the beginning of the Walker B motif cannot be completely excluded. Mutagenesis of Residue Met^{251}—The importance of the Met^{251} residue, which is situated at the beginning of the Walker B β-strand, was tested by combinatorial mutagenesis. Of the 59 mutants analyzed, 44...
(74.5%) were functional. DNA sequencing revealed that Arg, Glu, or Pro substitution resulted in a null phenotype (Fig. 4). Three double mutants were also recovered; of these, two mutants, A250V/M251G and M251T/F259L, showed the cs phenotype and the third mutant, A250T/M251G, showed a null phenotype. These data reaffirm the importance of hydrophobicity, β-strand structure, and the negative impact of the charged substitutions.

**The Asp^{255} Residue Is Critical for Function**—Of the 269 combinatorial mutants tested, only five (1.9%) exhibited a functional phenotype. Since there are two codons for aspartate (3.1% of the total triplet combinations), this frequency suggests that no substitutions other than the native aspartate were tolerated. Indeed, DNA sequencing showed that all four functional phenotypes contained one of the two aspartic acid codons.

### TABLE 1
**Novel and deviant Walker B motifs in large terminase proteins**

The conserved aspartate and catalytic glutamate in the classic Walker B motif are shown in purple. In the Deviant I and II Walker B motifs, the conserved aspartate is replaced with glutamate (blue) and serine (red), respectively. In the Deviant III motif, the conserved catalytic glutamate is replaced with aspartate (green). The deviant Walker A motifs (Deviant I, blue; Deviant II, red; Deviant III, green) have been reported earlier (34). These are included here in relation to the Walker B deviant motifs. Note the unique deviant in phage VP2 (last line under Deviant II), which shows swapping of the conserved Walker A serine and Walker B aspartate. Accession numbers are as follows: T1 (AAP49948); T5 (AAU10290); T4 (NP_049776); T3 (NC_003298); HK620 (NP_112076); PhilSST (NP_075901); VHML (NP_758915); k139 (NP_539648); Phi-CTX (NP_490600); P2 (NP_046758); 186 (NP_052251); HPH2 (NP_536821); L-413C (NP_839851); Mu (NP_050632); RM_378 (NP_835653); Hf2 (NP_542602); Lambda (P03708); 21 (AAU32340); WO (BAAY9621); 933W (NP_049511); HPV5 (NP_542306); PheC2 (AAQ54982); Phi-77 (AAAM49610); PhiPSA (NP_510983); Phi3626 (AAJ57722); A118 (CAB5788); MM1 (NP_150161); bIL67 (AAAY7327); P1605 (BAA36828); SP1 (NP_690654); c2 (NP_043650); Vp262 (AAAM2938); phiKMV (NP_877662); AP1e (AY370673); K1–5 (AY370674); APSE-1 (NP_050979); Xp10 (NP_658895); Bcep781 (NP_705653); TP901–1 (NP_112694); VP2 (NP_024418); F116 (AAAT45822). *, the default name given to the putative large terminase protein is TerL unless a gene or protein name is provided.
Ser, Thr, Arg, Lys, and (of particular interest) Glu, substitutions were recovered (Fig. 4).

Conservative Substitutions, D255N and D255Q, Resulted in a Loss of Function—Biochemical analysis of Walker B aspartate mutants in other systems indicated that a conservative Asn or Gln substitution can result in retention of some ATPase activity (18, 36). Although the frequency of functional substitutions in the Asp255 mutant screen signified with virtual certainty that D255N and D255Q should be null mutants, these were not recovered among the null mutants sequenced. We therefore constructed these mutants by splicing by overlap extension to definitively establish their phenotypes, as well as to purify and perform biochemical characterizations of the mutant proteins. A mutant library was constructed wherein the Asp255 residue was replaced with Asn, Gln, Lys, or His. A total of 37 random mutants were analyzed, and none displayed a functional phenotype. DNA sequencing showed that both the D255N and D255Q are null mutants, reinforcing the conclusions drawn from the Asp255 combinatorial mutant library.

The D255E/E256D “Flip” Mutant—Combinatorial mutagenesis of the adjacent Glu256 residue showed that no substitutions, including the highly conservative E256D, were tolerated (15). Would “flipping” the DE sequence into ED result in a loss of function? This mutant was also constructed by the splicing by overlap extension strategy. Testing of several independent clones revealed no positive marker rescue, demonstrating that the ED flip mutant is a null mutant.

The Asp255 Mutants Showed Reduced Nuclease Activity—Loss of one or more activities associated with gp17 (nuclease, ATPase, and in vitro DNA packaging) could account for the lethality of Asp255 mutations. A set of conservative (D255E, D255N, and D255Q) and less conservative (D255T and D255L) mutants and the ED mutant were selected for biochemical analyses.

gp17 exhibits a nonspecific nuclease activity (25, 28, 37), which is consistent with the terminase paradigm in dsDNA phages (2, 3). The gp17-nuclease activity was analyzed by in vivo nuclease toxicity and DNA cleavage assays (15, 25, 28, 37). When His-tagged gp17 recombinant plasmid is transformed into E. coli BL21(DE3), which expresses significant basal levels of gp17 due to the leaky T7 RNA polymerase (27), the nuclease-proficient constructs generate colonies that have a glassy, transparent (GT; sick) appearance, presumably due to toxicity of the expressed nuclease; on the other hand, the nuclease-deficient constructs generate round, healthy (RH; normal) colonies. Additionally, the transformation efficiency (number of colonies on BL21/number of colonies on BL21) of the latter is higher than that of the former (15, 25).

DNA packaging (Fig. 3) could account for the lethality of Asp255 mutations. A set of conservative (D255E, D255N, and D255Q) and less conservative (D255T and D255L) mutants and the ED mutant were selected for biochemical analyses.

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On the other hand, transformation of the non-His-tagged gp17 plasmid, which expresses a more active gp17 than the His-tagged gp17, results in lethality (12, 28).

FIGURE 2. Phenotypes of aa substitutions at the Tyr253 residue. A, the TAG amber codon was introduced at the Tyr253 residue and was suppressed by one of the 13 aa suppressors constructed by Kleina et al. (24). The effect of each aa substitution was assessed by plaque forming ability of Y253am on the corresponding suppressor strain. The aa substitutions below the native sequence resulted in a null phenotype, and the ones above resulted in a functional phenotype. Glu suppressor tRNA is reported to be charged with Gin about 20% of the time (24); thus, it is likely that the observed suppression was due to Gln substitution. B, secondary structure predictions of the mutant proteins using the Simpa96 program (35). The aa on the left correspond to the suppressor strain used to substitute for the Tyr253 residue (shown in boldface type).

FIGURE 3. Combinatorial mutagenesis of 252IYI254 residues. A, a 3-aa combinatorial library was constructed by randomizing the nucleotide sequence corresponding to the 252IYI254 residues. The substitutions below the native sequence resulted in null phenotype, and the ones above resulted in a functional phenotype. The sp and ts mutants are shown in italic type. B, secondary structure predictions of the mutants (35). The aa sequences on the left correspond to the respective mutant sequence that replaced the native IYI sequence (shown in boldface type).
The Asp255 mutants and control plasmids (positive control: His-tagged wild-type, D255D; negative control: His-tagged terminase defective mutant, pHisDK-3.1 (H436R)) (28) were transformed into BL21 and BL21(DE3) strains. Isopropyl 1-thio-β-D-galactopyranoside induction and SDS-PAGE showed that all of the constructs expressed gp17 at comparable levels (data not shown). In the BL21(DE3) background, the Asp255 mutants, like the wild type, showed the GT phenotype, which indicated that they did retain the nuclease activity. However, the transformation efficiency was up to 5-fold higher for the Asp255 mutants, indicating lower nuclease activity in the mutants (data not shown). The reduced nuclease activity of the Asp255 mutants was also evident under the same conditions as the mutants. It was used as a wild-type control, in addition to the independent pRL-H17 wild-type control that was constructed by amplification and cloning of gp17 directly from the wild-type T4 DNA (12). As would be expected, the D255D and pRL-H17 constructs showed the same functional behavior. Few of the null phenotypes were inferred from the marker rescue data. Few of the null phenotypes were inferred from the marker rescue data.

The Asp255 Mutants Lost the gp16-stimulated gp17-ATPase Activity

The basal and gp16-stimulated gp17-ATPase activities of D255D, D255E, D255N, and ED mutants were determined. Hydrolysis of ATP to ADP and Pi was analyzed by incubating the purified proteins with γ-32P-labeled ATP in the presence or absence of gp16. The results from numerous experiments using independent preparations showed that all of the mutants lost the gp16-stimulated ATPase activity (typical data shown in Fig. 7). No change in the very weak basal activity was observed.10 One notable exception is the D255E mutant, which showed a low (4-fold) stimulation of the ATPase activity, which is equivalent to about 20% of the wild-type gp17 (Fig. 7). To account for any possible alteration in the kinetic properties of the mutants, the ATPase assays were performed using different concentrations of the protein and/or ATP. No change in the ATPase activity pattern described as above was observed under any condition tested.

The Asp255 Mutants Are Defective for Binding ATP—Although the Walker B aspartate has been implicated in nucleotide binding, it cannot always be assumed that the loss of ATPase is directly related to a loss of ATP binding (38). The ability of purified Asp255 mutant proteins to bind ATP was tested using the ATP analog, [γ-32P]-azido-ATP. The proteins were incubated with the azido-ATP at 4 °C, and the bound compound was cross-linked by exposing to UV (360 μm). Data from a number of independent experiments showed that the mutants having no carboxyl group (D255T and D255N) showed no significant cross-linking, whereas the ones that retained the carboxyl group (D255E and ED) showed cross-linking to azido-ATP (Fig. 6B). Similar results were obtained using [γ-32P]2-azido-ATP (data not shown). No ATP hydroly-
Phage T4 DNA Packaging ATPase

TABLE 2

In vitro DNA packaging activity of Asp255 mutants

The recombinant plasmids were transformed into E. coli BL21(DE3) pLys-S and induced with isopropyl 1-thio-D-galactopyranoside for 2 h at 30°C. Overexpression of gp17 was confirmed by SDS-PAGE. The cells were harvested by centrifugation at 10,000 rpm for 5 min and concentrated 50-fold in 50 mM Tris-Cl, pH 7.4, containing 5 mM MgCl2, and 100 mM NaCl. The cells were lysed by French press treatment (20,000 pounds/inch²), and the cell debris was removed by centrifugation at 12,000 rpm for 15 min. Samples from both the supernatant and the pellet were subjected to SDS-PAGE to confirm that the constructs showed roughly equivalent amounts of gp17 in the soluble form. The supernatants (10 or 20 μl) were added to a freshly made E. coli extract following infection with 16am(N66)-17am(A66) wild-type phage. The extract is estimated to provide 10 to 20×10⁶ probes/assay. Other components of the reaction mixture include 1–2 μg of purified wild-type phage T4 DNA, 50 mM Tris-HCl, pH 7.0, 5 mM ATP, 3 mM β-mercaptoethanol, 5% polyethylene glycol, 6 mM MgCl2, 2 mM spermidine, and 100 mM NaCl, in a total volume of 100 μl. The reaction mixtures were incubated at 30°C for 90 min. Packaging was terminated by the addition of a drop of chloroform and 400 μl of 20 mM/mg pancreatic DNase I in phage dilution buffer (50 mM phosphate buffer, pH 7.0, 70 mM NaCl, and 1 mM MgSO4). The phage yield was determined by titration on E. coli NS5329 (26) and represented as the total number of plaques formed units produced per 100 μl of reaction mixture. The experiment was done three times with independent preparations, and typical data are shown.

| Construct | Phage yield |
|-----------|-------------|
| pDK-3.1  | 1.00        |
| D255D    | 0.17        |
| D255E    | 0.29        |
| D255N    | 0.75        |
| D255Q    | 0.83        |
| D255T    | 0.46        |
| D255L    | 0.73        |
| D255E/E256D | 0.63 |

A. Al-Zahrani and V. B. Rao, manuscript in preparation.
The identity of the Walker B motif that is required for DNA packaging is unknown. Sequence analyses show that the conserved T4 sequence, 25IYID, exhibits the important features of the classic Walker B motif. These include (i) distance from Walker A lysine (Lys166), (ii) strict conservation of the aspartate (Asp255) residue, and (iii) hydrophobicity and B-strand structure of the residues preceding Asp255 (Fig. 1). More than 200 large terminase sequences in the database, except for certain conservative deviations listed (Table 1), conform to these features, despite lacking overall sequence similarity (10) (Table 1). On the other hand, a second proposed sequence (21, 22), GYSVAKL, is unlikely to be the Walker B motif. The sequence is not conserved among the T4 family terminases or the phage/viral terminases, and the 277-aa distance between Asp473 and the Walker A lysine is unusually large. Furthermore, the N-terminal 360 aa residues of gp17 are sufficient to exhibit the full complement of stimulated ATPase activity (41).

The functional significance of the Walker B hydrophobic residues has not been analyzed in any ATPase motor. Our data show that both the hydrophobicity and integrity of the B-strand are critical for function. The rare functional phenotypes recovered from screening hundreds of mutants from the 25IYID combinatorial library provided the best demonstration of this point (Fig. 3). DNA sequencing revealed that the functional phenotypes retained the hydrophobicity and the wild-type B-strand length, whereas null mutants disrupted one or both of these features, many having a shortened predicted B-strand. Certain deviations are tolerated: for instance, a single substitution with a small side chain polar aa such as serine was tolerated at Met251, Ile252, or Tyr253, so long as the predicted B-strand structure was retained. However, only conservative hydrophobic substitutions such as valine, leucine, and methionine were tolerated at the Ile254 residue, which immediately precedes the critical Asp255 aspartate (Figs. 2–4). It appears that a greater restriction is imposed here presumably because the substitution disturbs the precise orientation of the adjacent Asp255 carboxyl in the ATPase catalytic pocket.

Charged aa substitutions, such as M251R, M251E, Y253R, and Y253K, were not tolerated. These probably disrupt the hydrophobic environment despite the fact that they do carry long aliphatic side chains. However, substitutions that altered the hydrophobicity in a more subtle way resulted in conditionally lethal phenotypes. For instance, the A250V/E259L mutant (enhanced hydrophobicity) showed a ts phenotype, whereas the I252S and 252LAL254 mutants (reduced hydrophobicity) showed a ts phenotype. Thus, the Walker B B-strand apparently resides in a hydrophobic core, which supports precise positioning of aspartate in the catalytic pocket. We speculate that perturbations alter this dynamic, increased rigidity in the case of the cs mutant and increased disorder in the case of the ts mutant, causing lethality at the restricted temperature. Incidentally, these mutants represent the first conditionally lethal Walker B mutants reported for any ATPase motor.

Extensive screening revealed that only 1.9% of the Asp255 combinatorial mutants exhibited a functional phenotype, each of which upon sequencing showed an aspartic acid codon at position Asp255 (Fig. 4). Individually constructed conservative substitutions, D255N, D255Q, and D255E/E2556 flip mutants, resulted in a null phenotype. Thus, subtle perturbations, such as increasing the side chain by one C–C bond (1.54 Å; D255E mutant) or changing the carboxyl group to an amide (D255N), resulted in a null phenotype. As evident from the azido-ATP cross-linking experiments, the mutants exhibited defects in ATP-Mg complex formation (see below). These data suggest that Asp255 is a critical catalytic residue, and its phenotypic behavior is in clear contrast to that of the preceding hydrophobic aa, wherein a number of substitutions are tolerated, and the tolerance is related to structure, not catalysis.

The Asp255 mutants lost both the gp16-stimulated ATPase and in vitro DNA packaging activities. The loss was probably due to defective ATP-Mg complex formation. This was evident in the case of D255N and D255T mutants, which failed to cross-link to azido-ATP (Fig. 6). The D255E and D255E/E255D mutants, particularly the latter, did cross-link to azido-ATP, yet they exhibited a loss of ATPase and in vitro DNA packaging activities. Thus, binding to ATP as such is not sufficient for catalysis; a precise three-dimensional positioning of the ATP-Mg complex must occur to support catalysis (17, 18, 38). Otherwise, a nonproductive complex would result, making the following in-line attack by an activated water molecule either inefficient (D255E) or impossible (D255E/E255D). Subtle changes such as increasing the length of the side chain while retaining the functional group (D255E, D255E/E255D) or changing the functional group (D255N) while retaining the size of the side chain, resulted in a profound loss of ATPase and DNA packaging activities. We predict that the effects of these modifications will be more severe in the case of the natural substrate, ATP, because gp17 exhibits about 70–560-fold lower affinity for ATP (Km = 280 μM) when compared with azido-ATP (Km = 0.5 μM) for 2-azido-ATP and 4 μM for 8-azido ATP.11

The Asp255 mutants lost the gp16-stimulated ATPase activity. About 1 μM of each purified gp17 was incubated either alone or in the presence of about 8 μM gp16 in a reaction mixture containing 500 μM ATP and 10 μCi (150 mCi/mmol) of (γ-32P)ATP (specific activity 3000 Ci/mmol; GE Healthcare), 50 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, and 5 mM MgCl2, for 20 min at 37 °C. The reaction was terminated by the addition of 50 μl EDTA, and thin layer chromatography was performed on a polyethyleneimine plate (Sigma). The figure shows the autoradiogram of the chromatography plate. Each mutant was assayed in duplicate as shown in adjacent lanes. Buffer only and gp16 only controls account for the background radioactivity. The schematic at the left shows the positions of ATP, ADP, and Pi on the autoradiogram.

FIGURE 7. The Asp255 mutants lost the gp16-stimulated ATPase activity. About 1 μM of each purified gp17 was incubated either alone or in the presence of about 8 μM gp16 in a reaction mixture containing 500 μM ATP and 10 μCi (150 mCi/mmol) of (γ-32P)ATP (specific activity 3000 Ci/mmol; GE Healthcare), 50 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, and 5 mM MgCl2, for 20 min at 37 °C. The reaction was terminated by the addition of 50 μl EDTA, and thin layer chromatography was performed on a polyethyleneimine plate (Sigma). The figure shows the autoradiogram of the chromatography plate. Each mutant was assayed in duplicate as shown in adjacent lanes. Buffer only and gp16 only controls account for the background radioactivity. The schematic at the left shows the positions of ATP, ADP, and Pi on the autoradiogram.

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It is interesting that, in the Walker B deviants of HK620, SPP1, and RM 378 terminases, the Asp255 equivalent is a glutamate, whereas in 933W, BPC5, and Bcep22 terminases, it is a serine (Table 1). From a functional standpoint, both glutamate and serine can coordinate with ATP-Mg2⁺ and fulfill the role of aspartate. In fact, the Walker A serine/threonine, a strict requirement in ATPases, similarly interacts with the ATP-Mg2⁺ complex (16, 17). It is thus probable that the structure of the catalytic pocket in these terminases is such that the three-dimensional positioning of the ATP-Mg complex is more optimal for catalysis with E or S than D. The unique deviant in phase VP2, which shows “swapping” of Walker A serine and Walker B aspartate, is very interesting, also supporting this reasoning. In essence, these exceptions do support the “rule” that the Asp255 in gp17 (and the analogous D in terminases) functions as the catalytic Walker B aspartate.

Recent evidence suggests that gp17 consists of two domains, an N-terminal ATPase domain and a C-terminal nuclease domain (41). The latter domain, which exhibits a non-specific nuclease activity, was implicated in the terminase activity that generates the ends of packaged DNA (25, 37). The reduced nuclease activity of Asp255 mutants (Fig. 5) implies that ATP occupancy at the N-terminal ATPase center allosterically influences the nuclease activity. Defective ATP binding apparently keeps gp17 in the unstimulated conformation that has very low nuclease activity, whereas the catalytic carboxylate (Glu256) mutants trap the protein in the correct ATP-bound conformation, enhancing the nuclease activity (15). These observations are consistent with the emerging theme in terminases that communication between ATPase and nuclease domains is necessary to orchestrate DNA packaging and termini generation. In phase λ, ATP binding, but not hydrolysis, is required for cos cleavage by the large terminase protein, gpA (42). Fidelity of cos cleavage is also enhanced in the ATP-bound conformation (43). In phase T3, ATP acts as an allosteric modulator and affects the specificity of DNA cleavage catalyzed by the large terminase (44).

This and the previously reported studies (14, 15) define the three basic components of the N-terminal ATPase catalytic center in gp17: Walker A, Walker B, and catalytic carboxylate. In light of the tight relationship observed between the ATP-Mg complex formation at the N-terminal ATPase site and DNA packaging, it is reasonable to speculate that the ATP-Mg complex is more optimal for catalysis with E or S than D. The unique deviant in phase VP2, which shows “swapping” of Walker A serine and Walker B aspartate, is very interesting, also supporting this reasoning. In essence, these exceptions do support the “rule” that the Asp255 in gp17 (and the analogous D in terminases) functions as the catalytic Walker B aspartate.

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