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Review

The terminase enzyme from bacteriophage lambda: a DNA-packaging machine

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Abstract. This review focuses on the biochemical, biophysical, and catalytic properties of terminase, an enzyme involved in bacteriophage λ genome packaging. The holoenzyme possesses ATPase, DNA strand-separation, and site-specific nuclease activities that work in concert to insert a viral genome into the confines of a preformed capsid. Moreover, the terminase subunits are part of a series of nucleoprotein complexes involved in genome packaging, including remarkably stable intermediates that transition to a highly mobile DNA packaging 'machine.' Models for the assembly and interconversion of these complexes are presented. Interactions between the catalytic sites in the enzyme complex, and modulation of these catalytic activities as it relates to the assembly and relative stability of the packaging intermediates are discussed. This ordered progression of nucleoprotein intermediates is a common theme in biology as demonstrated by mechanistic similarities between viral DNA packaging, the initiation of chromosomal replication, and the initiation of transcription. Terminase is thus part of a growing number of examples of biological 'machines' or molecular 'motors.'

Key words. Phage lambda terminase; DNA packaging; virus assembly; molecular motors.

Introduction

Complex double-stranded DNA (dsDNA) viruses such as adenovirus, herpesvirus, and the bacteriophages ∅29, T4, T7, and λ consist of viral DNA tightly packaged within an icosahedral protein shell known as the capsid [1, 2]. One of the final steps in the assembly of these viruses is the condensation, or 'packaging,' of viral DNA into the confines of the capsid [2–4]. Similar packaging pathways have been proposed for the dsDNA phage and the herpesvirus groups, in each of which, an enzyme known as terminase is responsible, at least in part, for viral DNA packaging. This review will focus on the biological, biochemical, and biophysical properties of the bacteriophage λ terminase enzyme, including a discussion of the multiple catalytic activities that must work in concert to effect genome packaging. Furthermore, models for the assembly and interconversion of stable nucleoprotein packaging intermediates, and the transition to a highly mobile packaging complex will be presented. Interactions between the catalytic sites in the enzyme complex, and modulation of these catalytic activities as it relates to the assembly and relative stability of the packaging intermediates are discussed.

Overview of bacteriophage λ development

Several excellent reviews of bacteriophage λ development have been published [5, 6] and only a brief summary is presented here. The virus is composed of a

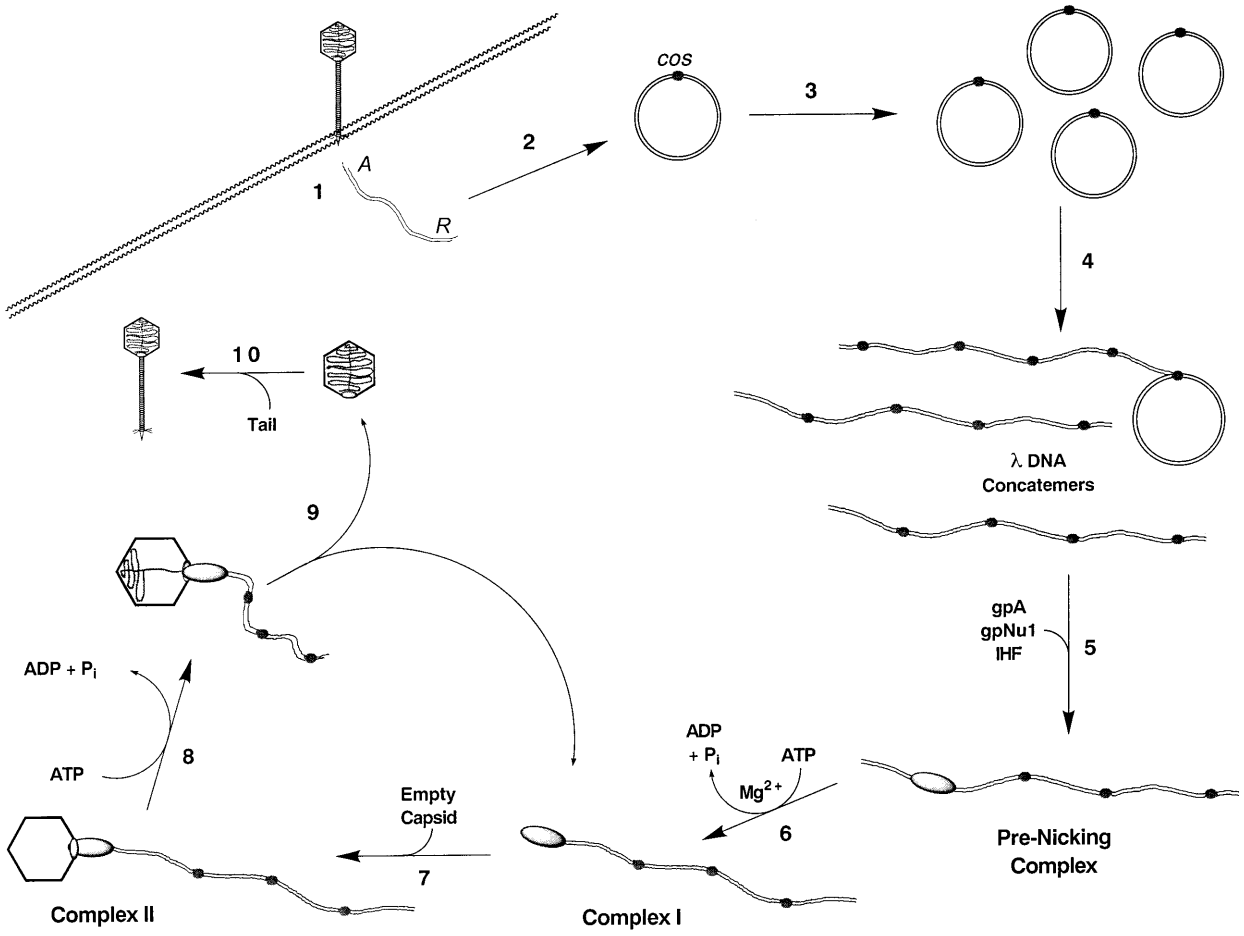

Figure 1. Bacteriophage λ development. (1) Attachment of the virus to the cell and injection of the dsDNA genome. The location of the first (*A*) and last (*R*) genes in the monomeric linear genome are indicated. (2) Circularization of the genome forming an intact *cos* site (dark oval). (3) Bidirectional, or θ replication yields daughter circles. (4) Rolling-circle, or σ replication, gives rise to linear concatemers of the viral genome. (5) Assembly of a multiprotein pre-nicking complex. (6) Terminase-mediated duplex nicking and strand separation yields complex I. (7) Procapsid binding yields complex II which (8) triggers the ATP-dependent translocation of the packaging proteins along the duplex. (9) The translocating complex encounters the next *cos* and again nicks and strand separates the duplex (terminal *cos* cleavage, downstream nicking reaction). This results in simultaneous release of the DNA-filled capsid and regeneration of complex I. (10) The addition of a tail, and in some strains tail fibers, yields a fully infectious virus. Details are presented in the text.

48.5-kb linear dsDNA genome tightly packaged within the viral capsid with a specialized structure known as the tail [7]. Infection initiates with the adsorption of the virus to the cell surface via interactions between the tail fiber gpJ protein and an *Escherichia coli* outer membrane protein involved in maltose/maltodextrin transport [8, 9]. Subsequent interactions lead to 'injection' of the viral genome into the cell through the tail tube (see fig. 1, step 1). Upon entry into the cell, the linear duplex circularizes via hybridization of 5′ complementary 12-base 'sticky' ends [10, 11] and the nicks are sealed by host ligase thus forming the cohesive end site (*cos*) of the viral genome (step 2) [6, 12–14]. Initially, genome replication occurs via bidirectional, or θ replication yielding daughter circles (step 3) [14–16]. During the latter stages of infection, DNA synthesis switches to a rolling-circle mechanism (σ replication) that gives rise to linear concatemers of the individual genomes linked in a head-to-tail fashion (step 4) [14, 16]. This concatemeric 'immature' DNA is the preferred packaging substrate [6, 14, 17, 18] and the replicated *cos* sites represent the concatemeric joint, or the junction between the left and right ends of individual λ genomes. One of the final steps in λ assembly requires the excision of a single genome from the concatemer and concomitant packaging of the monomer into an empty preformed capsid, a process known as DNA maturation [2–4]. This packaging pathway has been described [6,

18–21] and initiates with the assembly of the terminase enzyme at the *cos* site of a DNA concatemer forming a pre-nicking complex (step 5, fig. 1). Phage λ terminase consists of large (gpA) and small (gpNu1) subunits and is isolated as a $gpA_1{\cdot}gpNu1_2$ holoenzyme protomer [22, 23]. ATP and *E. coli* integration host factor (IHF) modulate the assembly and stability of the pre-nicking complex [22–28]. The magnesium-dependent endonuclease activity of the enzyme next introduces symmetrical nicks 12 bases apart within the *cos* site, a reaction whose activity and specificity is also modulated by ATP binding [29]. Terminase-mediated strand separation of the nicked, annealed duplex, driven by the hydrolysis of ATP (step 6), yields a stable enzyme·DNA intermediate known as complex I. This intermediate, originally isolated from phage-infected cells [30] and later characterized biochemically [23, 31], represents the mature (sticky) left end of the viral genome tightly bound by the terminase subunits. Complex I next binds to an empty viral procapsid (step 7) which triggers the release of terminase from *cos* thus forming complex II and initiating unidirectional, ATP-dependent translocation of terminase across the duplex[1] and active DNA packaging (step 8) [21, 32, 33]. The phage λ gpFI protein modulates the interactions between the procapsid and complex I and is required for efficient transition to complex II [34–36]. Upon encountering the next downstream *cos* in the concatemer (the end of the viral genome), terminase again introduces symmetrical nicks into the duplex and enzyme-mediated strand separation simultaneously releases a DNA-filled capsid and regenerates complex I for a second round of DNA packaging (step 9). The terminase-independent addition of a preformed tail to the DNA-filled capsid (step 10), and in some strains the addition of tail fibers [37] are the final steps in the assembly of a fully infectious virus. Processive genome packaging has been demonstrated [38–40] with two to three genomes packaged per terminase-DNA binding event (step 7, fig. 1) [41].

Phage λ terminase thus possesses an ATPase activity, an ATP-modulated site-specific endonuclease activity, an ATP-dependent strand-separation activity, and a putative ATP-driven DNA translocase activity that are required for DNA packaging and virus assembly. The enzyme is an integral part of several nucleoprotein intermediates that transition from highly stable, site-specifically bound complexes to a highly mobile packaging complex that must bind DNA tightly, but non-specifically. This ordered progression of nucleoprotein intermediates is a common theme in biology as shown by mechanistic similarities between viral DNA packaging, the initiation of chromosomal replication [42], and the initiation of transcription [43]. Within this context, terminase is part of a growing number of examples of biological 'machines' or molecular 'motors.'

[1] The description of genome packaging presented here presumes a stationary DNA duplex across which the terminase·capsid complex translocates. An alternate point of view considers the terminase·packaging complex as stationary, 'pushing' the duplex into the capsid.

Historical perspectives

cos, the terminase assembly site

Early studies coined the term 'ter' reaction (from termini) to describe the maturation of concatemeric viral DNA in vivo [44] and localized duplex cutting to a region between the *R* and *A* genes in the concatemer. These genes are found in the extreme right and left end, respectively, of mature (monomeric) λ DNA (see figs 1, 2) and this cleavage site thus corresponds to the junction between individual genomes in the concatemer. Later studies identified the cleavage site as the *cos* region of the viral genome [41, 45] and I will refer to this reaction as the *cos*-cleavage reaction rather than using the historical ter reaction.

Sequence analysis of *cos* revealed that the nicking site (*cosN*) possessed a twofold hyphenated symmetry (fig. 2), and it was suggested that the 'terminase' enzyme might bind as a dimer and introduce symmetric nicks into this site generating the 12-base single-stranded ends

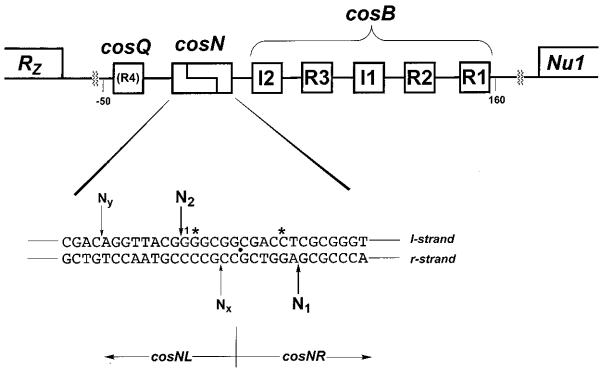

Figure 2. Structure of *cos*. The *cos* region of a λ concatemer is shown at top (not to scale). The *Rz* and *Nu1* genes represent the last and first genes, respectively, in an individual (mature) genome. The three subsites, *cosQ*, *cosN*, and *cosB* are indicated, as are the three gpNu1 binding elements (R1–R3) and the two IHF-binding elements (I1, I2) found within *cosB*. The sequence of *cosN* is expanded below with a dot indicating the center of symmetry within the element. The left (*cosNL*) and right (*cosNR*) half-sites of *cosN* are indicated. The first base in the genome [140] is indicated with a 1. Terminase normally introduces nicks at the N_1 and N_2 sites to yield the natural 12-base sticky end. Erroneous nicking occurs at N_x and N_y sites as discussed in the text. The positions of the G_2C and $C_{11}G$ *cosN* mutations discussed in the text are indicated with an asterisk.

of the mature viral genome [46, 47]. Further studies suggested that in addition to this nicking site, the region between base pairs -40 and ≈ 160 was also required for DNA maturation and efficient packaging in vivo (see fig. 2) [48–50]. A series of genetic and biochemical studies have demonstrated that the *cos* site is actually tripartite (see fig. 2) consisting of (i) *cosN* (bp -22 to $+24$), the site at which duplex nicking occurs [49, 51], (ii) *cosB* (bp $+51$ to $+160$), a region important to the assembly and stability of a packaging complex and the initiation of DNA packaging (steps 5–6, fig. 1) [49, 51], and (iii) *cosQ* (bp -34 to -18), a sequence involved in the terminal *cosN*-nicking reaction (step 9, fig. 1) [49, 51]. Each of these sites is discussed in more detail below.

Identification and isolation of the terminase enzyme

Demonstration of *cos*-cleavage activity in vivo [44] led to the search for the enzyme responsible for the reaction. Early studies demonstrated that the *cos*-cleavage reaction could be performed using a cell-free extract system, and genetic analysis showed that the product of the *A* gene (gpA) was required [17, 52, 53]. The in vitro reaction further required magnesium, ATP, and spermidine [54, 55]. A two-stage packaging system was subsequently developed by Becker, Gold, and co-workers [30, 56] which similarly utilized extracts of phage-infected and uninfected *E. coli* cells. These studies demonstrated that a stable intermediate, termed complex I, could be isolated from the first stage of the reaction using sucrose gradient centrifugation and size exclusion chromatography [30]. Complex I formation required λ DNA, gpA, magnesium, ATP, spermidine, and a then unidentified *E. coli* host factor. Moreover, experiments with non-hydrolyzable ATP analogs demonstrated that ATP hydrolysis was required for the formation of this intermediate [30, 55]. Based on these studies, complex I was proposed to be composed of gpA bound to the concatemeric DNA substrate, and that this stable nucleoprotein intermediate could subsequently be converted into infectious phage with the addition of capsid and tail components in the second stage of the reaction [30, 56]. Importantly, functional gpA was also critical to the second stage suggesting that this protein is important not only in the assembly of the packaging complex, but is also involved in active DNA packaging [30]. While these early studies clearly demonstrated that the *A* gene product was required for *cos*-cleavage both in vivo and in vitro, the role of the *Nu1* gene product in DNA packaging was suggested later [30, 57, 58].

The development of in vitro *cos*-cleavage assays directly demonstrated the nuclease activity of partially purified terminase [59]. These studies similarly showed requirements for magnesium, ATP hydrolysis, and spermidine, but only a stimulatory role for a host factor, later identified as IHF [22, 60, 61]. Early studies further suggested that a second *E. coli* protein (terminase host factor, THF) was necessary for phage λ development [62, 63]. THF was reported to be a 22-kDa, basic, heat-stable protein related to the other *E. coli* 'histone-like' proteins. More recent work has demonstrated that λ development shows a modest requirement for the *E. coli* HU protein [26]. While phage development is attenuated three- to fivefold in either IHF^- or HU^- cell lines, phage yield is decreased over 60-fold in IHF^-,HU^- double-mutant strains suggesting that either protein may suffice. Moreover, the latter study casts doubt on the nature and requirement of THF in phage development in vivo.

The terminase genes (*Nu1* and *A*) are poorly expressed in *E. coli*, likely due to poor translation [64, 65], and only ≈ 100 copies of the enzyme are found per phage-infected cell [66]. Nevertheless, the first purification of terminase holoenzyme (30–80% pure) was accomplished from phage-infected cells by the heroic efforts of the Becker and Gold laboratories [22, 53, 56]. These studies were the first to clearly identify gpNu1 as an integral part of the holoenzyme and suggested a $gpA_1{\cdot}gpNu1_2$ subunit stoichiometry. The partially purified enzyme possessed an ATP-stimulated *cos*-cleavage activity and a DNA-dependent ATPase activity. The construction of overexpression vectors [64, 67] led the way to the purification of large quantities of the holoenzyme and the isolated subunits, though this has been difficult due to the formation of inclusion bodies in *E. coli* [23, 64, 67–70]. Nevertheless, a variety of purification protocols for the holoenzyme and the isolated subunits have been published, and it is now possible to isolate milligram quantities of highly purified protein [23, 69–71]. Recently, vectors that express hexahistidine-tagged subunits have been constructed and the purification of these proteins in high yield is now possible in a single step [72].

The terminase enzyme

Genetic domains and primary sequence analysis

λ terminase is a hetero-oligomer consisting of the gene products of A (gpA, 641 residues, 73,298 Da) and *Nu1* (gpNu1, 181 residues, 20,440 Da) [22, 23, 69]. Elegant genetic studies by Feiss and co-workers have defined regions in the individual subunits that are responsible for specific protein·protein and protein·DNA interactions as follows (fig. 3). (i) An N-terminal domain of gpNu1 [103 amino acids] is required for specific DNA-binding interactions [73] and a putative helix-turn-helix DNA-binding motif has been identified by primary se-

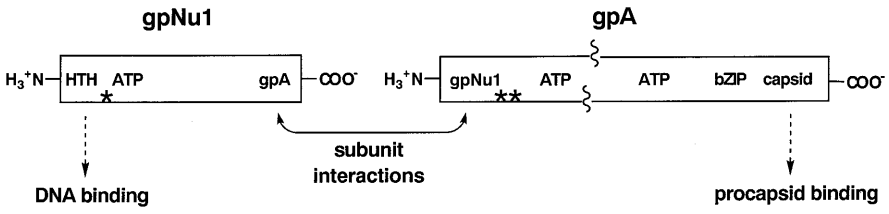

Figure 3. Proposed terminase domain structure. Genetically defined domains within the primary sequence of each protein (not to scale) are indicated: HTH, putative helix-turn-helix DNA-binding motif; ATP, putative Walker type-A ATP-binding (P loop) motifs. Asterisks indicate the location of residues modified with photoaffinity ATP analogs; gpA, the gpA interaction domain of gpNu1; gpNu1, the gpNu1 interaction domain of gpA; bZIP, putative bZIP protein dimerization motif; capsid, the region of gpA responsible for interactions with viral capsids. Based on genetic studies, subunit interactions are mediated by the ≈ 90 C-terminal amino acids of gpNu1 and the ≈ 40 N-terminal amino acids of gpA. See text for details.

quence analysis (Lys_5–Glu_{24}) [74; A. Becker, cited in ref 19]. (ii) Subunit interactions in the holoenzyme complex are mediated by domains located within the C-terminal 91 amino acids of gpNu1 and the N-terminal 48 amino acids of gpA [73, 75]. (iii) The C-terminal 38 amino acids of the gpA subunit are responsible for capsid binding with the penultimate 5 amino acids strictly required for these interactions [73, 76, 77].

Primary sequence analysis of the gpA polypeptide has revealed a region (Glu_{560}–Gly_{621}) with reasonable homology to the basic leucine zipper motif (bZIP) found in eukaryotic transcription factors [78, 79]. The presence of this protein dimerization motif combined with the symmetric nature of *cosN* has led to the suggestion that gpA binds to each *cosN* half-site as a functional dimer mediated by bZIP interactions [80]. Primary sequence analysis has further identified a reasonable match to the Walker A-type ATP-binding consensus sequence (P loop) in gpNu1 (Val_{29}–Gly_{36}) [81], and two weak matches in the gpA subunit of the enzyme [82, 83].

Purification and biophysical characterization of the terminase subunits

Expression of the isolated terminase subunits in *E. coli* yields soluble gpA that has been purified to homogeneity [69, 71, 72]. Structural studies of the isolated subunit have demonstrated that the protein is monomeric at low concentrations [69], but may associate into a soluble dimer upon concentration (C.E. Catalano, unpublished data). Circular dichroism (CD) analysis of the protein is consistent with a folded conformation that possesses significant α-helical and tertiary structures [71]. Thermal unfolding studies have further demonstrated that while denaturation is irreversible, unfolding of gpA is cooperative ($m_T = -144\ K^{-1}$) with a melting temperature (T_m) of 317 K [71].

The purification of gpNu1 has proven more difficult due to the insolubility of the expressed protein in *E. coli* [23, 68, 69, 71]. Recent studies have demonstrated that the protein may be solubilized from the inclusion bodies using either sarkosyl (a strong detergent) [69] or guanidinium hydrochloride (GDN) [71], and that gpNu1 may be refolded into an active conformation upon dialysis of the denaturant; however, the properties of the isolated gpNu1 subunit depend, to some extent, on whether sarkosyl or GDN is used to solubilize the protein from inclusion bodies [70]. Solubilization with sarkosyl yields a purified protein that possesses significantly less α-helical structures, has a greater tendency towards aggregation, shows a greater apparent stability to thermal and chemical denaturation, but that unfolds with very little cooperativity and a low free energy [$\Delta G(H_2O) \approx 1.6$ kcal/mol] [70]. Interestingly, however, thermal denaturation of the sarkosyl-purified protein followed by refolding at 4 °C yields a preparation with properties identical to the GDN-purified protein (see below). These data suggest that detergent may remain bound to the sarkosyl-purified protein throughout the isolation procedures and that it is released only upon denaturation of the protein [70].

CD analysis of the isolated gpNu1 subunit has shown that while the protein possesses significant α-helical structures, it possesses little, if any, tertiary structure [71]. Moreover, while thermally induced unfolding of the protein is fully reversible, the transition is broad and uncooperative ($m_T = -38\ K^{-1}$), and occurs at a significantly lower temperature than that of gpA ($T_m \approx 307$ K) [70, 71]. Consistently, chemical (GDN) denaturation experiments have shown that the free energy of unfolding of gpNu1 is relatively low [$\Delta G(H_2O) \approx 4.7$ kcal/mol]. The properties of the isolated gpNu1 subunit suggest that the protein exists in solution in a 'molten globule' or 'compact intermediate' state, and that gpA·gpNu1 interactions in the holoen-

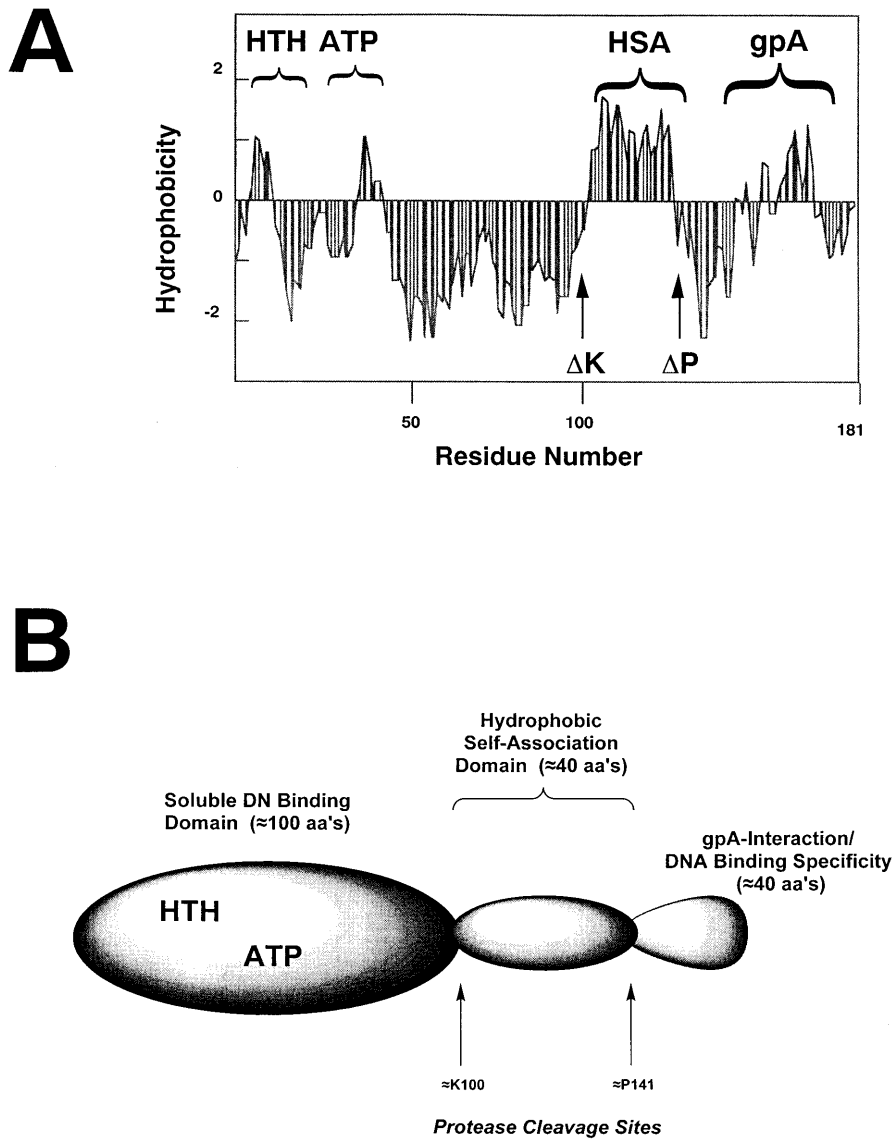

Figure 4. Structural domains of gpNu1. (*A*) Kyte-Doolitle hydropathy plot of full-length gpNu1. The locations of the putative helix-turn-helix DNA-binding motif (HTH) and the putative P-loop motif (ATP) are indicated. The hydrophobic self-association domain (HSA) and gpA-interactive region of the protein are also indicated. The locations of the C-terminal ends in the gpNu1ΔK_{100} (ΔK) and gpNu1ΔP_{141} (ΔP) constructs are indicated with arrows. (B) Proposed domain structure of gpNu1. The soluble DNA and nucleotide (DN)-binding domain and the hydrophobic self-association domain are indicated as ovals. Protease cleavage sites are indicated with arrows. Details are presented in the text.

zyme complex are required to stabilize the folded conformation of the smaller gpNu1 subunit [71]. Similarly, the isolated gpA subunit appears to be unstable in the absence of gpNu1 [69], suggesting that the stability of both subunits is enhanced in the holoenzyme complex. Importantly, terminase reconstituted from the individually purified subunits possesses nuclease, ATPase, and strand-separation activities found in wild-type holoenzyme [31, 69, 71, 72, 84–86].

Structural domains of the terminase gpNu1 subunit

Self-association domain

The isolated gpNu1 subunit shows a strong tendency to aggregate which has made structural studies on the protein difficult. The hydropathy plot for the protein (fig. 4A) reveals a strongly hydrophobic region extending between residues ≈ 100 and 140 of the primary sequence, and it was suggested that this region might define a self-association domain responsible for the ob-

served aggregation behavior of the protein [87]. To define structural domains of the protein more clearly, two deletion mutants were constructed and characterized. The first, gpNu1ΔP_{141}, comprises the N-terminal 141 amino acids of full-length gpNu1 (fig. 4). This construct shows an extremely strong tendency to aggregate (consistent with the presence of a hydrophobic self-association domain), but remains soluble and can be concentrated in the presence of 400 mM GDN. Interestingly, fluorescence and CD studies indicate that gpNu1ΔP_{141} is folded in low concentrations of GDN, possessing significant α-helical and tertiary structures [88]. A second construct, gpNu1ΔK_{100}, comprises only the N-terminal 100 amino acids of full-length gpNu1 (fig. 4). Deletion of the putative self-association domain indeed yields a fully soluble homodimer that shows no aggregation behavior, even at concentrations as high as 2 mM [87]. Similar to gpNu1ΔP_{141}, CD and nuclear magnetic resonance (NMR) analyses have demonstrated that gpNu1ΔK_{100} is folded in solution [87].

gpA-interaction domain

Genetic studies have demonstrated that the C-terminal half of gpNu1 is required for interactions with the gpA subunit of the enzyme [73] and, not unexpectedly, gpNu1ΔK_{100} does not physically interact with gpA, nor does it form a catalytically competent holoenzyme complex [87]. Interestingly, while gpNu1ΔP_{141} interacts weakly with gpA, it neither promotes *cos*-cleavage nor ATPase activities of the large terminase subunit [88]. Thus, the C-terminal 40 amino acids of gpNu1 appear necessary, at least in part, for efficient interactions with gpA and are critical to the formation of a catalytically competent holoenzyme complex. This region of the protein is therefore referred to as the gpA-interactive domain.

DNA-binding domain

Both of the gpNu1 constructs described above possess the putative helix-turn-helix DNA-binding motif found in the full-length protein, and *cos*-specific DNA-binding interactions have been demonstrated with each protein [87, 88]; however, while gpNu1ΔP_{141} binds to DNA with an affinity similar to that of the full-length protein, discrimination between specific and non-specific DNA substrates is impaired [88]. Thus, the $\approx$40 C-terminal amino acids of gpNu1 are also important in discrimination between *cos*-containing and non-specific DNA sequences. Further deletion of the hydrophobic self-association domain (as in gpNu1ΔK_{100}) results in a 1000-fold decrease in the affinity for DNA, but no further reduction in discrimination capacity [87, 88].

gpNu1 domain model

The genetic and biochemical data are consistent with the gpNu1 domain model presented in figure 4B. The region spanning Met_1–Lys_{100} defines a soluble DNA-binding domain [87], and NMR experiments have demonstrated that this domain also binds (but does not hydrolyze) ATP (N. Berton and C. E. Catalano, unpublished data). We therefore refer to this domain as the DNA and nucleotide (DN)-binding domain of gpNu1. The isolated domain binds DNA much less tightly than does the full-length protein, and discriminates only modestly between *cos*-containing and non-specific DNA substrates [87]. The residues between Lys_{100} and Pro_{141} constitute a self-association domain that is important for high-affinity DNA-binding interactions and that is required for cooperative assembly of gpNu1 at *cos* [88]. This hydrophobic domain is further responsible for the observed aggregation behavior of the protein at elevated concentrations. Finally, residues between Pro_{141} and Glu_{181} define a region required for efficient interactions with the terminase gpA subunit and critical to the formation of a catalytically competent holoenzyme complex. Importantly, while the self-association domain is required for *high-affinity* DNA-binding interactions, the C-terminal 40 amino acids of the protein play an important role in DNA-binding *specificity* [88]. Finally, it should be noted that while the isolated full-length subunit appears to be in a molten-globule state, both of the deletion mutants described here are folded in solution. These data suggest that the C-terminal gpA-interactive domain interferes with global folding of gpNu1 in the absence of gpA, but that deletion of this region allows the remaining domains to adopt a folded conformation.

DNA-binding activity of terminase and its subunits

gpNu1 and IHF DNA-binding interactions

While phage λ does not require IHF for development, burst sizes are about two- to fourfold lower in IHF$^-$ *E. coli* hosts [24, 26, 89, 90]. Studies on phage λ *cos*154, a mutant virus that is IHF dependent for growth, identified the mutation as a G to A transition at position 154 in the λ genome [61]. Inspection of this region revealed that the mutation fell within one of three repeated 16-bp 'R' sequences found within *cosB* (R3–R1; fig. 2). Two potential IHF-binding sites (I1 and I2) were also identified within the *cosB* region and a fourth, though weaker, R element (R4, now known as *cosQ*) was identified upstream from *cosN* [61, 91]. Studies on this and other IHF-dependent phage led to the suggestion that (i) the R elements represent gpNu1-binding sequences, that (ii) cooperative binding of gpNu1 at these sites is required for efficient DNA packaging, that

(iii) mutations introduced into these elements impair gpNu1 assembly at *cosB*, and that (iv) IHF binding to I1 and I2 promotes gpNu1 assembly at the R elements and becomes essential when gpNu1·R site interactions are impaired by mutation [61]. IHF binding to its recognition element in DNA introduces a sharp (140° to $>160°$) bend in the duplex [92, 93] and is thought to promote the assembly and stability of a variety of nucleoprotein complexes [94].

DNase footprinting studies have directly demonstrated IHF and gpNu1 binding to the *cosB* region of the λ genome. The isolated gpNu1 subunit indeed protects the three R elements in wild-type *cosB*, but not the upstream *cosQ* element [28, 68, 69, 95]. Identical footprints are observed in the R3 and R2 region of phage λ *cos*154, but no protection is observed at R1, consistent with the posit that the mutation alters the affinity of gpNu1 for that site [95]. Similar studies have demonstrated that IHF binds strongly to the I1 element in *cosB*, but only weakly to I2 [26, 91, 92], and that IHF promotes gpNu1 binding to the R elements [25]. Subsequent studies have demonstrated that point mutations in any of the R elements found within *cosB* also render the virus IHF dependent for development, with the severity of the mutations following the order R3 > R2 > R1 [96]. Insertions between R2 and R1 (7–20 bp) and/or deletion of R1 yield phage phenotypically identical to λ *cos*154 suggesting that IHF obviates the need for the R1 element rather than increasing the affinity of gpNu1 for the site. This is consistent with the observation that IHF does not stimulate gpNu1 binding to the R1 element of λ *cos*154 in vitro [25]. Conversely, while phage with point mutations in the R2 element are similarly IHF dependent for growth, deletion of this element is lethal even in IHF^+ hosts [96]. These data suggest that gpNu1·R2 interactions are critical to phage development and that IHF may overcome binding defects resulting from mutation of this site, but not its deletion. Tandem point mutations in the R3 and R2 elements are also lethal and unresponsive to IHF [96] suggesting that cooperative binding to R3–R2 is critical, and that IHF is insufficient to overcome the additive binding defect resulting from dual mutations.

These genetic studies are complemented by in vitro DNase footprinting studies demonstrating that while gpNu1 binds poorly to an isolated R element, cooperative assembly to adjacent R3 and R2 elements is quite efficient [25]. Cooperative binding between the R2 and R1 elements is not observed, however, and the data suggest that gpNu1 binding to R1 requires cooperative interactions across all three R elements [25]. Gel retardation experiments are further consistent with strong cooperative DNA-binding interactions across all three of the R elements in *cosB* [31, 38].

A number of revertants have been isolated that suppress binding defects induced by *cosB* mutations, again rendering them IHF independent for growth [24, 89, 97]. The mutations typically map to the terminase gpNu1 subunit, and each of the pseudorevertants suppresses a variety of *cosB* mutations, but to varying degrees. Several missense (ms) mutants have been isolated and characterized including *Nu1*ms1 ($L_{40}F$, also known as *ohm1*), *Nu1*ms2 ($L_{40}I$), *Nu1*ms3 ($Q_{97}K$), and *Nu1*ms4 ($A_{92}G$) [24, 89, 97, 98]. While the in vitro DNA-packaging activity and in vivo virus yield of λ ($Nu1^{WT}$, $cosB^-$)[2] phage are decreased 12- and 1000-fold, respectively, compared to λ ($Nu1^{WT}$, $cosB^+$) phage, λ ($Nu1^{ms}$) revertants are IHF independent and utilize $cosB^-$ DNA as efficiently as wild-type $cosB^+$ DNA. The nuclease activity of these mutants is similar to wild-type enzyme and they each possess wild-type DNA-binding affinities as determined by competition experiments [98]. Interestingly, however, the yield of λ ($Nu1^{ms}$, $cosB^+$) phage is reduced in the presence of IHF when compared to wild-type λ ($Nu1^{wt}$,$cosB^+$) phage [97, 98]. Taken together, the data suggest that the gpNu1-ms subunits have increased affinity for the R elements (both mutant and wild type), but that the increased affinity does not affect the assembly of gpNu1 at *cosB* or the nicking activity of the enzyme. Rather, strong binding interactions appear to be more important to a post-cleavage complex, presumably complex I (see fig. 1). While this complex must be sufficiently stable to prevent premature dissociation of the enzyme from the newly formed genome end, over-stabilization of complex I impedes terminase disengagement from *cos* and the transition to the active DNA-packaging complex (step 8, fig. 1) [98, 99]. The presumed increase in stability may be related to ATP-induced effects as these mutants have a two- to three fold decrease in the K_m for ATP hydrolysis at the gpNu1 subunit (but no change in the kinetic parameters at gpA) [98].

gpA DNA-binding interactions

Similar to *cosB* mutations, introduction of point mutations into *cosN* render the virus IHF dependent for growth [100]. Interestingly, however, while *cosN* exhibits twofold rotational symmetry, mutations introduced into the *cosNL* half-site are more severe than symmetric mutations in *cosNR* (see fig. 2) [100, 101]. Mutation of *cosNL* (G_2C) reduces the virus burst size tenfold, likely due to a *cos*-cleavage defect, while the symmetrically disposed *cosNR* ($C_{11}G$) mutation has no

[2] λ($Nu1^{WT}$, cosB^-) refers to a virus carrying a wild-type *Nu1* gene and a mutant cos*B* region. A variety of substrates containing single and/or multiple point mutations in the cos*B* R elements were examined [100]. The data presented here are for the cos*B*($R3^-R2^-R1^-$) substrate.

significant effect [100, 101]. Reversion mutants of *cosN*$^-$ phage have been isolated and all of the characterized isolates have missense mutations in the *A* gene [101]. Three isolates (gpA-$E_{515}G$, gpA-$R_{504}C$, gpA-$N_{509}K$) were further characterized and all three restored IHF-independent growth. Moreover, the pseudorevertants showed wild-type growth characteristics when introduced into a *cosN*$^+$ background, and in vitro *cos*-cleavage assays have demonstrated that the gpA-$E_{515}G$ mutation restores wild-type nuclease activity with both *cosN*$^+$ and *cosN*$^-$ substrates [101]. None of these pseudorevertants suppressed mutations in *cosB*, however, and the *Nu1*ms revertants that effectively overcome the severe *cosB*($R3^-R2^-R1^-$) mutation do not suppress the *cosN*$^-$ defect. These data provide support for the suggestion that gpA and gpNu1 interact at the *cosN* and *cosB* subsites, respectively. Unlike gpNu1, however, the isolated gpA subunit does not yield a detectable footprint of *cos*-containing DNA [69]. This is consistent with the weak and relatively non-specific DNA-binding activity observed in gel mobility shift experiments [31].

Terminase DNA-Binding interactions

Gel shift studies have shown that the DNA-binding affinity of each terminase subunit is significantly increased in the presence of the other, and that the gpNu1 subunit promotes *cos*-specific binding of gpA [31]. Consistently, DNase footprinting studies have demonstrated that gpNu1 binding to an R3 element promotes gpA DNA binding, even in the absence of an upstream *cosN* binding site [28]. These studies have further demonstrated that terminase holoenzyme provides R element protection similar to that observed with the isolated gpNu1 subunit, but only if ATP is withheld [28]. In the presence of ATP, the protection pattern is more extensive and extends from *cosQ* through R1 (see fig. 2) [28]. Deletion of a *cosN* half-site does not significantly alter the DNase protection of the alternate half-site, although *cos*-specific binding becomes ATP dependent if *cosNR* is deleted. Overall, the protection pattern suggests an organized nucleoprotein complex involving $\approx$ 200 bp DNA and multiple terminase protomers, the conformation of which is modulated by ATP.

Catalytic activities of λ terminase

ATPase activity

Characterization of the ATPase activity of phage λ terminase has demonstrated that magnesium (1–5 mM) is strictly required for the reaction, though other divalent metals including Mn^{2+}, Ca^{2+}, and Ba^{2+} will also support ATP hydrolysis to varying extents [84, 102]. The reaction is unaffected by polyamines and IHF, but is stimulated by salt, with maximal (twofold) stimulation observed at 200 mM NaCl [102]. Early studies suggested that the ATPase activity of λ terminase was DNA dependent [22], but more recent experiments have demonstrated a basal activity that is stimulated three- to fivefold by non-specific DNA [84, 102, 103].

The identification of putative ATPase phosphate-binding loop (P loop) motifs in each subunit of the enzyme [81, 82] suggested that both proteins might possess ATPase activity. A preliminary steady-state kinetic analysis of ATP hydrolysis by the enzyme revealed two kinetically distinct catalytic sites: a high-affinity site ($K_m \approx 5$ μM) and a low-affinity, DNA-stimulated site ($K_m \approx 1.3$ mM minus DNA, $K_m \approx 470$ μM plus DNA) [102]. Recent experiments have demonstrated that DNA also stimulates the high-affinity ATPase activity of the enzyme [72]. Affinity labeling studies have demonstrated that while both subunits in the holoenzyme may be covalently modified with photoreactive ATP analogs, much lower concentrations are required to label gpA than gpNu1 (apparent K_Ds of ≈ 3.5 μM and ≈ 500 μM, respectively) [28, 103]. These data suggested that the high- and low-affinity ATP sites were located in gpA and gpNu1 subunits, respectively. Peptides comprising Thr18–Lys35 in gpNu1 and Ala59–Lys84 in gpA have been shown to be covalently modified with photoactive ATP analogs [104]. Recently, photoadducts to Tyr46 and Lys84 in the gpA subunit have been directly demonstrated (Q. Hang, M. Tack and M. Feiss, unpublished data).

Studies on the ATPase activity of the isolated terminase subunits have demonstrated that gpNu1 possesses weak, if any, ATPase activity, while gpA possesses significant and DNA-stimulated activity (L. Woods and C. E. Catalano, unpublished data) [84, 87]. Reconstitution of terminase from gpA and sarkosyl-purified gpNu1 does not result in increased ATPase activity and it has been suggested that gpA alone is responsible for the bulk of the ATPase activity in the holoenzyme complex [84]. This is inconsistent with mutational and kinetic studies, however, that show significant activity at the gpNu1 catalytic site [102, 103]. Moreover, reconstitution of terminase from gpA and GDN-purified gpNu1 yields a holoenzyme with significantly enhanced ATPase catalytic activity [72, 87]. These data suggest that appropriate associations of gpNu1 and gpA in the holoenzyme complex are required for full-expression ATPase activity and that the sarkosyl-purified protein may be deficient in these interactions, perhaps due to the presence of tightly bound detergent molecules (see above).

Mutational analysis of the 'critical' P loop lysine [105] at position 497 in gpA was performed. These studies have clearly demonstrated that a high-affinity ATPase

site is located in the gpA subunit of the enzyme, and that mutations at this site (i.e., $K_{497}D$) do not affect catalysis (k_{cat}), but significantly decrease binding affinity ($\approx$15-fold increase in the K_m for ATP) [103]. Similar studies have shown that a low-affinity site is located in gpNu1 and that mutation of the putative P loop lysine (i.e., $K_{35}D$) results in both a threefold increase in the K_m for ATP and a tenfold decrease in the k_{cat} [103]. Interestingly, however, increasing the concentration of DNA in the reaction mixture restores wild-type ATPase kinetics to the gpNu1-$K_{35}D$ mutant, suggesting that ATP hydrolysis, per se, has not been affected by this mutation. Rather, communication between the ATPase catalytic site in gpNu1 and the DNA-binding domain(s) of the holoenzyme appears to be disrupted [103]. These studies have further demonstrated that introduction of mutations into the putative P loop lysine of one subunit has modest effects on ATP binding (but not hydrolysis) in the second subunit, suggesting allosteric communication between the ATPase catalytic sites in the holoenzyme complex [103].

GTPase activity: evidence for subunit communication

Preliminary characterization of the ATPase activity of λ terminase has demonstrated that GTP (and dGTP) stimulate the ATPase activity of the enzyme [102], and terminase-mediated GTP hydrolysis has been demonstrated [84]. An analysis of this reaction has demonstrated that unlike the ATPase activity of the enzyme, GTP hydrolysis is DNA *dependent* and that a single catalytic site is observed in the steady-state kinetic analysis of the reaction ($K_m \approx 500$ μM, $k_{cat} \approx 37$ min^{-1}) [106]. The optimized reaction conditions are, for the most part, similar for ATPase and GTPase activities, though while salt stimulates ATP hydrolysis, NaCl strongly inhibits GTP hydrolysis [106]. This is easily understood, however, as salt strongly inhibits DNA binding by the enzyme [31]. Of note is that GTP-mediated stimulation of ATPase activity does *not* require DNA, suggesting that while guanosine nucleotides can *bind* to the enzyme in the absence of DNA, GTP *hydrolysis* requires polynucleotide [106].

An interesting feature of the 'NTPase' activity of terminase is that while guanosine nucleotides significantly stimulate ATP hydrolysis by the enzyme, adenosine nucleotides strongly inhibit GTP hydrolysis [102, 106]. These studies suggest that ATP/ADP binding to the high-affinity ATPase site in the gpA subunit is responsible for the observed inhibition of GTPase activity, while GTP/GDP binding to gpNu1 stimulates ATP hydrolysis at gpA [106]. While the physiologic role of GTP in terminase-mediated virus assembly remains vague, these studies strongly suggest that the NTPase catalytic sites in the two enzyme subunits interact, confirming results obtained from mutagenesis experiments (see above). Moreover, work in our laboratory has demonstrated negative hysteresis in the ATPase activity of λ terminase with the observed rate of ATP hydrolysis decreasing to a steady-state rate over 15–20 enzymatic turnovers (L. Woods and C. E. Catalano, unpublished data). We have suggested that the ATP hydrolysis cycle in gpNu1 regulates the ATPase activity of the gpA subunit [106]. We further postulate that this communication is important in regulating the assembly and relative stability of the nucleoprotein packaging complexes and coordinating the transitions between the multiple intermediates required for the assembly of an infectious virus.

Endonuclease (*cos*-cleavage) activity

Terminase nicks λ DNA at *cosN* forming the 12-base single-stranded 'sticky' end of the mature λ genome (steps 6 and 9, fig. 1). In vitro kinetic analysis has demonstrated that the initiating *cos*-cleavage reaction (step 6) requires Mg^{2+} (or Mn^{2+}), is inhibited by salt ($>$50 mM NaCl) and polyamines ($>$5 mM), and has an alkaline pH optimum [23, 85]. The observed rate of *cos*-cleavage is stimulated by ATP, ADP, and GDP, but interestingly, not GTP [22, 29, 59, 85, 106]. IHF also stimulates the observed rate two- to threefold, but only when the terminase concentration is limiting [23, 85, 107]. The reaction kinetics are characterized by a single enzymatic turnover and the data suggest that two terminase protomers are required to efficiently nick the duplex [23, 107]. These data are consistent with the assembly of a gpA dimer bound at the *cosN* half-sites and in the absence of procapsids, the formation of the stable intermediate complex I (fig. 1).

Kinetic analysis has further demonstrated that the nuclease reaction time course is monophasic at low enzyme concentrations, but becomes clearly biphasic at elevated concentrations with the introduction of a rapid phase of *cos*-cleavage [23, 107]. The kinetic data are consistent with a model where the nuclease rate is limited by the slow assembly of the terminase subunits at *cos* ($k_{slow} \approx 0.03$ min^{-1}) followed by rapid duplex nicking ($k_{fast} \approx 0.3$ min^{-1}) [23, 107]. While the nicking reaction requires divalent metal, terminase binding does not [31] and pre-incubation of terminase with DNA obviates the slow assembly step and rapid duplex nicking is observed [107]. The assembly step is not affected by DNA length, and similar rates are obtained using *cos*-containing substrates from 0.266 to 12 kb in size. The stability of the pre-nicking complex appears to be weaker with short DNA substrates, however [107].

Mutational studies have suggested that the nuclease activity of terminase holoenzyme resides within the gpA subunit [79, 80]. These experiments have demonstrated that mutations introduced into the putative gpA bZIP

dimerization motif and/or into a second region that shows homology to conserved sequences in bacterial primase and DNA polymerase proteins (Leu_{395}–Gly_{416}) abolishes *cos*-cleavage in vitro and strongly attenuates the packaging of concatemeric DNA [79]. Packaging of mature DNA is not affected, however, suggesting that these mutants are defective in *cos*-cleavage, but retain DNA-binding, procapsid-binding, and DNA translocation activities. More directly, a *cosN*-specific nuclease activity has been demonstrated with elevated concentrations of the isolated gpA subunit [85, 107]. Interestingly, gpA titration studies have revealed a non-linear concentration dependence, again suggesting that gpA self-assembly may be important to DNA binding and efficient nuclease activity [85]. The nuclease activity of the isolated gpA subunit is strongly stimulated by gpNu1, suggesting that gpNu1 aids in the assembly of gpA at *cosN*. This idea is consistent with DNA-binding studies [31] that show that higher concentrations of DNA are required for efficient binding to gpA than to the holoenzyme complex [85, 107].

Role of ATP in the *cos*-cleavage reaction

The maturation of concatemeric DNA requires that terminase nicks the duplex appropriately at the N_1 and N_2 sites within *cosN* (see fig. 2). Several studies have demonstrated that ATP modulates both the rate and the fidelity of the nuclease reaction and revealed a complex interaction of the terminase subunits with *cosN*, *cosB*, and ATP in the assembly of a catalytically competent nicking complex of high fidelity [27, 29, 59, 96, 108, 109]. The results of these studies have been previously summarized in detail [21]. Overall, the data suggest that intrinsic binding interactions between the gpA subunit and the *cosNL* half-site play a dominant role in N_2 nicking fidelity (see fig. 2). Conversely, gpNu1·R3 interactions are important to the assembly of a gpA subunit at *cosNR*, though this complex shows limited nuclease fidelity. In fact, gpNu1 binding to the purine-rich r-strand of the R3 element (see fig. 2) promotes upstream gpA assembly and non-specific r-strand nicking ≈ 47 bases in the 3′ direction (upstream). The gpNu1·R3 interaction thus appears to be the dominant interaction driving the assembly of a nuclease-competent complex. In a complete *cos* sequence, the *cosN* and *cosB* subsites assemble a nucleoprotein complex efficiently with gpNu1 binding at R3 promoting gpA binding at *cosNR*, and gpA·gpA interactions assembling a second gpA subunit at *cosNL*. Dimerization at *cosN* is thus driven both by intrinsic gpA·*cosNL* binding interactions and protein-protein interactions presumably mediated by the putative gpA bZIP motif.

ATP affects the nuclease activity in two ways: (i) the observed nicking rate is increased $\approx$ tenfold with no apparent increase in the affinity for DNA, and (ii) the fidelity of the nuclease reaction is ensured with nicking appropriately taking place at N_1 and N_2. The exact mechanism remains unclear; however, the data suggest that an ATP-dependent conformational adjustment in the pre-nicking complex may be required for accurate duplex nicking, and that this conformational change is the rate-limiting step in the *cos*-cleavage reaction at elevated enzyme concentrations. Finally, it should be noted that *cosQ* (also known as R4) does not appear to play a role in the assembly of the nicking complex or the putative ATP-dependent conformational change, but is important to the downstream, terminal nicking reaction (step 9, fig. 1) [110]. This is described in more detail below.

ATP stimulates the rate of *cos*-cleavage at concentrations as low as 5 μM [22, 27] suggesting that ATP interactions at the high-affinity ATPase site in gpA affect the nuclease activity of the enzyme. Consistent with this suggestion, revertants of *cosN* mutations (see above) map in a cluster near the putative ATPase catalytic site in gpA and decreased ATPase activity is observed in these mutants [discussed in ref. 101]. Furthermore, mutation of the 'critical' lysine in one of the putative gpA P loop motifs (K_{497}) not only affects ATP binding, but abolishes *cos*-cleavage and strand-separation activities both in vitro and in vivo (Y. Hwang and M. Feiss, unpublished data) [83, 103]. The effects of this mutation are not restored by increasing the concentration of DNA or by ATP concentrations sufficient to fully saturate the altered ATP-binding site [83], and it has been postulated that ATP binding and/or hydrolysis modulates the nuclease activity of this subunit [103]. Interestingly, the ATPase and nuclease activities are restored in the gpA-(K_{497}D,E_{515}G) double mutant suggesting that the mutation at E_{515} restores communication between the ATPase and nuclease catalytic sites that was disrupted by the mutation at K_{497} [101].

It is clear that the gpNu1 subunit also plays a role in the *cos*-cleavage reaction. Mutations introduced into the 'critical' lysine of the putative P loop in gpNu1 (i.e., K_{35}D) are lethal and no *cos*-cleavage is detected in vivo [111]. Consistently, the in vitro ATPase and *cos*-cleavage activities of the gpNu1 mutant enzyme are severely impaired and the protein does not effectively discriminate between *cos*-containing and non-specific DNA substrates [103, 111]. Interestingly, however, increasing the concentration of DNA in the in vitro assays fully restores wild-type catalytic activities. The data suggest that the gpNu1 ATP-binding site is involved in site-specific DNA-binding interactions and the assembly of terminase at *cosB*.

Strand-separation activity

Subsequent to nicking of the duplex at *cosN*, terminase separates the nicked annealed strands yielding the mature left end of the λ genome protected in complex I (fig. 1). While this reaction has been described as a 'helicase' activity, terminase is not a helicase in the strict sense, and we refer to this activity as the strand-separation activity of the enzyme. In vitro analysis of this activity has demonstrated that the reaction is optimal at alkaline pH, requires divalent metal (Mg^{2+} or Mn^{2+}), and is inhibited by polyamines [84, 86]. Strand separation requires adenosine nucleotides with a hydrolyzable β,γ phosphate bond, though other hydrolyzable nucleotides support the reaction to varying degrees. Strand-separation activity has been observed with the isolated gpA subunit, and the reaction requirements parallel those observed for the holoenzyme [84].

IHF stimulates the strand-separation activity of the holoenzyme, especially when excess non-specific DNA is included in the reaction mixture [86]. The data suggest that IHF binding to the I1 element within *cosB* (see fig. 2) forms a binary protein·DNA complex readily distinguishable from bulk DNA [86]. Low concentrations of salt (< 50 mM) stimulate the strand-separation activity but inhibit the reaction at elevated concentrations [86]. This likely reflects the opposing effects of salt on ATP hydrolysis [102] and DNA binding by the enzyme [31]. The ATP concentration dependence of strand-separation activity revealed two ATP-binding sites with apparent K_Ds of 49 μM and 230 nM [86]. The extremely high affinity site identified in these studies represents a novel ATP-binding site distinct from the two ATPase catalytic centers characterized in the steady-state kinetic analysis of ATP hydrolysis by the enzyme [102]. The nature of this binding site remains obscure.

As described above, terminase is not a helicase in the strict sense, yet separation of the nicked duplex requires ATP hydrolysis [29, 69, 86]. We have suggested that the ATP hydrolytic cycle might drive alternating protein conformations, with ATP and ADP stabilizing conformations that differentially bind duplex and single-stranded DNA [86]. This model for terminase-mediated strand separation essentially represents a single turnover of the active rolling strand separation model proposed by Lohman and co-workers for helicase enzymes [112, 113].

Transition to complex II and active DNA packaging

Complex I is an extremely stable nucleoprotein intermediate with a half-life of > 8 h (in the absence of procapsids) [31]. The stability of this intermediate is presumably mediated by gpA interactions with the newly formed single-strand end and strong gpNu1·*cosB* binding interactions. Based on the gpNu1 DNA-binding studies described above, the latter may in fact be the predominant force stabilizing complex I. Whatever the case, procapsid binding to complex I triggers the release of terminase from *cos* and translocation of DNA into the capsid. The procapsid is a spherical structure composed of gpE, the major capsid protein, and a specialized vertex containing the collar and the connector which together make up the portal vertex [114]. The collar is composed of ten molecules of pX, a covalent modification of gpC and gpE proteins, and is thought to hold and position the connector within the capsid. The connector is a dodecameric ring-like structure composed of the viral protein gpB, and the gpA subunit within complex I is presumed to interact with the connector to initiate translocation. In fact, it is likely that some combination of the terminase proteins and the portal vertex proteins in the procapsid make up the active DNA-packaging machine.

Early studies demonstrated that a functional gpA protein is required during active DNA packaging [30], and important procapsid·gpA interactions have been localized to the five C-terminal amino acids of the terminase gpA subunit, particularly Leu_{636} (fig. 3) [77, 115]. Introduction of mutations into this region of gpA does not affect ATPase, *cos*-cleavage, or strand-separation activities of the holoenzyme, but abrogates procapsid-binding interactions and DNA packaging. Spontaneous revertants of C-terminal gpA mutations map to second sites in gpA and gpB, consistent with a gpA·portal vertex packaging complex [115, 116]. While the data strongly suggest that gpA is a component of the translocation machine, the role of the gpNu1 subunit in the active DNA packaging remains unclear.

The interaction of an empty viral procapsid with complex I results in disengagement of terminase from *cos* and the formation of a poorly characterized intermediate known as complex II (step 7, fig. 1) [30, 35, 56]. This interaction signals the transition to a mobile translocation complex and active DNA packaging. The translocase activity of λ terminase has been studied in vitro and has been shown to require procapsids, terminase, Mg^{2+} (but not Mn^{2+}), and ATP hydrolysis [32, 84]. The reaction is optimal at alkaline pH in the presence of polyamines and modest concentrations of salt [32]; however, while these reaction conditions yield optimal packaging activity, salt and polyamines diminish the capacity of the enzyme to discriminate between λ and non-specific DNA substrates. Conversely, IHF increases phage λ DNA packaging specificity and stimulates the rate of the reaction at low concentrations [32, 33]. Packaging activity increases linearly with terminase (0–100 nM) and DNA (0–0.8 nM) concentrations, but a sigmoidal concentration dependence is observed with procapsids [33].

It is commonly accepted that ATP hydrolysis drives the translocation apparatus, though this has never been directly demonstrated. Neither of the identified ATPase catalytic sites in the terminase holoenzyme has a turnover rate sufficient to accommodate that required to power translocation [102, 103]. This suggests either that (i) one of the identified ATPase catalytic sites is strongly stimulated in the translocation complex or (ii) a quiescent and as yet unidentified catalytic site in the packaging complex is activated. Whatever the case, the time course for ATP hydrolysis mirrors that of DNA packaging and the ATP concentration dependence of the reaction ($C_{1/2} \approx 3$ μM) suggests that a high-affinity ATPase site in the gpA subunit may be responsible for driving translocation [33, 83]. Consistently, low packaging activity is observed with the isolated gpA subunit while gpNu1 alone does not possess any detectable in vitro DNA-packaging activity [32]. Interestingly, however, while mutations introduced into the 'critical' lysine in a putative gpA P-loop motif (i.e., gpA-K_{497}D) are lethal and strongly affect ATPase and *cos*-cleavage activities of the holoenzyme (see above), these mutations only modestly affect in vitro virus assembly [83]. The data suggest that the ATPase catalytic site identified at K_{497} does not represent the packaging ATPase. Recent photoaffinity labeling studies using 8-azido-ATP have identified Tyr_{46} and Lys_{84} as residues proximate to a distinct ATP-binding site in the gpA subunit (M. Feiss, personal communication). Mutation of Tyr_{46} (i.e., gpA-Y_{46}F) yields a holoenzyme complex with wild-type strand-separation and *cos*-cleavage activities, but that no longer possesses a high-affinity ATPase catalytic site and that is deficient in DNA-packaging activity (M. Feiss, personal communication). These data suggest that this novel ATPase site may represent, at least in part, the packaging ATPase.

Role of gpFI

The phage λ gpFI protein is strongly expressed during phage infection to a level comparable to that of the major capsid proteins, though it is not a component of the mature capsid [66]. The protein is a 14.4-kDa monomer with an acidic pI (≈ 4.1) [117]. Interestingly, in vitro phage assembly occurs independent of gpFI, but only when elevated concentrations of terminase and procapsids are utilized [33, 34, 117, 118]. GpFI neither binds to DNA nor does it stably interact with procapsids or terminase [35]. The protein appears to increase the rate of DNA packaging [34] and to induce turnover in the *cos*-cleavage reaction under conditions of limiting enzyme concentration [36]. Based on in vitro studies, it has been proposed that gpFI stimulates the transition between complex I and complex II, i.e., procapsid binding to complex I, or that the protein stimulates terminase release from *cos* to initiate active DNA packaging [34–36]. Recent experiments suggest that in vivo *cos*-cleavage is defective in FI^- phage, and it has been proposed that procapsid·gpFI interactions may be required prior to complex I formation in vivo [119].

While gpFI is required for phage development in vivo, FI^- mutants are leaky suggesting that this protein plays an ancillary role [118, 120]. Pseudorevertants of FI^- phage, termed *fin* for FI independence, have been isolated and the second-site mutations have been mapped to two loci located either within the Nu1/A border, or a 26-amino-acid stretch of gpE termed the EFi domain [119, 121]. These mutations have further been classified phenotypically as either *fin*A, those that overexpress the terminase gpA subunit (about fourfold), and *fin*B, those that do not [120]. The characterized *fin*B mutants include second-site mutations in the EFi domain of gpE, or in the C-terminal end of the gpNu1 subunit [119, 120]. Moreover, overexpression of gpNu1 also yields a *fin* phenotype [122]. The exact nature whereby gpFI stimulates DNA packaging in vivo remains obscure.

Termination of packaging: the terminal *cos*-cleavage reaction

Translocation across the duplex drives DNA into the capsid. Upon reaching the next downstream *cos* site in the concatemer (the end of the viral genome), terminase again symmetrically nicks the *cosN* site and gpA-mediated strand-separation simultaneously releases the DNA-filled capsid and regenerates complex I (step 9, fig. 1). The terminal *cos*-cleavage and strand-separation reactions are formally identical to those performed by the nicking complex at the initiation of packaging (step 6, fig. 1); however, the complement of proteins performing these functions certainly differs. While the most obvious difference is that the translocating complex includes a viral capsid, it is quite likely that the composition of the terminase proteins in the pre-nicking complex and the translocating complex also differ. This supposition suggests that the DNA-binding elements involved in the terminal *cos*-cleavage event are distinct from those required for the initial assembly of the packaging complex. Within this context, genetic studies have demonstrated that sequences upstream from *cosN* are necessary for phage development and the critical sequence has been identified as *cosQ* (see fig. 2) [17, 39, 123]. Point mutations in this R-like element are deleterious to phage development and render the virus IHF dependent, while deletion of the element is lethal [123]. Importantly, *cosQ* mutations introduced into the upstream (initiating) *cos* of a tandem prophage are insignificant, while similar mutations introduced into the

downstream (terminal) *cos* are detrimental to phage development [123].

Based on in vivo *cos*-cleavage experiments and the examination of capsids isolated from *cosQ*$^-$ phage, it has been suggested that while packaging may be initiated at a *cos* sequence bearing a mutation in *cosQ* (step 6, fig. 1), similar mutations in a terminal *cosQ* element result in aborted packaging due to the inability to cleave at the downstream *cos* site to complete the packaging process (step 9, fig. 1) [123]. More detailed studies have demonstrated that the initial and terminal *cos*-cleavage reactions possess distinct, and nearly opposite requirements for the DNA elements surrounding *cosN* [110]. Terminal *cos*-cleavage shows a strong dependence on *cosQ*, is relatively insensitive to mutations in the *cosNL* half-site, and while *cosB* is required for efficient nuclease activity, the R elements do not appear to play a major role in this reaction [110]. Moreover, the I2 element within *cosB* and the spacing between *cosN* and *cosB* play an important role in the terminal, but not the initial *cos*-cleavage reaction.

Pseudorevertants of *cosQ* mutants have been isolated and fall into three classes [96]: (i) phage with secondary mutations in *cosQ*, (ii) phage with mutations in the *B* gene encoding the connector protein, and (iii) phage with increased genome length. Unlike revertants of mutations in the *cosB* R elements that regain IHF independence, spontaneous revertants of *cosQ* lesions remain dependent on IHF. Moreover, *cosQ* revertants do not suppress the packaging defects observed with similar *cosB* mutations [96, 99]. Consistently, gpNu1-ms revertants that effectively suppress mutations in *cosB* do not suppress similar mutations in *cosQ* [96, 99]. Taken together, the data strongly suggest that while *cosQ* shows sequence homology to the R elements found in *cosB*, their roles in DNA packaging are distinct: *cosB* R elements are required for the assembly and stability of initiating packaging complexes while *cosQ* is important in the recognition of the terminal *cosN* by the translocating complex (see fig. 1) [99].

The *cosQ* mutation studies further suggest a connection between genome length and the terminal *cos*-cleavage event. This has, in fact, been appreciated for some time. Upon packaging of $\approx 10-50\%$ of the viral genome, the procapsid undergoes an expansion process whereby the 25-nm spherical capsid expands to a radius of 32 nm, roughly doubling its volume and acquiring its mature icosahedral shape [6; cited in ref. 18]. The virally encoded gpD protein joins the capsid at this point serving to stabilize the expanded structure [124]. Early studies on phage development demonstrated that significant deletions within the 'non-essential' *b* region of the λ genome are deleterious to virus growth, and suggest that efficient DNA packaging is sensitive to genome length [125–127]. These studies further suggest that the terminal *cos*-cleavage reaction is impaired with the shorter packaging substrates.

A model for DNA packaging: an ordered progression of nucleoprotein complexes

Phage λ terminase possesses multiple catalytic activities and is an integral part of an ordered series of nucleoprotein complexes involved in the maturation and packaging of viral DNA. During virus development in vivo, each complex quickly transitions to the next and virus assembly proceeds rapidly. The accumulation of some of these complexes has been observed in vivo during infections with packaging-deficient phage, and others have been characterized in vitro, as discussed above. Models for individual steps in the packaging pathway have been proposed by a variety of research groups [20, 21, 28, 31, 98, 99, 106, 107], and based on these models and the recent studies presented here, a model for the assembly of and transition between these nucleoprotein packaging complexes can be proposed (fig. 5).

Step I: assembly of a pre-nicking complex

Genome packaging initiates with binding of gpNu1 to the *cosB* R3 element of a concatemer, driven by strong cooperative interactions at R2 and R1. Occupation of R3 is both necessary and sufficient to initiate complex assembly. IHF binding to I1 (and possibly I2) provides a nucleoprotein scaffold that is more conducive to cooperative gpNu1 assembly at *cosB*, and obviates the need for the R1 element. While normally dispensable, IHF becomes essential for λ development when mutations are introduced into any of the R elements. GpNu1 bound at R3 promotes specific gpA binding to *cosNR*, and protein-protein interactions, presumably mediated via the putative bZIP protein dimerization motif, combine with intrinsic gpA·*cosNL* binding interactions to assemble a symmetrically disposed gpA dimer at *cosN*. This stable pre-nicking complex ($T_{1/2} > 12$ h) may be formed in the absence of divalent metal, though its assembly is protein concentration dependent, and at low terminase concentrations may represent the rate-limiting step in the *cos*-cleavage reaction.

Step II: transition to an activated nicking complex

Addition of Mg^{2+} to the pre-nicking complex results in gpA-mediated nicking of the duplex at *cosN*. It is clear that ATP modulates the nuclease activity of the enzyme, but the nature of this interaction remains obscure. While ATP does not affect the overall affinity of the terminase subunits for DNA (i.e., assembly of the

pre-nicking complex), Mg^{2+}·ATP alters the conformation of the pre-nicking complex, perhaps inducing wrapping of the duplex, and increases the rate and fidelity of the nuclease reaction. Mutation of ATPase catalytic sites in either enzyme subunit affects not only ATP binding and/or hydrolysis, but also abrogates *cos*-cleavage activity. The role of ATP at each enzyme subunit differs, however. ATP binding to gpA appears to directly affect the nuclease activity of the subunit, suggesting allosteric communication between the ATP- and DNA-binding sites in the protein. Conversely, mutation of the gpNu1 ATPase catalytic site does not appear to affect the nuclease activity of the gpA subunit nor the apparent affinity of either subunit for DNA, though *cosNR* nuclease fidelity is altered and discrimination between *cos*-containing and non-specific DNA is adversely affected. To rationalize these results, we suggest that the gpNu1 ATPase catalytic site is involved in promoting a conformational reorganization of the terminase subunits assembled at *cos* to yield the activated nicking complex, a catalytically competent nuclease complex of high fidelity (fig. 5). This putative ATP-dependent conformational change may be rate limiting at elevated enzyme concentrations.

Steps III and IV: the transition to complex I

Mg^{2+}-dependent duplex nicking by the gpA subunit in the activated complex yields the nicked, annealed complex and gpA-mediated strand separation discharges *cosNL* from this intermediate generating complex I (fig. 5). This strand-separation activity of the enzyme is driven by ATP hydrolysis, likely at the gpA subunit of the holoenzyme. It is noteworthy that the pre-nicking complex and complex I are both extremely stable, even though the bound DNA is quite different in each (intact duplex vs 12-base 5′ single-stranded overhang). Given that *cosB* mutations appear to predominantly affect the stability of complex I, it is likely that the gpNu1 nucleoprotein complex assembled at *cos*B remains intact in the transition from the pre-nicking complex to complex I. Moreover, it is likely that strand separation subsequent to duplex nicking divides the symmetrically disposed gpA subunits bound at the *cosN* half-sites. It has

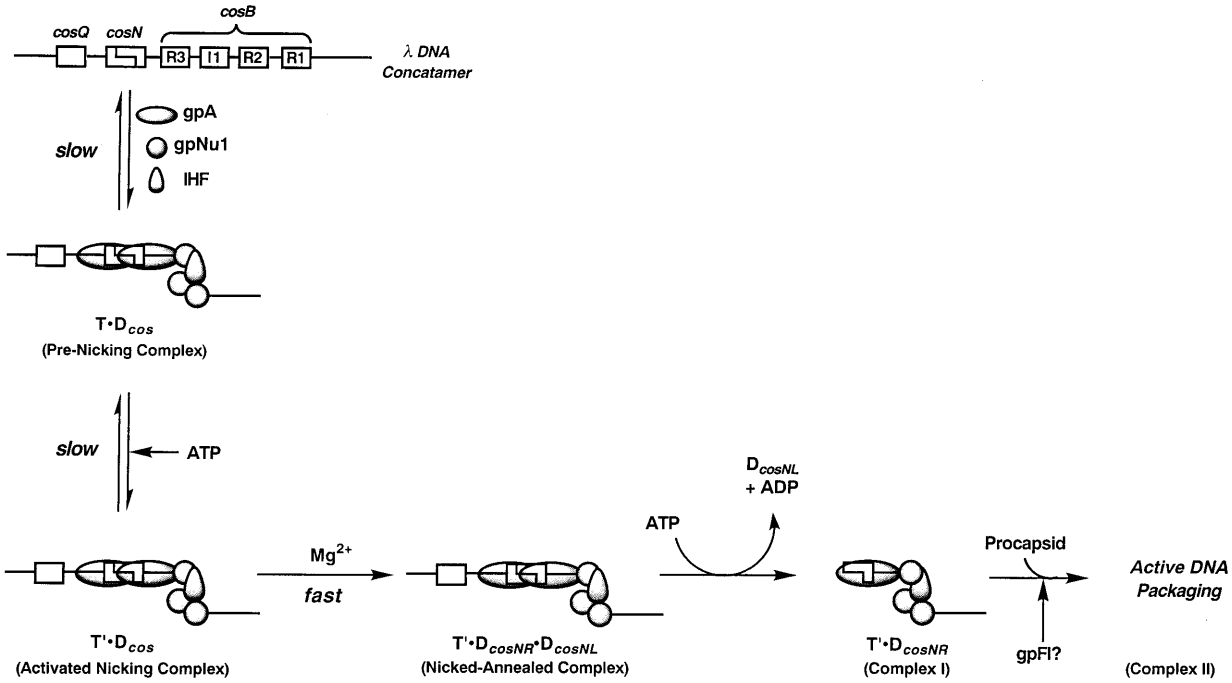

Figure 5. Model for the initiation of λ DNA packaging. The terminase subunits and IHF assemble at a *cos* site in the concatemer forming a pre-nicking complex ($T{\cdot}D_{cos}$). ATP induces a conformational reorganization of the nucleoprotein complex yielding an activated nuclease complex ($T'{\cdot}D_{cos}$) of high fidelity that, in the presence of Mg^{2+}, site-specifically nicks the duplex at *cosN* (*cos*-cleavage reaction) yielding the nicked-annealed complex ($T'{\cdot}D_{cosNR}{\cdot}D_{cosNL}$). ATP-dependent strand-separation yields the stable intermediate complex I ($T{\cdot}D_{cosNR}$). Binding of an empty procapsid yields complex II and initiates the ATP-dependent insertion of viral DNA into the capsid (active DNA packaging). The stoichiometry of the terminase subunits assembled at *cos* remains speculative. Details are presented in the text.

been suggested that the translocation complex is actually composed of a gpA hexamer [21] similar to that proposed for bacteriophage T3 [128, 129], and it is possible that additional gpA subunits are recruited to complex I prior to or concomitant with procapsid binding. Alternatively, gpA multimers may actually assemble at each *cosN* half-site in the pre-nicking complex. Whatever the case, it is likely that the complement of gpA assembled at *cosN* is altered in the transition from the pre-nicking complex to complex I.

Step V: procapsid binding and transition to an active DNA packaging machine

One of the most fascinating and least understood aspects of the packaging pathway is the procapsid-mediated transition from the extremely stable complex I to the rapidly translocating packaging complex. Though not directly demonstrated, genetic, mutational, and biochemical studies all suggest that the translocase activity of λ terminase is centered in the gpA subunit of the enzyme. DNA packaging by gpA initially bound at *cosNR* in complex I requires transit through the gpNu1 nucleoprotein complex assembled at *cosB* (see fig. 5). In fact, gpNu1 may provide a 'molecular roadblock' function that prevents premature gpA translocation from *cos*. The transition to an active DNA-packaging machine thus requires, among other things, that gpNu1 switches from a high-affinity, site-specific DNA-binding protein to a non-specific DNA translocation protein. Alternatively, gpNu1 may completely dissociate from *cosB* in a manner analogous to bacterial sigma factors that are required for the assembly of a transcription complex, but that are ejected after the transition to the elongation phase of RNA synthesis [130]. Whatever the case, the mechanism responsible for this molecular switch remains unclear; however, communication between the ATPase catalytic sites in gpA and gpNu1 has been suggested based on kinetic [106] and mutational [103] studies. ATP-regulated DNA binding is common in biology [131] and it is not unreasonable to suggest that the ATP hydrolysis cycle in one or both of the terminase subunits regulates the stability of complex I, and provides the molecular switch to the translocating complex. In this model, procapsid interactions with complex I may affect ATP hydrolysis cycles in one or both enzyme subunits and ultimately allow terminase release from *cos*. This latter posit suggests mechanistic similarities between λ terminase and the ATP-regulated DNA-binding activities of recA protein [132, 133] and DNA helicases [112, 113], as well as the ATP-modulated transitions between stable and mobile nucleoprotein complexes involved in DNA replication [42, 134] and transcription [43, 135].

Procapsid binding to complex I mediates terminase release from *cos* and transition to an active DNA-packaging machine that translocates across the duplex. A variety of models for an ATP-powered translocation machine have been proposed [summarized in ref. 21], but there is no conclusive experimental evidence that supports one model over another. Studies on phage $\varnothing$29 and T3 packaging systems have demonstrated that approximately two base pairs of DNA are packaged per ATP hydrolyzed [82, 129]. Given that an entire λ genome may be packaged in less than 5 min in vitro, the translocation ATPase can be estimated to possess a turnover rate of at least 80 s^{-1}, a rate significantly greater than that observed with terminase holoenzyme under optimal conditions. Procapsid interactions with complex I must therefore activate a latent ATPase activity, presumably located in the gpA subunit, that powers DNA packaging. Though the precise location of this catalytic site has not been defined, mutational and affinity labeling studies suggest that Tyr_{46} in the gpA primary sequence plays an important role. Finally, the observed negative hysteresis in the ATPase activity of the holoenzyme suggests that the ATP catalytic cycle in the gpNu1 subunit may regulate turnover at the gpA catalytic site. We speculate that this negative regulation may be related to the inhibition of the gpA translocase ATPase in complex I. This model suggests that gpNu1 bound at *cos*B serves not only as a 'molecular roadblock' to gpA translocation, but also as a 'catalytic brake' to the gpA translocase ATPase activity. Procapsid binding to complex I presumably alters the ATP hydrolysis cycle at both catalytic sites resulting in release of the catalytic brake on gpA translocation. Alternatively, the translocase activity of the packaging machine may be partially (or totally?) contained within the procapsid portal vertex proteins with the gpA ATPase catalytic site(s) providing only regulatory roles.

Step VI: termination of packaging (terminal *cos*-cleavage)

Upon encountering the next downstream *cos*-site, the translocating terminase complex nicks the duplex at *cosN* and separates the strands to complete genome packaging (see fig. 6). Assuming uniform movement from initiation to termination, the translocation rate may be estimated to be >160 base pairs/s [21]; however, it is likely that the translocating complex slows as it approaches the end of the viral genome due to steric and electrostatic repulsive forces resulting from DNA condensation within the capsid. It has been postulated that the rate of the translocating complex must be sufficiently slowed in order to trap the complex at the terminal *cosN* site efficiently [96]. In this model, the *cosQ* element may act as a molecular 'speed bump' to

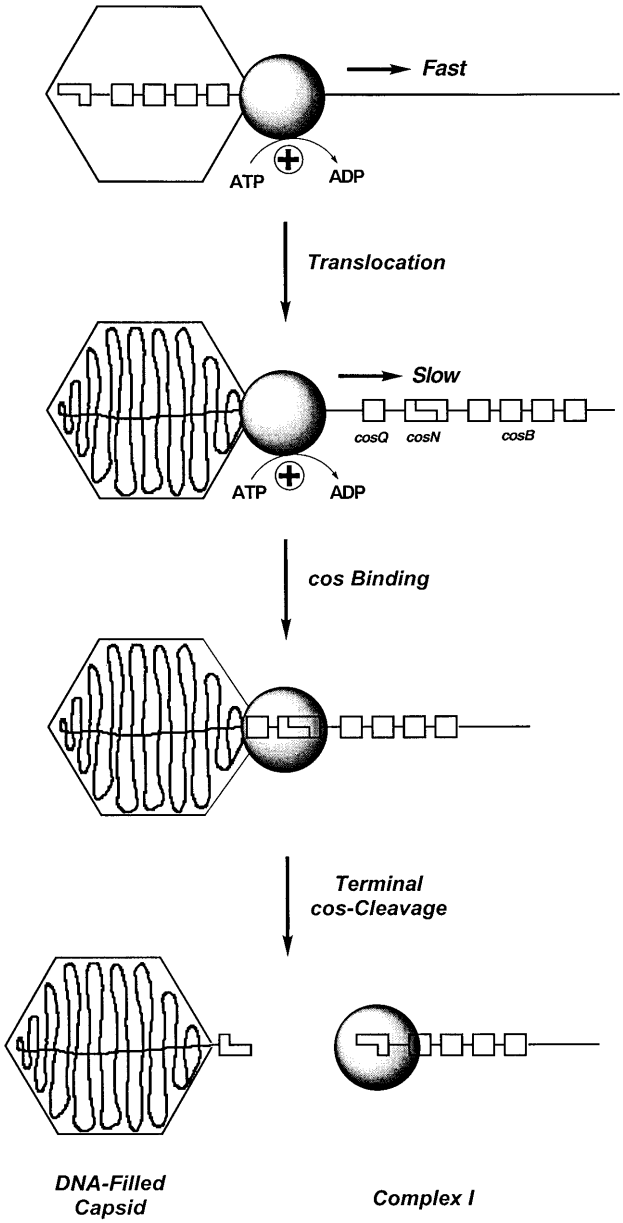

Figure 6. Model for the termination of λ DNA packaging. The translocating packaging complex slows as it reaches the terminal *cos*-site. *cosQ*, *cosN*, and *cosB* work in concert to trap the translocating complex. Terminase then nicks the duplex and separates the strands. Complex I thus formed binds another empty capsid to initiate a second round of packaging. Details are presented in the text.

provide an additional obstacle to terminase translocation. While *cosQ* may be necessary, it is not sufficient, as even multiple copies of *cosQ* are incapable of trapping a translocating complex prior to reaching *cosN* in a natural-length genome [110]. We suggest that this postulated decrease in translocation rate (k_{trans}) as the packaging complex approaches the terminal *cos* site ultimately becomes competitive with the 'on-rate' (k_{on}) for DNA binding. The *cosQ*, *cosN*, and I2 elements work in concert to capture the slowing complex, and together provide an efficient stop signal for *cosN* binding, nicking, and termination of DNA packaging.

An interesting problem arises when one considers the symmetry of the translocating complex and that needed for *cos*-cleavage. Assuming that the gpA·portal interactions required for transition to complex II are maintained in the translocating complex, then the polarity of gpA in this complex is such that the C-terminal end of the protein is directed towards the capsid. GpA-mediated nicking of the terminal *cosN* site presumably requires that symmetrically disposed gpA subunits bind to each *cosN* half-site, however, and some mechanism is required to 'depolarize' the unidirectionally disposed gpA subunit(s?) in the translocating complex. While depolarization models have been proposed [110], limited information on the nature of the translocation complex hinders our ability to describe this process in any detail.

Summary

We have made great strides in our understanding of DNA packaging in phage λ and, in particular, the biochemical, biophysical, and catalytic properties of phage λ terminase. Typically, however, accompanying each advance is a new series of questions and mysteries. While each of the identified catalytic activities of the enzyme has been examined in some detail, the interaction between the catalytic sites remains vague. Clearly, complex interactions exist between the NTPase catalytic sites in the holoenzyme, between nucleotide and DNA binding, and between ATPase and nuclease activity. Studies that scrutinize the allosteric interactions among these multiple catalytic sites, their role in the assembly of packaging intermediates, as well as mediating the transitions between the intermediates remains a fertile and exciting area of research.

Structural domains within each enzyme subunit responsible for protein·protein, protein·nucleotide, and protein·DNA interactions have been inferred for quite some time. Only recently, however, has a physical characterization of these putative domains been accomplished, and these studies are in their infancy. Similarly, a biophysical description of the protein·protein and protein·DNA interactions required for cooperative nucleoprotein complex assembly is in a primordial state. Nevertheless, we are now poised to define rigorously the thermodynamic principles that underlie the assembly and stability of these packaging intermediates. Each of these nucleoprotein complexes likely possesses distinct catalytic activities, and altered catalytic activity in each intermediate likely drives one transition to the next. A

wealth of information thus remains buried in the delicate interplay between catalytic activity and thermodynamic stability, and this too provides an exciting arena for continued research.

Our understanding of phage λ DNA packaging has been and will continue to be supported by parallel studies in other phage systems, most prominently bacteriophage $\varnothing$29, bacteriophage T3, and bacteriophage T4 [3, 136–138]. While many of the details will undoubtedly differ, the basic biochemical and biophysical principles remain the same, and lessons learned in one system will advance our understanding of the others. Importantly, this will be invaluable to developing viable models for DNA packaging in the ill-characterized eukaryotic viruses such as the herpesvirus groups [139]. Finally, the nucleoprotein complexes involved in DNA packaging bear mechanistic similarities to a variety of nucleoprotein complexes involved in diverse biological functions such as DNA replication, DNA transcription, and recombination [131]. Moreover, terminase enzymes are part of a growing number of examples of biological 'machines' or molecular 'motors,' and an understanding of the biochemical, biophysical, structural, and catalytic properties of this packaging machine will yield insight into the more general properties of large multiprotein enzyme complexes.

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