A viral genome packaging ring-ATPase is a flexibly coordinated pentamer

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Multi-subunit ring-ATPases carry out a myriad of biological functions, including genome packaging in viruses. Though the basic structures and functions of these motors have been well-established, the mechanisms of ATPase firing and motor coordination are poorly understood. Here, using single-molecule fluorescence, we determine that the active bacteriophage T4 DNA packaging motor consists of five subunits of gp17. By systematically doping motors with an ATPase-defective subunit and selecting single motors containing a precise number of active or inactive subunits, we find that the packaging motor can tolerate an inactive subunit. However, motors containing one or more inactive subunits exhibit fewer DNA engagements, a higher failure rate in encapsidation, reduced packaging velocity, and increased pausing. These findings suggest a DNA packaging model in which the motor, by readjusting its grip on DNA, can skip an inactive subunit and resume DNA translocation, suggesting that strict coordination amongst motor subunits of packaging motors is not crucial for function.
DOUBLE-STRANDED DNA-CONTAINING BACTERIOPHAGES ARE THE MOST WIDELY AND ABUNDANTLY DISTRIBUTED "ORGANISMS" ON EARTH. BACTERIOPHAGE (PHAGE) T4, A PHAGE WITH A CONTRACTILE TAIL THAT INFECTS ESCHERICHIA COLI, BELONGS TO MYOVIRIDAE FAMILY AND IS ONE OF THE MOST WELL-CHARACTERIZED VIRUSES. IT HAS BEEN PROPOSED THAT EACH SUBUNIT TAKES PART IN A PROCESSIVE MECHANICAL MOVEMENT DURING PACKAGING IN WHICH ATLEAST 100 SUBUNITS ARE INVOLVED.

Results

The actively packaging T4 motor is a pentamer. We used single-molecule TIRF to count the number of gp17 subunits present in an actively packaging T4 motor, i.e., the motor's stoichiometry. A functional T4 motor was re-constituted by mixing empty T4 heads (capsids) and gp17 in the presence of the poorly hydrolyzed analog ATP-γS. A 120-μM unlabeled DNA was also added to allow efficient assembly of packaging-competent motors, which requires both ATP-γS and DNA. The assembled motors were then attached to a polyethylene glycol (PEG) passivated surface via anti-T4 polyclonal antibody (Fig. 1a and Supplementary Fig. 2a; see "Methods"). The motor comprised of active gp17 subunits into which a fluorescent label was introduced by fusing a 20-kDa SNAP-tag at the N-terminus and labeling it with SNAP-Surface® 549 fluorescent substrate (hereafter referred to as "Cy3" because it has similar fluorescent properties as Cy3) (Supplementary Fig. 1a–c; see Methods). Each packaging motor was imaged as a single fluorescent spot consisting of m Cy3 labels on n subunits, with m depending on labeling efficiency and photoactivity. Each motor’s packaging activity was monitored from the fluorescence of encapsidated short, 45-bp Cy5-labeled double-stranded DNA molecules (Fig. 1a). Correspondingly to each Cy3 spot, a colocalyzed Cy5 spot indicated packaged Cy5-DNA, certifying the motor’s activity (Fig. 1b inset). Using a bulk DNA packaging assay, we verified that Cy3 SNAP-tagged motors were nearly as active as the WT motors (Supplementary Fig. 1c).

The appearance of Cy3 and Cy5 signals required the presence of all the components of an immobilized packaging motor, substrate, the dynamics of DNA engagements at the time of packaging initiation, and how the firing of motor ATPases is coordinated. Based on a structural model of the T4 motor, it was proposed that each subunit takes part in a processive mechanical movement during packaging in which at least 100 subunits are involved.

Molecular motors belonging to the additional strand conserved E (glutamate) superfamily are responsible for a myriad of cellular activities, including DNA replication involving DNA helicases, transcription regulation by transcription termination factors such as Rho, protein degradation by chaperones and proteasomes, and cargo transport by dyneins. These molecular machines are ring-NTPases powered by energy from NTP hydrolysis, and many translocate their substrates through the ring’s central channel. The packaging motors, such as the large "terminase" gp17 of phage T4, share this common characteristic of being homologous ring-NTPases that utilize ATP energy to encapsidate viral genomes. Thus, they are a useful model for studying conserved biological mechanisms of ring-NTPases such as motor coupling and coordination of chemical events for processive mechanical movement.

Since the basic packaging mechanism is well-conserved in eukaryotic viruses such as herpes viruses, genome packaging also presents a special target for antiviral drug discovery.

Phages employ powerful molecular motors to translocate DNA into a pre-assembled capsid shell. They generate extraordinary forces, as high as 80–100 pN, >25 times that of myosin, to overcome the mechanical and repulsive electrostatic forces from the DNA condensed inside the capsid. In T4, a ring of gp17 molecules assemble on an empty prohead at the unique portal vertex of the icosahedral capsid and encapsidate ~171 kb (headful) of newly synthesized concatemeric genomic DNA. Genetic and biochemical analyses have established that gp17 possesses ATPase, nuclease, and translocase activities essential for cutting the concatemeric genome and translocating a unit length DNA genome into the virus capsid.

Packaging at a rate of up to ~2000 bp/s and generating a power density of ~5000 kWe/m³, about twice that of a car engine, the T4 motor is the fastest and the most powerful packaging motor reported to date.

The atomic structure of phage T4 packaging motor protein gp17 and of the analogous proteins from other phages and herpes viruses have been determined. These display a common architecture containing two major domains connected by a linker or hinge domain. The N-terminal ATPase domain powers DNA translocation whereas the C-terminal nuclease domain makes cuts in the concatemeric DNA at the time of packaging initiation and termination. The C-terminal domain is also required for DNA binding and translocation functions. The motor protein of phage φ29 also retains this common architecture, despite lacking nuclease activity because it packages a unit-length genome and does not require initiation and termination cuts.

Extensive structural and functional studies of packaging motors support a common mechanism, and several models have been proposed. However, many aspects of the packaging mechanism remain unresolved or are controversial. Cryo-electron microscopic (cryo-EM) reconstructions have determined a pentameric stoichiometry of capsid-assembled motors in T4 and φ29 phages. However, the motor in the case of T4 was in a passive state, not associated with DNA. In the case of φ29, counting of fluorescently labeled packaging RNAs associated with the φ29 packaging motor, in both passive and active states, gave a distribution consistent with a hexameric stoichiometry. Furthermore, little is known about how the motors engage with the DNA
was with distributions with $n$ blue line and Supplementary Fig. 1d). In contrast, the binomial distribution was used to describe the experimental distribution, with two $fi$ distribution (Fig. 1c). The binomial distribution was used to quantify the stoichiometry of the T4 motor (# of subunits forming the motor). The presence of the Cy5 signal indicates that the motor is capable of packaging DNA. The number of Cy5 photobleaching steps, indicating number of packaged DNA, can range from 1 to many. Inset: CCD images of the Cy3 and Cy5 channels from which the representative trajectories were selected. c The distribution of Cy3 photobleaching steps pooled from separate experiments and a binomial fit (blue curve) to the distribution with $n$ = 5 subunits (stoichiometry of the motor) and $p$ = 0.66 (Cy3 labeling efficiency). Error bars represent std. The number of trajectories used is indicated by $n$ = #.

Fig. 1 Single-molecule fluorescence assay determines a pentameric stoichiometry for the actively packaging T4 motor. a Schematic of the assay. Homomeric subunits constituting the motor are labeled with Cy3 and 45-bp dsDNA molecules are labeled with Cy5. b Representative fluorescence time trajectories showing Cy3 and Cy5 signals, which indicate T4 motor and packaged DNA, respectively. The indicated Cy3 photobleaching steps are used to quantify the stoichiometry of the T4 motor (# of subunits forming the motor). The presence of the Cy5 signal indicates that the motor is capable of packaging DNA. The number of Cy5 photobleaching steps, indicating number of packaged DNA, can range from 1 to many. Inset: CCD images of the Cy3 and Cy5 channels from which the representative trajectories were selected. c The distribution of Cy3 photobleaching steps pooled from separate experiments and a binomial fit (blue curve) to the distribution with $n$ = 5 subunits (stoichiometry of the motor) and $p$ = 0.66 (Cy3 labeling efficiency). Error bars represent std. The number of trajectories used is indicated by $n$ = #.

Previous crystal structures$^{34}$ show that the Q163 residue is not in direct contact with the bound ATP, suggesting that the Q163A mutant is capable of binding ATP but deficient in a post-ATP binding step. The Q163A mutant binds to T4 heads with the same affinity as WT gp17 and can also assemble into a pentamer (Supplementary Fig. 3b–g). We doped the packaging reaction with a varying fraction of inactive subunits. Since gp17 exists as a monomer in solution and assembles as a pentamer on the portal vertex, doping led to an ensemble of hetero-pentameric motors containing between 0 and 5 inactive subunits. Figure 2a, b show that the packaging activity in bulk, as determined from the amount of DNA protected from DNase I digestion by encapsidation (see “Methods”), decreases as the fraction of inactive subunits increases (Fig. 2b). A similar trend was also observed at the single-molecule level, where the number of Cy5 spots (each indicative of 1 or more packaged DNAs) decreased with an increasing fraction of inactive subunits (Fig. 2c–e).

Based purely on the statistical probabilities, we determined the relative populations of motors containing 0–5 inactive subunits. These populations were used to predict the ensemble activity at varying levels of coordination (see “Methods”). In a strictly coordinated motor, firing by one subunit depends on the firing of an adjacent subunit$^{21,28,29}$. Therefore, the presence of one inactive subunit should stall the motor, and only those motors with 5 active subunits would exhibit packaging (Fig. 2e, solid black line). Conversely, in a completely uncoordinated motor, the packaging activity would continue with even 1 active subunit. As a result, the measured packaging activity should depend linearly on the fraction of active subunits (Fig. 2e, solid gray line). At intermediate coordination levels, a minimum of 2–4 active subunits would be required for packaging, with a motor containing 4 active subunits exhibiting twice the activity of a motor with 2 active subunits (Fig. 2e, dashed and dotted lines). Comparing the experimental data to these models shows that the packaging activity exceeds that predicted for a strictly coordinated motor but is also lower than that predicted for a completely uncoordinated motor (Fig. 2e). Notably, this comparison suggests validating the sensitivity and accuracy of our assay (Supplementary Fig. 2b).

To quantify stoichiometry of the motor, we first added 1 mM ATP and 2.5 nM Cy5-DNA into the imaging chamber and allowed packaging to proceed on the surface for ~10 min. Then packaging was terminated by adding DNase I to digest any DNA that was not encapsidated. Next, Cy3–Cy5 were imaged until complete photobleaching to visualize the intensity of Cy3 and Cy5 spots as a function of time in the form of single-molecule trajectories (Fig. 1b, c). For each of the trajectories exhibiting both Cy3 and Cy5 signals, indicating an active motor, the photo-bleaching steps of Cy3 intensity were counted to determine the number of Cy3-labeled gp17 subunits per motor (Fig. 1b). These Cy3 counts from multiple trajectories were pooled together into a distribution (Fig. 1c). The binomial distribution was used to describe the experimental distribution, with two fitting parameters: the number of subunits in the motor $n$ and the Cy3 labeling efficiency $p$ of the gp17. The best fit of the distribution was with $n$ = 5 and $p$ = 66%, also in agreement with independent measurements of labeling efficiency (see “Methods”; Fig. 1c, solid blue line and Supplementary Fig. 1d). In contrast, the binomial distributions with $n$ = 4 or $n$ = 6 were improbable. Many trajectories with counts of 5 were observed invalidating the $n$ = 4 and no trajectories with a count of 6 photobleaching steps (among 486 trajectories analyzed) were found invalidating $n$ = 6 (Supplementary Fig. 3a). Thus, this distribution and their fits indicate the stoichiometry of the actively packaging T4 motor to be five gp17s.

The T4 motor can tolerate inactive subunits. Next, we investigated the coordination of the five gp17 subunits by analyzing the packaging behavior of motors containing inactive subunits. For the inactive subunit, we selected a gp17 mutant in the Walker A P-loop, Q163A$^{32,33}$, which is impaired in ATP hydrolysis (Supplementary Fig. 1e) and shows no detectable levels of DNA packaging activity in ensemble assays (Supplementary Fig. 1c).
that the presence of at least one inactive subunit does not abolish motor activity and that the motor is not strictly coordinated.

To further probe into the mechanism of packaging for motors containing inactive gp17 subunits, we selected single inactive-subunit doped (ISD) motors containing a precise number of inactive subunits and analyzed their packaging behavior. We used a single-molecule fluorescent assay, similar to the one above, but with Cy3 labeling of only the inactive Q163A gp17 subunit, which was mixed with unlabeled WT gp17 to assemble motors with varying numbers of inactive subunits. The number of Cy3 photobleaching steps indicated the number of inactive subunits in each motor (# Cy3), and the Cy5 photobleaching steps indicated the number of packaged DNA molecules (# packaged DNA). Packaging was initiated by flowing Cy5-DNA and ATP into the chamber containing the surface-immobilized motors and terminated by addition of DNase I after ~10 min. Strikingly, many spots colocalized both Cy3 and Cy5 fluorescence, providing direct confirmation that motors containing the inactive subunit(s) were able to package Cy5-DNA (Fig. 2f, g; 2405 trajectories were analyzed). Due to <100% labeling efficiency and photoactivity, a

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

**Fig. 2 Motors containing inactive subunits exhibit impaired, but not abolished, activity.** a Schematic of in vitro bulk packaging assay. After allowing the packaging to proceed, the unpackaged DNA was digested by DNase I and the packaged DNA was retrieved from the capsids by digesting the capsid and DNase I with proteinase K. b Agarose gel electrophoresis of DNA packaged by motors constituted with increasing numbers of inactive subunits. The WT gp17 and the inactive Q143A mutant gp17 (inactive subunit) were pre-mixed at different ratios and added to the reaction mixtures containing capsids, ATP, and linearized plasmid DNA. The uncut gel image is shown in Supplementary Fig. 14. c Schematic of single-molecule fluorescence assay. The assay is similar to the one in Fig. 1a but performed with increasing percentage of inactive subunits (black sphere). d No. of Cy5 spots with increasing percentage of inactive subunits. In the inset are representative CCD images of the Cy5 channel, showing the number of Cy5 spots (each containing 1 to n # of packaged DNA) indicate the packaging efficiency with increasing percentage of inactive subunits. e DNA packaging activity quantified from the agarose gel in b (cyan squares). Also shown are predicted activities where the minimum number of active subunits required for packaging is 1 (no coordination; black) to 5 (strict coordination; gray)). f Representative single-molecule trajectories of Cy5 photobleaching steps (red), indicating the number of DNA molecules packaged inside the capsid, and Cy3 photobleaching steps (green), indicating the number of inactive subunits in the motor. Inset: Schematic of the assay, similar to the one shown in c, with inactive subunits (black sphere) labeled with Cy3. g Distribution of the counts of packaged DNA molecules from motors reporting 0–3 Cy3 photobleaching events (indicative of the count of inactive subunits). The average number of packaged DNA is indicated by f. The number of trajectories used is indicated by n = #.
small fraction of the photobleaching steps in the analysis shown in Figs. 2 and 3 may represent more inactive subunits than the ones represented by photobleaching steps. No. of.

The number of packaged DNA molecules for varying number of Cy3 inactive subunits were pooled from multiple trajectories to generate a distribution of the number of packaged DNA as a function of the number of Cy3-labeled monomers (Fig. 2g). While the ISD motors containing 1 or 2 inactive subunits could package multiple Cy5-DNA molecules (Fig. 2f, g), packaging overall was impaired when compared to WT motors. Although we could not determine how often WT motors (which are unlabeled) failed to package DNA (# packaged DNA = 0) since such events generate no Cy3 fluorescence signal, the differences in the distributions of number of packaged DNA molecules for WT and ISD motors was significant. While the WT motors could be found packaging as many as ≥8 DNA molecules within the ~10 min allotted, this number rarely exceeded 2 or 3 for ISD motors (Fig. 2g). Strikingly, the number of packaged DNA was not proportional to the number of inactive subunits, indicating that packaging is impaired by the presence of an inactive subunit but not necessarily proportional to the number of inactive subunits (Fig. 2g).

Motors with inactive subunits show a reduced propensity to engage DNA and a reduced packaging efficiency. To probe the mechanism by which the motors engage with DNA and initiate packaging, we modified the single-molecule assay described in Fig. 2c to monitor the Cy5 fluorescence intensity in real time, immediately after the addition of ATP and Cy5-DNA (Fig. 3a and Supplementary Figs. 4–6; see “Methods”). The Cy5 intensity trajectories for single packaging motors showed sharp upward steps, indicating real-time DNA engagements by the motor, as reported previously. The sharp downward steps in the Cy5 signal indicated either dissociation of the DNA or Cy5 photobleaching. At the end of each trajectory, we counted the number of inactive subunits in each motor from Cy3 photobleaching steps (Fig. 3a; 2888 trajectories were analyzed). The dwell times between the Cy5 upward steps represent the time for DNA to engage the motor (Fig. 3a). For motors with 0, 1, and 2 inactive subunits, we collected individual dwell times from multiple trajectories and calculated the average lifetime ($\tau_{avg}$). $\tau_{avg}$ was ~12 s for WT motors and rose to ~40 s for motors with 1 or 2 inactive subunits, highlighting a reduced propensity (or ability) of the ISD motors to engage the DNA (Fig. 3b). Consistent with previous studies, the distribution of these dwell times was best described by a double exponential function, with two lifetimes, $\tau_1$ and $\tau_2$ ($\tau_{avg}$ being the amplitude-weighted average of $\tau_1$ and $\tau_2$; Supplementary Fig. 7). It was previously shown that the T4 motor alternates between an “DNA engaging” state competent to engage and package DNA in rapid bursts and a long-lived “quiescent” state that does not engage DNA at all. $\tau_1$ corresponds to the time for DNA to bind to the motor in its active state, whereas $\tau_2$ represents the lifetime of the quiescent state. $\tau_1$ did not change appreciably with inactive subunits (Fig. 3b), indicating that the time for DNA engagement in the active state was unaffected. In contrast, $\tau_2$ more than tripled in the presence of inactive subunits (Fig. 3b), indicating that the ISD motors had a longer-lived quiescent state.

We also estimated the lifetimes of the DNA engagements from the dwell time between each upward step and the subsequent downward step in Cy5 fluorescence intensity (see “Methods”). We observed different characteristic types of DNA engagements. Many DNA engagements were transient, lasting ~2 s (Fig. 3a, purple), much shorter than the average photobleaching lifetime of Cy5 under the imaging conditions (expected to be ~40 s). We interpreted the downward steps in fluorescence during these transient engagements as dissociations of Cy5-DNA from the motor, i.e., DNA engagements where packaging failed. In contrast, we also observed long-lived engagements lasting >10 s (Fig. 3a, cyan). The downward steps ending these engagements were consistent with Cy5 photobleaching, and such events likely represent successful packaging events with completed encapsidation of 45 bp long DNA. Lastly, we detected engagements of intermediate lifetime, “semi-transient” engagements that lasted between 2 and 10 s (Fig. 3a, gray). These could plausibly represent engagements in which packaging was initiated but failed before complete encapsidation occurred. Distributions of DNA engagement lifetimes were best fit by a three-exponential model, validating our classification of DNA engagements into three characteristic types (Supplementary Fig. 8). The frequency of all three types of engagements is plotted for motors with 0–2 inactive subunits (Fig. 3c). For all motors, the frequency of transient and semi-transient engagements was significantly higher than those of long-lived engagements. This observation signifies that all motors, irrespective of the number of inactive subunits, tend to fail frequently in attempts to package DNA (from an end). In addition, there was a significant difference in the frequency of long-lived DNA engagements between the WT and ISD motors (Fig. 3c). The fraction of DNA engagements that were long-lived was higher for WT motors than for ISD motors (Fig. 3d), indicating an impaired mechanism of successful packaging in the ISD motors.

We estimated the total number of DNA molecules packaged by a motor from the cumulative number of long-lived engagements until the endpoint of the trajectory. These numbers, from multiple trajectories, were pooled together into a distribution for motors with 0, 1, and 2 inactive subunits (Fig. 3e). These distributions show that, in the first 4 min, the WT motor can package an average of 0.81 Cy5-DNA molecules when compared to 0.27 and 0.32 for motors with 1 and 2 inactive subunits, respectively. Markedly, there were multiple instances of WT motor being able to package as many as 5–7 DNAs. However, this number rarely exceeded two packaged DNA molecules for motors containing inactive subunits.

We also observed that the engagements of the same type tended to cluster together in the time trajectories. We estimated the probabilities of each type of engagement following the engagement of each particular type. The probability of a transient engagement following another transient engagement was quite high (~0.9) (Supplementary Fig. 9s). The pattern was similar for the long-lived and semi-transient engagements, confirming that motors (in DNA engaging state) undergo persistent phases of repeated successful DNA engagements or unsuccessful DNA engagements. Detailed comparisons between the WT motor with and without the SNAP tag, further confirmed that SNAP tag did not affect the motor function (Supplementary Fig. 10).

Motors doped with inactive subunits show frequent pausing and reduced packaging velocity. The above measurements provide a picture for how the T4 motor engages DNA to initiate packaging. To probe the steps following initiation, we employed a dual-trap optical tweezers assay to monitor individual motors packaging long (3400-bp) DNA substrates. Motors were pre-assembled on the free end of DNA molecules attached to micrometer-sized polystyrene beads in the presence of 1 mM of poorly hydrolyzed ATP analog, ATP-γS (see “Methods”). Due to the lack of an energy source, the motors could bind (and grip) DNA but could not package it. The pre-assembled motors on beads were then flushed into the optical trap sample chamber and captured in one trap, and a tether was formed by binding a second trapped
Fig. 3 Motors with inactive subunits have less frequent and less productive engagements with DNA. a Representative trajectories showing Cy5-DNA engaging with the motors in real-time and Cy3 photobleaching steps, indicating the number of the inactive subunits in the motor. The time point at which Cy5-DNA was introduced is shown as a pin and is manifested as a small increase in Cy5 intensity. The upward spikes in Cy5 intensity correspond to DNA engagements with the motor. Examples of three types of DNA engagements are shown: transient (purple shaded area and zoom-in), semi-transient (gray area and zoom-in), and long-lived (cyan area). b Dwell times between DNA engagements for motors with 0, 1, or 2 inactive subunits. The survival probability distributions for dwell times from multiple trajectories were fit to a double exponential function (Supplementary Fig. 7), as observed previously\(^3\), yielding two lifetimes: \(\tau_s\) for dwell times between DNA engagements occurring in short bursts (middle), \(\tau_l\) for those occurring with long delays (right). \(\tau_{avg}\) is the amplitude-weighted average of \(\tau_s\) and \(\tau_l\) (left). c Number of transient (left), semi-transient (middle), and long-lived (right) DNA engagements per minute for motors with 0, 1, or 2 inactive subunits. d Fraction of DNA engagements that are transient (left), semi-transient (middle), and long-lived (right) for motors with 0, 1, or 2 inactive subunits. e Distribution of the number of packaged DNA in the first 4 min for motors with 0, 1, or 2 inactive subunits. The average number of packaged DNA is indicated by \(f\). The number of trajectories used is indicated by \(n = \#\).
bead to the 1T4 capsid (Fig. 4a). This tether was stretched to a constant force of 5 pN in a channel containing 0.4 mM ATP-γS (see “Methods”). As the motor–DNA grip remained stable under applied force, we referred to these motors as “arrested” because initial engagement of the DNA by the motor was successful but translocation was stopped. The trapped and tethered bead pair was then moved from the ATP-γS channel to a channel containing 2 mM ATP (Fig. 4b) to start packaging against a constant load force. The packaging was measured from the decrease in DNA extension between the two beads, as DNA was translocated into the capsid by the motor (Fig. 4b). Different dynamic properties of individual motors were derived from these raw extension trajectories.

We investigated both WT and ISD motors. Arrested ISD motors were prepared using a 1:1 mixture of WT and Q163A gp17, in which 97% of the motors would be expected to have at least one inactive subunit (47% with 1 or 2 inactive subunits), and 3% of motors would have no inactive subunit. We selected packaging trajectories from 44 WT and 62 ISD motors for which the DNA substrates exhibited the correct length and elastic behavior (see “Methods”).

![Image of packaging assay](https://via.placeholder.com/150)

**Fig. 4 Motors containing inactive subunits exhibit slower start times, lower packaging speeds, and more pausing.**

- **a** Schematic of the optical trapping assay to measure the packaging dynamics of a single T4 motor. A multi-stream laminar flow chamber (left) is used to manipulate samples and buffer. A bead coated with anti-T4 antibodies is captured in one trap in a flow stream containing ATP (green stream; step ①). Another bead conjugated with arrested T4 capsid–gp17-DNA motors is captured in a second optical trap in the stream containing non-hydrolyzable analog ATP-γS (red stream; step ②). The motor bound to DNA in the presence of ATP-γS cannot initiate packaging due to a lack of energy source. To form a tether between the bead pair, the beads are moved close for 1 s and separated apart to detect binding of capsid to antibody (step ③). After a constant force (F = 5 pN) load is applied to the tether, the traps are moved into ATP and packaging is detected as the decrease of DNA extension when DNA is translocated into the capsid by the motor (step ④).

- **b** Extension of unpackaged DNA vs. time, under constant force load. Packaging activity is observed within seconds of entering the ATP stream. Representative packaging trajectories with WT (blue) and ISD motors (orange) are shown.

- **c** Distribution of the start times of WT (blue, n = 44) and ISD motors (orange, n = 62), defined as the dwell time between entry into the ATP stream and the start of packaging.

- **d** Distribution of mean pause-free packaging velocity derived from each packaging trace. ISD motors (orange) show a lower packaging velocity than WT motors (blue).

- **e** Histogram of the logarithm of pause frequency, defined as the number of pauses per kb packaged. ISD motors (orange) exhibit more pauses than WT motors (blue) on average. Motors with long start times (black stars; c–e) have a lower packaging velocity and higher pause frequency. The number of trajectories used is indicated by n = #.
of WT motors, it is still much larger than the 3% expected if only motors containing only WT subunits can package. This observation is consistent with our bulk and single-molecule fluorescence data described above which showed that inactive subunits are tolerated for packaging. Specifically, the 50% decrease in packaging efficiency between WT and ISD motors is in good agreement with the expected fraction of complexes with 0, 1, or 2 inactive subunits.

We then compared the start times, determined by the time elapsed between crossing the interface between the ATP-yS and ATP streams (Fig. 4b; \(t = 0\) s) and the first instance of packaging. The start time represents the time required for the motors to come into correct alignment with the already threaded DNA and persistently hydrolyze ATP but not the time required for DNA engagements and threading. The average start times of the arrested motors were 4.8 ± 1.1 s (mean ± SEM) for WT (Fig. 4b, blue) and 7.0 ± 1.3 s for ISD motors (Fig. 4b, orange). Significantly, while most ISD motors showed a similar start time distribution as WT motors, 20% of ISD motors restarted after >11 s, longer than all but one WT motor (Fig. 4c, black). These long start times account for the difference in means between ISD and WT motors.

Upon a successful start to packaging, the DNA extension decreased (Fig. 4b). For both WT and ISD motors, active packaging was occasionally paused, which we defined as any non-packaging interval of duration >0.1 s (see "Methods"). We determined the mean pause-free packaging velocity for each motor to assess the effect of inactive subunits. Histograms of the mean pause-free packaging velocity of WT motors (blue) and ISD motors (orange) are shown in Fig. 4d. The average velocity for ISD motors was 650 ± 30 bp/s (mean ± SEM), significantly lower than 960 ± 50 bp/s for WT motors. Mean packaging velocities of WT motors varied widely from 100 to 2000 bp/s (the standard deviation in velocity represents 35% of the mean), consistent with a previous study. While ISD motors exhibited a similar variation in mean packaging velocities (40% of the mean), a smaller fraction than the WT packaged at high speeds. Differences between WT and ISD motors were thus manifested in differences in mean velocities. We could not ascribe variability in packaging velocity to the number of inactive subunits in ISD motors since WT subunits themselves account for the majority of this variability.

We also estimated pause duration and pause frequency, the latter from the number of pauses per kb packaged. On average, pauses were longer and more frequent for ISD motors (1.5 ± 0.6 s, 2.3 ± 0.5 kb\(^{-1}\)) than WT motors (0.8 ± 0.3 s, 1.2 ± 0.4 kb\(^{-1}\); see Supplementary Fig. 11a and 4e). For both types of motors, we observed a negative correlation between mean packaging velocity and pause frequency (Supplementary Fig. 11b), indicating that pausing is more likely to occur when translocation is slowed. Furthermore, the 20% of ISD motors that started packaging the slowest (>15 s) also showed a lower packaging velocity (460 ± 60 bp/s; Fig. 4d) and a significantly higher pause frequency (4.9 ± 1.7 s\(^{-1}\)) than the other 80% of ISD motors (690 ± 40 bp/s and 1.7 ± 0.4 s\(^{-1}\)), the latter close to the value for WT motors; Fig. 4e). These correlations suggest that ISD motors that are more likely to be defective in starting also show greater packaging impairment.

Lastly, we observed that pauses either consisted of plateaus, during which packaging stopped, or gradual release or “unpackaging” of DNA, which is different from “slipping” where the motor completely loses grip on DNA (Fig. 4b; enlarged insets at \(t = 10\) s, 22.5 s). Both types of pauses have been previously reported in optical trap studies under low ATP conditions, which was attributed to reduced grip on DNA. We analyzed the velocity during all pauses to determine if there was more unpackaging in ISD vs. WT motors. The majority of ISD motors showed a similar distribution as WT motors, but ~10% showing significantly faster unpackaging events (velocity ranging from −100 to −500 bp/s), indicating motors with a poorer grip on DNA (Supplementary Fig. 11c). Furthermore, comparing these ISD motors to the other 90%, there was no significant increase in pause frequency, pause duration, or decrease of packaging velocity.

### Discussion

Our newly developed single-molecule assays allowed us to analyze the DNA packaging process through multiple stages, starting with the initial interactions between DNA and motor, engagement of DNA by the motor, translocation, and complete encapsidation. By studying individual motors with a precise number of active/inactive subunits, we could connect the above dynamic behaviors to motor stoichiometry and coordination. The determined pentameric stoichiometry of packaging-active T4 motors (Fig. 1c) is consistent with that of phages φ29 and T4 observed in cryo-EM reconstruction in which the motor was in a passive state, i.e., not engaged with DNA. That these independent approaches have arrived at the same pentamer stoichiometry is significant and also in functional alignment with the 10-bp helical pitch of DNA and ~2-bp motor step-size.

We discovered, by three different assays (ensemble, single-molecule fluorescence, and optical trap) that motors with an inactive subunit are able to encapsidate DNA (Figs. 2–4). Furthermore, in single-molecule fluorescence assays where the number of inactive subunits in a motor could be counted, the data showed that two, or even three, inactive subunits were tolerated (Figs. 2g and 3e), providing a clear indication that the coordination behavior of the T4 motor is distinct from that observed in φ29 and λ packaging motors. In the case of phage φ29, based on statistical probabilities of packaging activity in ensemble measurements of motors doped with a Walker A mutant in the large terminase subunit, it was concluded that the motor is strictly coordinated (100% coordination). Thus, the presence of a single mutant subunit rendered the motor inactive. In the case of phage λ, using a trans-arginine finger mutant in the terminase and single-molecule optical tweezers assays, it was found that one inactive subunit is tolerated. However, such motors systematically pause every 10 bp in the dwell phase of each translocation cycle, resulting in a tenfold reduction in the rate of packaging. Furthermore, φ29 motors containing more than one defective subunit are completely inactive. The above behaviors were not observed in T4 ISD motors. In contrast, randomly occurring pauses and unpackaging events were observed (Fig. 4b), and motors containing two or even three inactive subunits are functional (Fig. 2g). However, our data also do not support a completely uncoordinated mechanism because the ISD motors manifest impaired packaging, as evident from reduced DNA engagements and fewer encapsidations (see below). Packaging of DNA is a complex process requiring carefully orchestrated steps of ATP binding, DNA engagement, ATP hydrolysis, and directional DNA movement, which have to be precisely coordinated among the motor’s subunits.

In the initial stages of packaging, T4 motors alternate between a quiescent state, which is free of DNA association, and a DNA engaging state, which is competent to engage and package DNA (Fig. 5a). The inclusion of a single inactive subunit leads the motor to spend ~4× longer time in the quiescent state (Fig. 3b, middle panel). The molecular basis for the quiescent state is unclear but most likely due to impaired binding and/or hydrolysis of ATP of the inactive subunits that interferes with the transition to the DNA engaging state. Once in the engaging state, however, the rate of DNA engagements is largely unaffected by inactive
Fig. 5 A model for DNA translocation and subunit coordination by phage T4 DNA packaging motor. a The pentameric gp17 DNA packaging motor (five brown circles) alternates between a quiescent state and a DNA engaging active state when exposed to short 45-bp oligonucleotide DNA substrate (gray circles), with the ISD motor spending longer time in quiescent state than the WT motor (shown by blue deleterious symbol). b The pentameric motor adapts to DNA helix by interacting at ~2-bp intervals, leading to different subunits binding at different strengths, as shown by increasing color intensity with increasing strength of the grip. The surface view of the putative DNA-binding C-terminal domain of gp17 is used to illustrate DNA engagements by the motor decreases with inactive subunits (Fig. 3b, right panel). But the overall frequency of DNA engagements in the active state fail to lead to encapsidation regardless of motor composition (Fig. 3d, left panel). These transient events must correspond to DNA coming into loose contact with the motor and then dissociating. In order for productive packaging to initiate, an additional transition must occur for the DNA to engage tightly with the motor channel, preventing its dissociation against opposing diffusive forces. A decrease in the fraction of long-lived engagements in ISD motors (Fig. 3d, right panel) suggests that this transition is ATP dependent. Furthermore, the requirement for tight DNA engagement poses a significant barrier when occurring from a free DNA end, as it is more likely to slip out of the motor than a stretch of DNA that is already stabilized by multiple subunit contacts. This may account for the high failure rate of initiation. The higher diffusion coefficients of shorter DNAs may also explain why shorter DNAs tend to be less efficiently packaged than longer DNAs even in bulk assays (Supplementary Fig. 12).

It is also possible that these initial events are facilitated by the “small terminase” gp16. Gp16 and other phage small terminases are essential for packaging in vivo because the newly replicated viral genome in most cases is concatemeric and requires a double-stranded DNA cut in order to generate an end and initiate DNA translocation. The small terminases are reported to interact with DNA in vitro because the packaging substrate is already cut, it might still be important for the most efficient DNA engagements.

After successful packaging initiation, the presence of inactive subunits does not abolish packaging, suggesting that they can be repeatedly bypassed. Nevertheless, this bypassing causes defects in...
packaging, such as slower start, reduced packaging velocity, and more frequent pauses, and unpackaging events than in the WT motor (Fig. 4). How ISD motors bypass inactive subunits rather than arresting translocation, as is known to occur in strictly coordinated motors, remains an interesting question.

Recent structural data provide important constraints on the mechanism of DNA translocation in packaging motors. X-ray and cryo-EM structures show that gp17, and other packaging motor proteins from phages and viruses, consists of two domains, an N-terminal ATPase domain and a C-terminal DNA binding domain connected via a flexible hinge16,19,20,34. ATP hydrolysis in gp17 causes a ~7-Å conformational transition between the two domains, proposed to drive a ~2 bp movement of the bound DNA into the capsid11. In this model, the motor subunits around the ring take turns and sequentially hydrolyze ATP, causing the domains move in a piston-like fashion, akin to a five-cylinder engine, translocating DNA into the capsid (Video in ref. 11). Recent cryo-EM data indicate that the packaging subunits of Φ29 are arranged in a helical configuration22 tracking the DNA helix, similar to that observed in the structures of ringed hexameric helicases38,39. As a result, multiple subunits around the ring are likely to make contacts with the DNA every 2 bp, at the same or similar DNA elements. This is consistent with our recent studies on DNA gripping by single T4 motors, which suggest that multiple subunits, probably all five, when occupied with ATP, bind DNA to generate the grip necessary to hold onto it35,36. Thus, the motor–DNA grip consists of one pentameric motor and one helical length of DNA (Supplementary Video).

Based on the above considerations and the single-molecule measurements reported here, we propose a model for DNA translocation (Fig. 5b, c and Supplementary Video). The model requires that the motor subunits must be flexible enough to adjust and generate an asymmetric “nucleosome-like” helical motor–DNA complex; the helical organization is also expected to lead to different motor subunits binding DNA with different strengths. The asymmetric interactions at the symmetry-mismatched portal vertex, a conserved feature in phages and viruses, would also impart flexibility to attain this configuration10,41. Consequently, one of the motor subunits (#1) binds DNA with the largest affinity and the rest in decreasing order with subunit #5 with the least affinity due to the expected strain on the binding elements. Subunit #1 with its strongest grip on DNA is triggered to fire ATP hydrolysis, resulting in 2-bp translocation of DNA. Subunit #2 then attains the strongest affinity, while subunit #1 in an ATP-free apo state with the least affinity to DNA re-loads ATP. Subunit #2 then fires, causing another 2 bp of packaging, repeating the next cycle of translocation, ATP re-loading, and DNA gripping steps.

Whenever the DNA encounters an inactive subunit (Fig. 5d, e), we propose that the T4 motor shows flexibility to pause and to re-adjust the DNA registry, through micro-disengagement that may also involve backward DNA movement, until it recovers the right DNA grip and conformation to continue translocation. These micropauses and grip re-adjustments are likely too fast to resolve individually in our assays but are reflected as an overall reduction in the mean packaging velocity. Furthermore, on some occasions, the pauses are longer and lead to detectable unpackaging, a phenomenon characteristically observed with the ISD motors but not with the WT motors. Similar unpackaging events were frequently observed in our previous studies when one or more motor subunits are unoccupied with ATP, which appear to be equivalent to ATPase impaired subunit(s) in the present study35. A Monte Carlo simulation based on this model was able to faithfully recapture the motor’s packaging velocities with/without inactive subunits (Supplementary Fig. 12). The simulation also showed that lower quanta of average DNA slips minimizes the difference between the motor with varying number of inactive subunits (Supplementary Fig. 12). We suspect that unpackaging represents an event where the motor partially loses its grip on the DNA in the process of readjustment that, under an applied force, leads to loss of DNA registry with the motor and DNA release. When the DNA grip and registry is restored, the motor is reset and packaging continues. Similar unpackaging events were frequently observed in our previous studies35 when one or more motor subunits are unoccupied with ATP, which appear to be equivalent to ATPase impaired subunit(s) in the present study.

Our study highlights that, while the basic packaging mechanism and the structural and functional features of genome packaging motors are well conserved in phages and viruses42, significant distinctions exist in certain mechanistic details, such as the motor coordination. While the Φ29 motor uses a highly coordinated, dwell-burst, two-phase mechanism that is controlled at the whole motor level, the T4 motor seems to have adapted a flexible mechanism that is controlled at the individual subunit level. Consequently, while the T4 motor is able to skip one, two, or even three inactive subunits by re-adjusting its DNA grip, the Φ29 motor could at best tolerate one inactive subunit by making it special non-translocating subunit and re-setting the dwell-burst cycle accordingly30. Any more than one inactive subunit leads to arrest of packaging since both ATP binding and ATPase firing of a motor subunit are strictly dependent upon coordinated activities of the previous subunit. Thus T4 phage has apparently evolved a flexible motor by introducing a degree of independence for each subunit such that it can carry out the central tasks—ATP loading, DNA gripping, and ATP hydrolysis—without strict dependence on its neighbor. While this mechanism can lead to more frequent pauses and slips, as has been observed (Fig. 4)15,35,42, we speculate that it would eliminate the dwell phase and make T4 motor a faster motor that is able to skip subunits as well as pause and re-adjust when obstacles are encountered, such as a defective subunit or when packaging the newly replicated T4 DNA, a highly concatemeric and branched structure. Importantly, this mechanism would confer survival advantages to T4 phage such as packaging its ~171-kb genome, ~8.5 times longer than that of the Φ29, in about the same amount of time as the Φ29 phase43.

### Methods

#### Preparation of T4 heads

Empty phage T4 heads (capsids) for packaging experiments were prepared according to the protocol described previously44. Briefly, *E. coli* ϕP301 (sup-) cells (500 ml) were infected with 10am15am mutant T4 phage and lysed in 40 ml of 20 mM NaPO4/68 mM NaCl/22 mM KH2PO4/1 mM MgSO4, pH 7.5 containing 10 μg/ml DNase 1 and chloroform (1 ml) and incubated at 37°C for 30 min. The emptied heads thus produced were purified by differential centrifugations involving low-speed (6000 × g for 10 min) followed by high-speed (34,000 × g for 45 min) centrifugations. The final heads pellet was resuspended in 200 μl of Tris-Mg buffer (10 mM Tris-HCL, pH 7.5 / 50 mM NaCl/5 mM MgCl2) and purified by CsCl density gradient centrifugation. The major band containing empty capsids, appearing at about 1/3 from the bottom of a 5-ml gradient, was extracted and dialyzed overnight against Tris-Mg buffer. The capsids were further purified by DEAE-Sepharose ion-exchange chromatography. The peak fractions containing pure capsids were concentrated and stored at −80°C.

#### Cloning and expression of gp17 mutants

The T7 promoter containing *E. coli* expression plasmid vector pEF28b (Novagen, MA) was used to construct all the gp17 WT and mutant clones45. DNA fragments containing the gene of gp17 or ATPase defective mutant Q163A-gp17 were amplified by the polymerase chain reaction (PCR) using specific primers containing appropriate restriction site(s). Appropriate mutations were introduced using mutant primers by the overlap PCR strategy described previously45. The sequences of the primers, along with that of 45-bp DNA used in single-molecule imaging, is available in Supplementary Table 1. The PCR products were purified, digested with appropriate restriction enzymes (Thermo Scientific, Waltham, MA), and ligated with pET-28b vector DNA digested with the same restriction enzymes. The ligated DNAs were transformed into *E. coli* strain XL10-Gold (Agilent Technologies, Santa Clara, CA) and mini prep plasmid DNAs were prepared from single transformants. The accuracy of the cloned DNA was determined by DNA sequencing of the entire
insert and the flanking sequence (Retrogen, San Diego, CA). The resulting plasmids had gp17, Q163A-gp17 coding sequences fused in-frame with the 23 aa vector sequence. The presence of inactivated SNAP-tag DNA inserts and their orientation with respect to the promoter were tested using restriction enzyme digestion and/or amplification with insert-specific primers. The accuracy of the mutant DNA was confirmed by sequencing the entire insert DNA (Retrogen, San Diego, CA). The mutant plasmids were then transformed into E. coli BL21 (DE3) pLysS-competent cells (Strategene) for protein expression and purification. All the recombinant DNAs were then purified using E. coli codon-plus BL21 (DE3) RIPL (Strategene) for IPTG-induced overexpression of the recombinant proteins.

**Protein expression and purification.** The recombinant proteins were purified according to the basic protocol described as follows45. E. coli strain BL21 (DE3) pLysS or E. coli codon-plus BL21 (DE3) RIPL cells containing His-tagged WT-gp17 and Q163A-gp17 mutant clones were induced with 1 mM IPTG at 26–30 °C for 2–3.5 h. The solubility of the overexpressed proteins was tested using the B-PER reagent (Pierce Biotechnology). The presence of DNA inserts and their orientation with respect to the promoter were tested using restriction enzyme digestion and/or amplification with insert-specific primers. The accuracy of the mutant DNA was confirmed by sequencing the entire insert DNA (Retrogen, San Diego, CA). The mutant plasmids were then transformed into E. coli BL21 (DE3) pLysS-competent cells (Strategene) for protein expression and purification. All the recombinant DNAs were then purified using E. coli codon-plus BL21 (DE3) RIPL (Strategene) for IPTG-induced overexpression of the recombinant proteins.

**SNAP labeling of T4 motors.** Frozen aliquots of SNAP-gp17 and ATPase-inactive SNAP-Q163A-gp17 proteins in gel filtration buffer (50 mM Tris·HCl pH 7.5, 100 mM NaCl, 1 mM DTT) were put on ice and mixed with SNAP-tag reagent (Pierce). The IPTG-induced E. coli cells were harvested by centrifugation (4000 x g for 15 min at 4 °C) and resuspended in 50 ml of HisTrap binding buffer (50 mM Tris·HCl pH 8.0/200 mM imidazole/300 mM NaCl). The cells were lysed by an Amino French press (Thermo Fisher Scientific, Inc., Waltham, MA). The cell lysate was centrifuged at 34,000 x g for 20 min at 4 °C and the supernatant containing the His-tagged fusion protein was loaded onto a HisTrap column (AKTA-prime, GE Healthcare). The protein was eluted with a buffer containing 20–500 mM linear imidazole gradient. The peak fractions containing pure gp17 were concentrated and further purified using size-exclusion chromatography using Hi-Load 6/60 Superase (pre-grade gel filtration column) (GE Healthcare) in a buffer containing 20 mM Tris·HCl (pH 8.0) and 100 mM NaCl. The SNAP-gp17 and SNAP-Q163A-gp17 were overexpressed and purified using the same protocol as above except that SNAP-gp17 purification required the addition of reducing reagent such as 1 mM dithiothreitol (DTT) or 0.5 mM TCEP to prevent nonspecific disulfide bond formation. The peak fractions were concentrated by Amicon ultra-15 centrifugal filtration (MW 10 K cut off, Millipore, Temecula, CA) and stored at ~80 °C.

**SNAP labeling of T4 motors.** Frozen aliquots of gp17 and ATPase-inactive SNAP-Q163A-gp17 proteins in gel filtration buffer (50 mM Tris·HCl pH 7.5, 100 mM NaCl, 1 mM DTT) were put on ice and mixed with SNAP-tag fluorophores (SNAP-Surface 549 purchased from New England Biolabs) dissolved in 1 mM dithiothreitol (DTT) or 0.5 mM TCEP to prevent nonspecific disulfide bond formation. The peak fractions were concentrated by Amicon ultra-15 centrifugal filtration (MW 10 K cut off, Millipore, Temecula, CA) and stored at ~80 °C.

**Mathematical model of the ensemble packaging activity of the hetero-pentameric T4 motor.** A binomial distribution of the form shown below was used to describe the distribution of hetero-pentameric motors containing inactive subunits:

\[
\frac{\binom{n}{k} \cdot \frac{p}{k} \cdot \frac{1}{1-p}}{1} 
\]

where \( k \) is the probability that a pentameric motor (n = 5) contains k active subunits, given the probability p that an inactive subunit is present in the motor. The inactive subunits and WT subunits are presumed to have the same probability of being present in the motor, and the packaging activity of each subunit was calculated as the molar ratio of inactive subunits to all subunits present in the assay:

\[
\frac{\binom{n}{k} \cdot \frac{p}{k} \cdot \frac{1}{1-p}}{1} 
\]

If the subunits are strictly coordinated, then one inactive subunit is sufficient to abolish packaging activity entirely, and \( k_{\text{min}} = 5 \). If the subunits are completely independent, then a single active subunit is sufficient to drive packaging, and \( k_{\text{min}} = 1 \). Intermediate coordination mechanisms require 2–4 active subunits. The ensemble packaging activity for different p values was compared to these different models of coordination (Fig. 2e).

**Preparation of channels and constitution of T4 motor for single-molecule fluorescence imaging.** The T4 motor complex containing the empty head and motor was assembled on a PEG passivated surface for imaging its activity (Supplementary Fig. 2a). A buffer condition optimal for packaging activity i.e., 50 mM Tris·Cl (pH 7.5), 5 mM MgCl₂, 1 mM spermidine, 1 mM putrescine, 60 mM NaCl, and 5% (wt/vol) PEG, 0.2 mg/ml bovine serum albumin (BSA), 3 mM Trolerox, and an oxygen scavenging system (0.8% dextrose, 0.1 mg/ml glucose oxidase, and 0.02 mg/ml catalase) was used as the imaging solution for all the experiments, also referred as “Imaging Buffer-BSA.” To achieve specific surface immobilization of T4 motors, we used immobilization at the scale of single motors, i.e., the quartz slides and glass coverslips were surface-passaged with PEG (Layson Bio, Arab, AL) and 1% (wt/wt) biotinylated PEG (Layson Bio, Arab, AL). After preparing the channels using the quartz slides and glass coverslips, NeutrAvidin (Thermo Scientific) was added to the channel surface (0.2 mg/ml), followed by incubation with biotinylated protein-G (25 μM; Rockland Immunoclonal, Pottstown, PA) for 30 min. Following surface immobilization of protein-G, mouse polyclonal anti-T4 antibody (15 μM) was added to the channel surface and incubated for 30 min, which allowed for the binding of the anti-T4 antibody. Anti-T4 antibodies were raised in mice in compliance with all relevant ethical regulations for animal testing and research recommended by NIH (the Guide for the Care and Use of Laboratory Animals) and approved by the Institutional Animal Care and Use Committee of the Catholic University of America (Washington, DC) (Office of Laboratory Animal Welfare assurance number A4431-01). The channel was then washed with Imaging Buffer-BSA to remove the unbound antibodies.

**In vitro DNA packaging.** In vitro DNA packaging assays were performed by a previous described procedure46. The purified gp17 protein (~1.5 μM) was incubated with empty capsids purified as above (~2 x 10⁸ particles) and DNA (300 ng of 30- to 766-bp ladder DNA (New England Biolabs, Ipswich, MA) or linearized 2.8- or 6-kb plasmid DNA) and packaging buffer containing 30 mM Tris·HCl (pH 7.5), 100 mM NaCl, 3 mM MgCl₂, and 1 mM ATP for 5 min of short time incubation, or for 30 min of long-time incubation at 37 °C. Unpackaged DNA was degraded by adding DNase I (Sigma-Aldrich) by incubating this mixture for 30 min at 37 °C, and the encapsidated DNase-I-resistant DNA was released by treatment with 0.5 M EDTA. The retrieved encapsidated DNA was then analyzed by polyacrylamide (4–20% gradient) or agarose (0.8%) gel electrophoresis. Each experiment included one to several negative controls that lacked one of the essential packaging components: gp17 or ATP. A standard lane containing a small amount of the DNA substrate used in the packaging reaction allowed for quantification of the packaged DNA.

**Activity**

\[
\text{Activity}(k_{\text{min}}, p) = \frac{\binom{n}{k} \cdot \frac{p}{k} \cdot \frac{1}{1-p}}{1} 
\]

where f(k,p) is the probability that a pentameric motor (n = 5) contains k active subunits, given the probability p that an inactive subunit is present in the motor. The packaging efficiencies were calculated as the molar ratio of inactive subunits to all subunits present in the assay:
Single-molecule fluorescence detection and data analysis. The fluorescence emission from the Cy3-labeled, immobilized motors carrying out Cy5-DNA packaging was refocused onto an EMCCD Andor camera. The final emission was split into two separate channels for imaging Cy3 (for Cy3-labeled subunits) and Cy5 (for Cy5-labeled DNA). Cy3–Cy5 imaging was carried out in one of two ways. (1) Motors were incubated a few minutes in the dark with the addition of 1 mM ATP and 2.5 nM Cy5-DNA into the chamber to allow packaging to proceed for a sufficient time. DNAse I was then added to digest and freeze the completed unligated DNA, followed by Cy3–Cy5 imaging. (2) Alternately, imaging was done in real time upon the addition of 1 mM ATP and 2.5 nM Cy5-DNA. Cy3 imaging for Cy3 stoichiometry was done with Cy5 imaging either before or after it. The order of Cy3/Cy5 imaging did not affect the experiments. The time trajectories of Cy3 and Cy5 fluorescence intensities were extracted from the movies and then further analyzed using customized MATLAB programs. For some analysis, the trajectories of Cy3/Cy5 imaging did not affect the experiments. The time trajectories of Cy3 and Cy5 stoichiometry was done with Cy5 imaging either before or after it. The order of the addition of 1 mM ATP and 2.5 nM Cy5-DNA. Cy3 imaging for emission from the Cy3-labeled, immobilized motors carrying out Cy5-DNA fluorescence time trajectories showing Cy5 intensity spikes, showing real-time detection and data analysis.

Preparation of antibody-coated beads for optical trapping assay. Anti-T4 antibody-coated beads were used to bind T4 heads. In all, 1% w/v 800 nm Protein G-coated polystyrene beads (Spherotech Inc.) were washed in the same way as the assay of other beads. Ten micrometers of the purified beads was incubated with 2 μl of undiluted serum containing polyclonal anti-T4 antibodies and incubated at room temperature for 45 min, followed by 3 washes with 50 μl 1× PBS buffer. The antibody-coated beads were finally resuspended in 10 μl 1× packaging buffer and stored at 4°C for up to 1 week. For each experiment, 2 μl of antibody-coated beads were diluted into 500 μl 1× packaging buffer and transferred into an empty Hamilton syringe.

Dual optical traps and laminar flow chamber. Single-molecule optical trap packaging was conducted on high-resolution dual-trap optical tweezers, as previously described. The instrument and data collection were controlled by custom Labview programs and MatLab code.

In order to control the assembly of the packaging motors and initiation of packaging, we used a laminar flow chamber with multiple channels. The chamber was constructed by melting a patterned piece of paraffin between two glass coverslips, one of which had holes drilled for entry and exit of samples by syringe pumps. Sample chambers were designed with 4 channels (Fig. 4a). 2 inner channels that merged together but maintained a sharp interface (±0.5 μm) due to the laminar flow, and 2 outer channels separated by paraffin but each connected to the middle channels by a glass capillary to allow for exchange from one channel to the other. Streptavidin beads coated with arrested motors and anti-T4 antibody-coated beads were pumped through the two outer channels, respectively, and leaked into the inner channels. The inner channels contained 466 μl 1× packaging buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂), 5 μl Pyranose Oxidase oxygen scavenging system, 25 μl 20% glucose, and 4 μl of substrate: either ATP at a final concentration of 2 mM in the top inner channel or ATP-γ-S at a final concentration of 0.4 mM in the bottom inner channel (Fig. 4a). The oxygen scavenging system, used to reduce photodamage to the packaging motors and to ensure the DNA tethers had long lifetimes, was prepared by dissolving 2.9 mg of Catalase (Aspergillus niger) and 5.8 mg of Pyranose Oxidase (Coriolus sp.) in 200 μl of T30 buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl). All samples and buffer stored in Hamilton syringes were flowed into the chamber via polyethylene tubing and controlled by syringe pumps.

Optical trap assay procedure. In a typical experiment, an anti-T4 antibody-coated bead was first trapped near the top capillary in the ATP channel (Fig. 4a, step 0). Then the traps were moved to the lower capillary in the ATP-γ-S channel, and a bead coated with arrested packaging motors on DNA was captured in the other trap (Fig. 4a, step 2). A tether was formed when beads were moved close to each other (Fig. 4a, step 1) but maintained a sharp interface (<0.5 mm)52 due to the rise in force (>35 pN) as the beads were moved apart (Fig. 4a, step 0). After a correct tether formed, a constant force of 5 pN was applied in order to keep DNA extended between the beads. Then the trapped bead pair was moved from the ATP-γ-S channel to the ATP channel. Packaging could start at a constant load force and was measured from the decrease in DNA extension between the two beads, as DNA was translocated into the capsid by the packaging motor (Fig. 4a, step 0).

Once a tether formed, its elastic response (force–extension curve) was measured prior to initiating packaging. Traces were selected or discarded based on inspection of the trap calibration parameters and the elastic behavior of each tether. Specifically, each force–extension curve was compared to the extension–force–force profile of the bead54 with a persistence length55 of 50 μm, a stiffness of 1000 nN/μm, a distance per base pair of 0.34 nm, and contour length of 1156 bp. Due to the uncertainty of the size of beads and the orientation of the heads bound to beads, we found there could be an extension offset between this force–extension curve and the model. Typically, we found that most tethers had force–extension curves within –100 nm to 50 nm of the model, and tethers outside this range were discarded. In addition, we calculated the residuals between the extension-offset-corrected force–extension curve and the model above. Tethers for which the extremum and variance in the residuals exceeded a global threshold were discarded from analysis.

Packaging dynamics analysis. Custom MatLab code was written to distinguish pausing from packaging and to determine dynamic parameters for each selected trajectory. Each data file was processed from the first data point over a running 0.1-s window. A velocity threshold was assigned according to the variance in velocity, below which the time points were marked as potential pauses.
Time intervals of duration >0.1 s in which all time points were below the velocity threshold were recorded as a pause. The pause frequency was determined by dividing the number of pauses by the sum of packaging lengths during each packaging interval. Then the pauses were removed from the trace and the remaining packaging intervals were cateden. The mean packaging velocity was determined as the average of the velocity of the cateden trace.

**Statistics and reproducibility.** For all the experiments reported with error bars, 2–5 replicates and replicable experiments were performed to get statistics of mean and standard deviation. Values represent mean. Error bars throughout represent standard deviation.

**Modeling and simulation.** The packaging and slipping of the DNA into the capsid by the T4 motor was modeled as a one-dimensional (1-D) walk. The kinetic Monte Carlo simulations of such a 1-D walk was carried out using the Gillespie Algorithm. At any step of the simulation, if the subunit with the strongest grip (responsible for controlled 2-bp packaging) is not an inactive unit, the motor had a probability of packaging by 2 bp \((p_{\text{package}})\) indicating the controlled and ATP hydrolysis-driven event. Alternatively, the motor had a probability of pausing and slipping (un packaging) by \(n\) bp \((p_{\text{pause}}\))\). Since slipping is an uncontrolled event, the extent of each slip \((s\) bp\)) was Poisson-distributed and the value of \(s\) was randomly chosen from the exponential distribution of the form: \(\exp(\mu)\), where \(\mu\) is the average length of the slip, taken to be 0.5. At each step in the simulation, there were only two possibilities: DNA moving forward in the form of packaging (given by \(p_{\text{package}}\)) or backward in the form of slipping (given by \(p_{\text{pause}}\)). \(p_{\text{package}} = \frac{n}{k_{\text{package}}} + \frac{s}{k_{\text{package}}}\) \(p_{\text{pause}} = \frac{s}{k_{\text{pause}}}\)\)Where \(k_{\text{package}}\) and \(k_{\text{pause}}\) are the rates of packaging and pausing & slipping, respectively, as described in Fig. 5. The value of \(p_{\text{pause}}\) depended on the number of active subunits/inactive subunits in the motor. For the simulations, \(p_{\text{package}} = 0.65, 0.75, 0.85, 0.95\) and \(0.99\) for the motor with 0, 1, and 2 inactive subunits to match the experimental data of mean packaging velocity (Fig. 4) and fraction of successful encapsidation events (Fig. 3). If the subunit with the strongest grip is inactive, then \(p_{\text{package}}\) was taken to be 0. At each step of the simulation, a random number was generated between 0 and 1. For the motor with 0 or 1 or 2 inactive subunits, if the random number was \(p_{\text{pause}}\), i.e., indicating packaging, then the length of the unpackaged DNA length was reduced by 2 bp. If the random number was \(p_{\text{pause}}\), i.e., indicating pause and slipping, then the length of the unpackaged DNA length was increased by \(n\) bp where the value of \(n\) was randomly chosen from the exponential distribution as described above. At each step of the simulation, the time expended in the packaging or pause&slip was also taken to be exponentially distributed and taken from the exponential distribution of the form: \(\exp(\mu)\) or \(\exp(\mu)\). \(\mu\) package\(p_{\text{pause}}\) was the average time to package 2 bp and \(p_{\text{pause}}\) was the average time of pausing and slipping. The values \(p_{\text{package}}\) and \(p_{\text{pause}}\) did not significantly impact the simulated trajectories, in contrast to \(p_{\text{pause}}\) which was far more critical. Trajectories (unpackaged length vs. time in arb. units) whose unpackaged DNA length reached 0 indicated successful packaging. The trajectories whose unpackaged DNA length exceeded the length of the DNA indicated failed packaging. Such trajectories imply that the entire DNA would have slipped enough to unpackaged DNA length exceeded the length of the DNA indicated failed packaging. Such trajectories imply that the entire DNA would have slipped enough to

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V.B.R. and Y.R.C. directed research. L.D., D.S., V.I.K., S.L., R.V., Y.R.C. and V.B.R. designed experiments. L.D. and D.S. performed TIRF experiments. S.L. and V.I.K. performed optical trap experiments. M.M. and V.I.K. produced capsids and performed bulk packaging experiments. L.D., D.S., V.I.K., S.L., R.V.. and V.B.R. analyzed and interpreted data. D.S., L.D., Y.R.C. and V.B.R. wrote the paper.

Competing interests
The authors declare no competing interests.

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