Supplementary Figure 1: Schematic of experimental setup.

(a) Side view of experimental apparatus. A Teflon tube filled with KCl solution connects two wells, labeled cis and trans, also filled with KCl solution. The cis well is connected to negative terminal of the amplifier (ground) via a Ag/AgCl electrode while the trans well is connected to an Axopatch amplifier that supplies the driving voltage. The output of the amplifier is then filtered and digitally sampled. (b) Expanded view of the cis end of the Teflon tube. A bilayer is established upon the 20 µm aperture as described in to the Online Methods.
Supplementary Figure 2: Example of temporal resolution of SPRNT.

Raw data (grey) and automatically found level medians (black) for a phi29 DNAP moving sequence B through MspA. Notice that current levels less than 1 ms in duration are well defined and resolvable. Generally during phi29 DNAP progression along DNA, levels were greater than 1 millisecond in duration.
Supplementary Figure 3: Examples of DNA repositioning.

(a) As in Fig. 1h, we plot current levels taken at different voltages (indicated by symbols within the legend). Because current scales with voltage, we apply a linear scale and offset to the values so we can easily compare their current level patterns. To account for the DNA shifting positions under different applied forces we add a horizontal offset. A spline of the 180 mV current values is shown in black dashes. The predictive ability of the spline for the 180 mV levels illustrates that the different voltages reposition DNA within MspA’s constriction. (b) The voltage-induced changes to the DNA’s contour length normalized by the length at 160 mV plotted against the applied voltage normalized by 160 mV. We model the DNA’s elongation as an extensible freely jointed chain (Ex-FJC) (dashed line) and fit it to the observed elongation ratios, as further discussed in the Supplementary Discussion 1. While the Ex-FJC is derived for DNA in non-confined areas, for the forces that we are applying (~20-40 pN) the single parameter model describes the data well and suggests that the DNA repositioning within the pore is due to DNA elongation.
Supplement Discussion 1: Elongation of DNA

To test the hypothesis that ssDNA elongates within MspA with increasing force, we compared our results to the extensible freely jointed chain (Ex-FJC). The Ex-FJC is an experimentally validated model of DNA elongation under an applied force\(^20\), denoted \(F\). At forces in the 5-40 pN regime, the Ex-FJC gives the end-to-end distance of DNA, \(x\), by the following expression:

\[
x = L \cdot \left(1 - \frac{k_B T}{F b}\right).
\]

(Eqn. 1)

where \(L\) is the contour length of DNA, \(k_B\) is the Boltzmann constant, \(T\) is the temperature, and \(b\) is the Kuhn length \((1.45 \text{ nm})^{20,21}\). In our system, the end-to-end distance of DNA between the enzyme Phi29 DNAP and MspA’s constriction, \(x\), is fixed. The contour length, \(L\), changes with different applied forces. We assume that the force on the DNA is proportional to the applied voltage \(F = \alpha^* V\), giving

\[
x = L \cdot \left(1 - \frac{k_B T}{\alpha^* V b}\right).
\]

(Eqn. 2)

At a different voltage \(\beta^* V\), DNA is elongated by a different amount \(\omega^* L\). \(\omega\) is the ratio of contour lengths of the DNA between the enzyme and phi29 DNAP’s constriction at the two different voltages. We substitute \(V \rightarrow \beta^* V\) and \(L \rightarrow w^* L\), in [Eqn. 2] giving

\[
x = L \cdot \omega \cdot \left(1 - \frac{k_B T}{\beta^* V b}\right).
\]

(Eqn. 3)

Solving Eqns. 2 and 3 for \(\omega\) gives,

\[
\omega = \beta \cdot \frac{b \alpha^* V - k_B T}{b \alpha^* V \beta - k_B T}.
\]

(Eqn. 4)

The fractional elongation \(\omega\) can be recast as \(\omega = N/(N-\delta)\), where \(\delta\) is measured as in figure 1g and \(N\) is the number of nucleotides between phi29 DNAP and MspA’s constriction DNA at the initial force \(F\). From Ref. Manrao et al. \{Manrao, 2012\} we estimate \(N = 14\). We fit our data to (Eqn. 4.), as shown in Supplementary figure 3b, showing that a single parameter fit with \(\alpha = -1.02 \pm 0.12 \text{ e/nm}\) describes the data well. At 180 mV, with \(\alpha=-1.02\) and using Eqn. 4, we estimate the DNA to be to be 92% of fully stretched.

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Using $F = \alpha V$ and solving for $F$ in Eqn. 4 gives

$$F = \frac{k_B T}{b} \cdot \frac{1 - \frac{\omega}{\beta}}{1 - \omega}.$$  

(Eqn. 5)

Using Eqn. 5 with our data (Supplementary Fig. 3b) we find $F = 38 \pm 7$ pN at 160 mV. The uncertainty originates from (a) the measurements at different voltages and (b) allowing $N$ to vary by +2 or -2. This force is higher than the anticipated forces of around 10-20 pN\textsuperscript{12}, requiring further exploration to directly calibrate the force applied to DNA within MspA using SPRNT. Assuming that SPRNT operates well under voltages from 80 mV to 240 mV, then the force range of SPRNT is roughly 20-50 pN.
Supplementary Table 1:
A list of the DNA strands and complements used in this study. Complementary sections are underlined. Sequence A was the sequence used in the main text.

<table>
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<tr>
<th>DNA Construct</th>
<th>Sequence</th>
</tr>
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| sequence A                | 5' PIACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTAC...
Supplementary Table 2:

A summary of the statistics for all of the experiments performed in this study. Rows starting with “ATP titration” refer to experiments where we varied only [ATP] to determine the ATP dependent and independent steps, and the reaction kinetics of Hel308 during translocation of DNA. Rows starting with “repetitive pattern” refer to a sequence containing repeated current levels with high current difference. Rows starting with “Voltage repositioning” refer to phi29 DNAP experiments where the voltage was changed to study the effects of force repositioning the DNA within MspA. The final six rows summarize the total number of events for each of the DNA constructs examined using Hel308 or phi29 DNAP. Asterisks indicate that some data was used in Refs. (12, 16).

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<th>Voltage (mV)</th>
<th>Temperature (°C)</th>
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Supplementary Figure 4: Sequence B consensus  A combination of information in figures 2a, 2b, 2e and 2f of the main text but for DNA sequence B. (a) The observed level patterns for phi29 DNAP moving sequence B through MspA. Data was taken with 150 mM [KCl] in the cis well and 500 mM [KCl] in the trans well. (b) The observed level patterns for Hel308 translocating DNA sequence B through MspA (black lines). The automatically generated consensus levels for sequence B are aligned to the sequence and level pattern found for phi29 DNAP. Data was taken with 400 mM [KCl] buffers in both cis and trans wells. The difference in salt conditions accounts for the difference in current values between (a) and (b). A gap indicates a position where a level was missing due to degeneracy. (c) The number of times that a given level shown in (b) was observed. (d) The median duration of the levels with the current shown in (b) while using 10 and 50 µM [ATP] (black line) and using 1 mM [ATP] (red line). Level durations depend partially on sequence context. (e) Ratio of level durations for observations using low [ATP] (10 and 50 µM) and for high [ATP] (1 mM). We indicate odd-numbered levels that depend on [ATP] with an orange diamond and even-numbered levels that do not depend on [ATP] with a blue circle. Levels that could not be identified as ATP-dependent or independent due to degeneracy of nearby current values are indicated with a red ‘x’. Comparing the duration at different values of [ATP] removes level-duration dependence on sequence context.
Supplementary Figure 5: Sequence C consensus

A combination of information in figures 2a, 2b, 2e, and 2f of the main text but for DNA sequence C. (a) The observed level patterns for phi29 DNAP moving sequence B through MspA. The sequence was designed to have repeating pattern with high contrast between adjacent current levels. Data was taken with 150 mM [KCl] in the cis well and 500 mM [KCl] in the trans well. (b) The observed level patterns for Hel308 translocating DNA sequence C through MspA (black lines). The automatically generated consensus levels for sequence C are aligned to the sequence and level pattern found for phi29 DNAP. Data was taken with 400 mM [KCl] buffers in both cis and trans wells. The difference in salt conditions accounts for the difference in current values between (a) and (b). A gap indicates a position where a level was missing due to degeneracy. (c) The number of times that a given level shown in (b) was observed. (d) The median duration of the current levels shown in (b) while using 500 µM [ATP]. We identified odd-numbered levels as ATP-dependent and even-numbered levels as ATP-independent levels for sequence C based on the results of figure 2e, indicating that ATP independent levels will generally have a longer median duration at 500 µM [ATP].
Supplementary Figure 6: Current patterns for Hel308 and phi29 DNAP

Figure caption is the same as Fig. 2c in the main text but for different DNA sequences B and C displayed in (a) and (b), respectively. For both (a) and (b) the gray curve represents the spline of the levels observed with phi29 DNAP (black points) moving the DNA through MspA.

Means of current levels recorded with Hel308 actuated DNA movement (orange and blue symbols) were scaled to match the spline of phi29 DNAP levels. Points indicated with orange diamonds or blue circles were horizontally offset in order to best match the spline of levels taken with phi29 DNAP. Levels that were found to be depend on [ATP] are shown with gold diamonds, and levels that are independent of [ATP] are shown with blue circles.
Supplemental Discussion 2: Hel308 step sizes measurement using DNA position

We find the position of a given sequence of DNA when translocated by Hel308 in comparison to the same sequence of DNA translocated by phi29 DNAP. First, we find a consensus of Hel308 current levels and a consensus of phi29 DNAP current levels for the same DNA strand (Supplementary Discussion 2). Next, we aligned the Hel308 current levels to the DNA sequence. For levels observed with Hel308 we generate a spline interpolant for even-numbered levels and a separate spline for odd-numbered levels. Next, a linear scale and offset was applied to the phi29 DNAP current level values to compensate for different salt conditions between the phi29 DNAP and hel308 experiments. For both the spline of even-numbered and of odd-numbered Hel308 levels, we shift the horizontal position of the spline curves, and take a sum of square differences between the splines of the Hel308 data and the spline for levels observed with phi29 DNAP. The shift leading to the smallest sum of square differences is taken as the DNA position for the set of Hel308 levels relative to the phi29 DNAP levels. The error on the DNA position measurements was calculated using the following Monte-Carlo simulation. 1000 perturbed sequences of the Hel308 and phi29 DNAP levels were produced randomly using the known errors on the current levels. The above calculation was repeated for each of the 1000 sequences to generate a distribution of DNA position measurements. The standard deviation of this distribution is the error on the DNA position measurements. For each set of levels taken with Hel308 (even or odd numbered levels) we found the mean step position relative to phi29 DNAP. For DNA sequences A, B, C we found the distance between steps to be 0.54 ± 0.04 nt, 0.54 ± 0.04 nt and 0.44 ± 0.05 nt, respectively. See Supplementary figure 6 for additional information.
Supplemental Discussion 3: Determining ATP dependent steps from level duration information

We determined whether level durations are dependent on ATP concentration by comparing helicase events at various ATP concentrations. We started by aligning the levels for helicase events taken at each ATP concentration to the consensus of current levels for a given sequence. For each ATP concentration we determined the median duration, $\tau_{1/2}$, for the levels aligning to each level of the consensus of levels. To account for off-pathway enzyme mechanisms, such as back-steps, we examined only the durations of aligned levels that also moved to the next level. The error on the median, $\delta \tau_{1/2}$, is the standard deviation of the distribution of $\tau_{1/2}$ values determined by bootstrapping.

We determined the ATP dependence by comparing $\tau_{1/2}$ of each level between two different ATP concentrations. In figure 2f of the main text and Supplementary figure 4e we took the ratio of $\tau_{1/2}$ at 10 µM ATP to $\tau_{1/2}$ at 1000 µM ATP. If the ratio was significantly different from 1, then we defined the level as being ATP dependent.
Supplemental Discussion 4: ATP titration of hel308

To examine the nature of the two states of the Hel308 hydrolysis cycle we systematically varied the ATP concentration from 10 μM to 3 mM. We found, consistent with figure 2f, that the even levels did not change in duration with ATP concentration and that the odd levels did. Supplemental figure 7 shows the median reaction time, averaged over all levels as a function of the 1/[ATP] for the odd (gold) and even (blue) steps. We fit these curves to lines and find that

\[ \tau_{1/2} = [(4.1 \pm 0.4) \, \mu\text{M}/[\text{ATP}] + (0.05 \pm 0.01)] \, \text{s} \] for the ATP-dependent step, and

\[ \tau_{1/2} = [(0.08 \pm 0.8) \, \mu\text{M}/[\text{ATP}] + 0.16 \pm 0.01] \, \text{s} \] for the ATP-independent step. Because the error is much larger than the value of the slope, we conclude that this step does not depend on the [ATP].

An inset shows the inverse plot of rate vs. [ATP]. We find that the ATP dependent step follows the Michaelis-Menten equation (rate = \( V^* \) [ATP] / (K + [ATP])), with \( V^* = 15.5 \pm 3.5 \, \text{s}^{-1} \) and \( K = 92 \pm 22 \, \mu\text{M} \).

We also analyzed the distribution functions for each individual level in figure 2e/2f of the main text. We find that each of the ATP independent levels are well-described by an exponential (supplemental Fig. 8). The half-life varies from level to level in a statistically significant way, possibly due to sequence dependence. To analyze the ATP dependent levels, we first examined the high [ATP] data, where the rate limiting step dominates. We find that these distributions are again mostly well-described by a single exponential (supplemental Fig. 9).
Supplementary Figure 7: ATP titration of hel308

The median duration for the ATP dependent step, averaged over all ATP-dependent levels (gold) and the ATP independent step, averaged over all ATP-independent levels (blue) as a function of 1/[ATP], with best fit lines drawn as dashed lines over both. The inset shows the inverse plot of rate (rate = ln(2) / τ_{1/2}) vs. [ATP].
Supplementary Figure 8: Temporal distributions for ATP-independent steps

Probability distributions for the durations of each ATP-independent step (even numbered levels) shown in figure 2e/2f. The y-axis is logarithmic. Histograms were constructed by compiling the data for each ATP-independent level across each ATP concentration experiment. Each histogram has >180 counts. Error bars represent the 1σ Poisson errors. The red line is the best fit exponential to the data. Each data set is well described by a single exponential. The half-life and 1σ confidence intervals are displayed above each individual distribution.
Supplementary Figure 9: Temporal distributions for ATP-dependent steps at high [ATP]

Probability distributions for the durations of each ATP-dependent step (odd numbered levels) shown in figure 2e/2f at high [ATP]. The y-axis is logarithmic. Histograms were constructed by taking each measurement of the duration of a given level from the data at 1000 µM and 3000 µM [ATP]. At these concentrations of ATP, the reaction rate is at > 97% of the saturation rate, justifying the inclusion of both data sets. Error bars represent the 1σ Poisson errors. The red line is the best fit exponential to the data. Most data sets are well described by a single exponential. The half-life and 1σ confidence intervals are displayed above each individual distribution. Longer tails are believed to be caused by misalignments of the ATP-independent steps.
Supplementary Figure 10: Proposed Mechanisms of observed sub-nt steps during Hel308 translocase activity. (a) Illustration of DNA (black) moving within MspA (gold) during Hel308 (green) translocase activity. Hel308 starts in the conformation shown in (i). When ATP binds, the physical structure of Hel308 changes, altering how it sits on the rim of MspA, as in (ii), and/or repositioning the DNA within the Hel308, as in (iii). Yellow arrows indicate a DNA binding motif within the enzyme that can move the DNA relative to MspA’s constriction. (b) The image from Buttner et al. compares domains 1 and 2 between two crystal structures of Hel308 and another Ski2 like helicase, one without ATP bound, another with ATP bound. Büttner et al’s analysis indicates that ATP binding induces a conformational rotation of domain 2 by 20 degrees, and consequently moves the DNA binding motif IV closer to domain 1. (c) We take the illustration in (b) and highlight the DNA (black) and motifs Ia helix (orange) and motif IV helix (green), for the ATP unbound helicase (i) and for the ATP bound helicase (ii). The remaining 5’ end of the DNA is threaded through MspA’s constriction. Upon ATP binding, motif IV helix (magenta) repositions the DNA upwards towards domain 1, while the DNA-binding domain Ia helix (purple) remains nearly unmoved. Because the anticipated contact points between the helicases and MspA’s rim (horizontal dashed line) does not change the position of the helicase considerably, the helicase will move DNA relative to MspA constriction by an amount Δ. See Supplemental discussion 2 for more information on step size measurement. The remainder of the ATP hydrolysis cycle returns the Hel308 to its original conformation while completing the translocation of the DNA by one nucleotide through the pore.
Supplementary Figure 11: Cartoon of Hel308 translocase

An illustration of DNA being moved through MspA (gold) by the helicase Hel308 (green) in a lipid bilayer (purple). (a) Hel308 loads onto the exposed 3' end of the template DNA strand (black). The complement DNA strand (red) was designed to leave an 8 base overhang on the template strand 3' end on which the helicase loads. A cholesterol is attached to the 3' end of the compliment DNA strand to concentrate DNA onto the bilayer. (b) Hel308 partially unwinds the complement DNA strand until the voltage quickly dissociates the remainder of the complement DNA strand from the 5' end while pulling the template strand through MspA. (c) Hel308 functions as a 3' to 5' ssDNA translocase, drawing the DNA out of the pore. As Hel308 progresses along the DNA we observe discrete changes in current, indicative of Hel308 translocase activity.