Dynamic Interconversions of HCV Helicase Binding Modes on the Nucleic Acid Substrate

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Supporting Information

ABSTRACT: The dynamics involved in the interaction between hepatitis C virus nonstructural protein 3 (NS3) C-terminal helicase and its nucleic acid substrate have been the subject of interest for some time given the key role of this enzyme in viral replication. Here, we employed fluorescence-based techniques and focused on events that precede the unwinding process. Both ensemble Förster resonance energy transfer (FRET) and ensemble protein induced fluorescence enhancement (PIFE) assays show binding on the 3′ single-stranded overhang of model DNA substrates (>5 nucleotides) with no preference for the single-stranded/double-stranded (ss/ds) junction. Single-molecule PIFE experiments revealed three enhancement levels that correspond to three discrete binding sites at adjacent bases. The enzyme is able to transition between binding sites in both directions without dissociating from the nucleic acid. In contrast, the NS3 mutant W501A, which is unable to engage in stacking interactions with the DNA, is severely compromised in this switching activity. Altogether our data are consistent with a model for NS3 dynamics that favors ATP-independent random binding and sliding by one and two nucleotides along the overhang of the loading strand.

KEYWORDS: hepatitis C virus (HCV), RNA helicase, DNA helicase, fluorescence resonance energy transfer (FRET), protein induced fluorescence enhancement (PIFE)

Helicases are ubiquitous enzymes that catalyze the unwinding of nucleic acids through a variety of mechanisms. Several families of viruses encode helicases that play essential roles in the viral life cycle. The hepatitis C virus (HCV), which is an important human pathogen, has a 9.6 kb positive-stranded RNA genome that encodes a nonstructural (NS) protein, NS3, with N-terminal protease and C-terminal helicase activities. Although both the protease and helicase domains of NS3 modulate each other’s enzymatic activities, both domains can be expressed and are active on their own in vitro.1−5

The helicase domain of NS3 (NS3h) is a well-studied, prototypical member of the DExH subgroup of motor proteins,6 within the helicase superfamily 2 (SF2) based on the original classification scheme of Gorbalenya and Koonin.7,8 NS3h couples ATP binding and hydrolysis to conformational changes in the enzyme that are mechanistically linked to the unwinding process.9,10 The helicase activity is essential for viral replication.11 It has been suggested that NS3h may resolve RNA secondary structures and double-stranded (ds) intermediates and/or displace RNA binding proteins.12−14

Although HCV is an RNA virus, in vitro NS3h is able to unwind both RNA and DNA with a 3′ single-stranded (ss) overhang on the loading strand, unwinding with a 3′−5′ directionality.15−17 Several crystal structures of NS3h as well as full-length NS3 have been solved, indicating key interactions between the enzyme and bound ss nucleic acid.10,18−25 Static conformational snapshots during ATP binding and hydrolysis indicate unwinding of the nucleic acid duplex with a minimum enzyme translocation on the nucleic acid of a single base per

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ATP hydrolysis event and suggest that NS3h may act as a ss nucleic acid translocase.\textsuperscript{10,19} As a result, NS3h would step along one strand of the nucleic acid and actively displace the second strand of the duplex, as opposed to binding at the junction and taking advantage of transient melting at the ss/ds junction to exclude the complementary strand through Brownian motion (a Brownian motor).\textsuperscript{20,26,27}

Significant progress has been made in the understanding of the interactions between NS3h and the nucleic acid. It has been shown that the rate of unwinding by both full-length NS3 and the isolated helicase domain, NS3h, are sensitive to the stability of the duplex, with increased dissociation upon encountering more stable base pairs at the ss/ds junction.\textsuperscript{28,29} During unwinding there is evidence to support an interaction between not only the tracking strand but also the displaced strand of the duplex with full-length NS3.\textsuperscript{13,30,31} Binding studies have indicated that when the 3’ ss binding region is shorter than the footprint of the enzyme, binding of NS3h results in partial melting of the duplex.\textsuperscript{26} Chemical footprinting has suggested that NS3 binds rapidly to ssDNA, followed by slower binding to the duplex, and that binding at the ss/ds junction is preferred.\textsuperscript{32} More recently, Reynolds et al. have proposed an updated model in which NS3 slides on the ss overhang in the absence of ATP, and upon encountering the ss/ds junction, NS3 may form specific interactions with the complementary strand, strengthening the interaction with the junction.\textsuperscript{33} However, a detailed understanding of the dynamic events that precede unwinding of the duplex at the ss/ds junction remains elusive. Employing fluorescence-based single-molecule and ensemble assays, here we demonstrate the existence of distinct populations of DNA-bound helicase molecules that can interconvert in the absence of dissociation events.

\section*{RESULTS}

\textbf{Experimental Approach.} To better understand how the isolated HCV helicase, NS3h, interacts with partial duplex DNA, we utilized the complementary distance-based fluorescence techniques of Förster resonance energy transfer (FRET) and protein-induced fluorescence enhancement (PIFE). FRET involves the nonradiative energy transfer between two fluorescent dyes in a distance-dependent manner and, in this context, requires labeling of both the nucleic acid and the protein.\textsuperscript{34–36} PIFE refers to a photophysical effect whereby the emission intensity of a fluorescent dye attached to a nucleic acid is enhanced when a protein binds in close proximity.\textsuperscript{37–41} The increase in fluorescence intensity of the dye (e.g., Cy3) results from a drop in Cy3 trans→cis photoisomerization and concomitant formation of the non-fluorescent Cy3 cis isomer, due to the steric constraint/increased viscosity provided by the bound protein.\textsuperscript{42} Whereas the Cy3/Cy5 FRET efficiency has a distance dependence of approximately 3–6 nm (10–20 bp), PIFE has a distance dependence of approximately ≤3 nm (<10 bp), with a short-range distance resolution of a single bp.\textsuperscript{37,41,45,44}

For the FRET assay we designed a series of Cy3-labeled DNA duplexes with varying length ss overhangs and utilized maleimide–thiol coupling chemistry to generate Cy3-labeled NS3h by modification of cysteine(s). This allowed us to measure binding of NS3h to the DNA by ensemble FRET between Cy3 and Cy5. Of the 14 cysteines present in NS3h, we identified 8 residues as being potentially solvent exposed on the basis of their location in the crystal structure (Figure 1). By performing the labeling reaction with an equimolar amount of Cy5-maleimide relative to enzyme, we obtained labeling efficiencies of <1 Cy5 per NS3h. In efforts to identify the Cy5-modified cysteine(s) on the labeled NS3h, we generated cysteine to serine mutations and tested their effects on the labeling and FRET efficiencies (Figure S1A–C in the Supporting Information (SI)). We also performed tryptic peptide mass spectrometry to identify Cy5-modified peptides from NS3h-Cy5 (Figure S2 and Table S1). Although we were unable to conclusively identify the labeled cysteine residue(s) generating FRET, the results point to C622 as a key residue (SI text).

For the PIFE assay, we designed a new set of Cy3-labeled DNA duplexes with varying length ss overhangs; however, the location of the Cy3 dye was moved to the ss/ds junction to take advantage of the shorter distance dependency of PIFE compared to FRET as illustrated in Figure S1D.

\textbf{Tracking Binding of NS3h to the Overhang by Varying the Length of ss and ds Regions.} We used a strategically designed series of Cy3-S19/X′mer FRET substrates with a fixed-length helicase loading strand of 19 nucleotides (Cy3-S19) and increasing length ds regions (a = 5–19), which in turn shortens the ss overhang (Figure 2A and Table 1). Increasing the number of bases in the duplex region would distance the bound NS3h-Cy5 from the Cy3 dye at the S’ terminus of the Cy3-S19 strand as depicted in Figure 2A and should result in a reduction of the FRET efficiency. NS3h-Cy5 was tested in the FRET assay to correlate binding of the enzyme to the different Cy3-S19/X′mers. Upon excitation at 520 nm, fluorescence emission spectra for the different substrates in the presence of NS3h-Cy5 were recorded. Assay validation experiments were performed to verify that the FRET signal was due to specific and reversible binding of NS3h-Cy5 to the DNA substrate, where competition with increasing concentrations of unlabeled NS3h or increasing ionic strength in the assay buffer resulted in a decrease in the FRET signal (Figure 2B and Figure S3). The ratiometric intensity-based FRET efficiencies (E_{\text{FRET}}) for NS3h-Cy5 binding to the substrates with different length duplex regions were calculated from the ensemble fluorescence intensities recorded at 565 and 670 nm (Figure 2C). NS3h-Cy5 exhibited the highest E_{\text{FRET}} of ~0.15 with the Cy3-S19/X′mer, the substrate with the shortest duplex region (Figure 2C). It is noteworthy that the FRET efficiency is not closer to the theoretical maximum with a distance of 5 base pairs separating the Cy3 dye from the helicase loading region. However, it is important to consider that the labeling efficiency of NS3h is less than one dye per...
Figure 2. Tracking DNA binding of NS3h using FRET and PIFE approaches. (A) Schematic illustration of the employed FRET assay. The X’mer varies in length so that the duplex formed with Cy3-S19 varies from 5 to 19 bp of dsDNA. (B) Emission spectra in relative fluorescence units (RFU) of 400 nM NS3h-Cy5 with 200 nM Cy3-S19/X’mer at different concentrations of added unlabeled NS3h. Addition of unlabeled NS3h results in the increase in Cy3 and the decrease in Cy5 emission. (C) Calculated ratiometric, intensity-based FRET efficiency (E_FRET) for NS3h-Cy5 binding to the Cy3-S19/X’mers as a function of the length of the ds region (which is equal to “a”). The inset displays a plot of E_FRET for NS3h-Cy5 with the Cy3-S19/X’mer that has 8 base pairs and 2 mismatched bases at the junction, as well as Cy3-S19/X’mers (a = 8, 9, and 10) for comparison. All values represent the mean ± SD (n = 3). (D) Schematic illustration of the employed PIFE assay. Cy3-S19 is annealed to complementary Cy3-S19/Yc’mers yielding ss overhangs of 0–24 bases (c = number of bases composing the ss overhang). (E) RFU and normalized average fluorescence intensity (to [NS3h] = 0) of Cy3-S19/Yc’mer at 565 nm in the presence of different concentrations of NS3h. All values represent the mean ± SD (n = 2). (F) Normalized fluorescence intensities of 200 nM Cy3-S19/Yc’mers in the presence of 600 nM NS3h. All values represent the mean ± SD (n ≥ 3).

protein, that the position of attachment of the Cy5 dye on NS3h is not necessarily adjacent to the duplex in an ideal orientation for FRET, and that the binding of NS3h is not necessarily directly at the ss/ds junction. All of these factors may contribute to a lower FRET efficiency as observed in the assay. Another consideration is that as the experiments were performed at room temperature, the shorter duplexes (Cy3-S19/X’mer, a = 5, 6 and 7) may be shifted toward the ss form. However, the greater E_FRET on these three duplexes compared to the ss Cy3-S19mer supports the idea that NS3h does not preferentially bind to the 5’ termini of ss Cy3-S19.

As the length of the duplex increased, the E_FRET decreased, indicating that NS3h bound on average farther from the Cy3 dye. With the completely double-stranded Cy3-S19/X’mer, the resulting E_FRET was reduced to 0.03–0.04 (referred to as the residual value hereafter). This residual E_FRET was due to the bleed-through of the Cy3 signal into the Cy5 channel, which was observed with Cy3-DNA alone. Notably, the E_FRET dropped by 30% as the ds region extended from 9 to 10 bp, and decreased to the residual E_FRET when the duplex was longer than 14 bp, or correspondingly when the ss loading region was shorter than 5 bases. The ss Cy3-S19 strand and NS3h-Cy5 also yielded an appreciable E_FRET of ~0.13, confirming that NS3h could also bind to the ssDNA lacking a ss/ds junction.

A substrate with eight paired and two additional unpaired bases (X’8+2’mer) at the ss/ds junction was designed to mimic the local melting of the duplex at the junction (Table 1). We hypothesized that if NS3h preferentially binds to the ss/ds junction, then it would localize at the junction and subsequently displace the two unpaired bases on the Cy3-S19/X’8+2’mer, leading to similar E_FRET values for the Cy3-S19/X’8+2’mer and the Cy3-S19/X’mer. However, when the E_FRET between NS3h-Cy5 and the Cy3-S19/X’8+2’mer was plotted along with the two Cy3-S19/X’mers with the closest E_FRET values, the E_FRET fell somewhere between those of the Cy3-S19/X’mer and the Cy3-S19/X’8+2’mer, rather than being closer to that of the Cy3-S19/X’mer (Figure 2C inset). Consequently, the unpaired bases at the ss/ds junction may limit the proximity of binding by NS3h relative to the junction.

To clarify whether NS3h binds preferentially to the ss/ds junction, we next utilized the complementary fluorescence technique of PIFE. Here, we designed a new series of Cy3-S19/Yc’mers partial duplexes with Cy3 attached at the ss/ds junction and varying length single-stranded overhangs as depicted in Figure 2D. The S19/Yc’mers consist of a constant 19 bp DNA duplex formed by the Cy3-S19 sequence annealed to varying length complementary strands 19–43 bases in length (Table 1), resulting in ss overhangs ranging from 0–24 bases (i.e., c = 0–24). Initially, NS3h was titrated against the Cy3-S19/Yc’mer. Because the overhang length of 6 bases approximates the footprint of NS3h, binding of NS3h to the Cy3-S19/Yc’mer would restrict the binding to be adjacent to the ss/ds junction. Upon addition of increasing concentrations of NS3h up to 600 nM, NS3h induced an increasing fluorescence enhancement of Cy3 on the substrate by up to 1.4-fold (Figure 2E). A validation experiment was performed to verify that the PIFE signal was due to a specific and reversible electrostatic binding interaction between NS3h and the DNA substrate, similar to what was done for the FRET assay (Figure S3C).

We next investigated whether the ensemble PIFE assay could be used to track if NS3h preferentially binds to the ss/ds junction on longer overhangs or rather would sit randomly along the ss overhang. On the ss overhangs of Cy3-S19/
Yc19mers shorter than 5 bases (c = 0−5) no PIFE was observed (Figure 2F). This finding was consistent with the aforementioned observation in our FRET assays, supporting NS3h not readily binding to ss DNA overhangs shorter than 5 bases. As the positions of the fluorescent labels in the FRET and PIFE assays are very different, these identical results from complementary techniques support that labeling of the DNA is unlikely to be a confounding parameter limiting NS3h binding. Once the length of the ss overhang increased to 6 bases (c = 6), PIFE increased to a fluorescence intensity approximately 1.2-fold that of the substrate alone. As the length of the ss overhang further increased, PIFE gradually decreased on the 7−9 base overhangs (c = 7−9) and eventually became insignificant on overhangs longer than 9 bases (c > 9). As we observed FRET between NS3h-Cy5 and substrates with 9−19 base ss overhangs, the lack of PIFE by NS3h on the overhangs longer than 9 bases is not likely due to a lack of binding between NS3h and the Cy3-S19/Yc19mers, but rather would be explained by the location of binding by NS3h being farther from the Cy3 dye than the short distance dependence required for PIFE. To confirm this, we performed an electrophoretic mobility shift assay (EMSA) to compare the overall binding of NS3h with the Cy3-S19/Yc19mer (the substrate with the longest overhang) versus the Cy3-S19/Y6mer (the substrate with the highest PIFE). The EMSA confirmed that the fraction of Cy3-S19/Yc19mer-NS3h complexes was indeed greater than that of Cy3-S19/Y6mer-NS3h complexes at equivalent NS3h concentrations (Figure S4A). To confirm that the presence of the Cy3 dye at the ss/ds junction did not affect binding to the overhang, we also performed an EMSA where the Cy3 dye was replaced with a 32P radiolabel and similar results were obtained (Figure S4B). It should be noted that the ssDNA overhang of the Cy3-S19/Y2419mer is not predicted to form significant hairpin structures or self-dimers. Together, the data from our ensemble PIFE assay indicate that NS3h can bind to the Cy3-S19/Yc19mers adjacent to the ss/ds junction on the shorter overhangs of 6−9 bases, approximately one enzyme footprint in length, but farther from the junction as the length of the overhang exceeds 9 bases.

Using smPIFE To Dissect the Binding Modes of NS3h at the ss/ds Junction. To further investigate the positioning and dynamics of NS3h binding relative to the ss/ds junction, we applied smPIFE to investigate whether the decreased PIFE on longer overhangs was due to a homogeneous population with a low fluorescence enhancement or a heterogeneous mixture of fluorescence enhancement levels on a given overhang length. To test this hypothesis, we performed smPIFE on the Cy3-S19/Y6mer (c = 6, 7, 8, and 24) substrates with a biotin modification to enable surface immobilization (Figure 3A). Single molecule imaging enabled

### Table 1. DNA Sequences for Substrates in FRET and PIFE Assays

<table>
<thead>
<tr>
<th>Name</th>
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<tbody>
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</tr>
<tr>
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<td>5'−T GCT AGA TTT CCA CAC TG CG CG CG CG−3'</td>
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*a* The underlined red bases are not complementary with Cy3-S19. *b* Cy3 is conjugated to the S′ terminus of S19 (in green) via a phosphoramidite linker. *c* The Y019mer is identical to the X19mer (forms a fully dsDNA with Cy3-S19).
us to probe discrete binding modes of NS3h otherwise hidden in the above-mentioned ensemble-averaged PIFE studies.

Figure 3. NS3h induced smPIFE at the ss/ds junction. (A) Schematic illustration of the smPIFE substrates with the Cy3 dye located at the ss/ds junction and ss overhang lengths of 6, 7, 8, or 24 bases. To surface-immobilize the substrates via a streptavidin linkage, a 5’ biotin modification is included on the loading strand. (B) Subensemble histograms that were constructed from the individual normalized intensity—time trajectories for the Cy3-S19/Y24mer (c = 6, 7, 8, or 24) acquired in the presence of 100 nM NS3h WT.

We used total internal reflection fluorescence (TIRF) microscopy to simultaneously monitor in real-time the fluorescence enhancement of individual surface-immobilized Cy3-DNA substrates induced by binding of NS3h as it was flowed into the imaging chamber.45 Individual substrate traces from a given acquisition showing PIFE were analyzed as of 5 s after the earliest initial fluorescence enhancement time recorded in the given acquisition. This window provided sufficient time for enzyme to flow into the chamber.45 This approach ensured that NS3h was accessible in solution to all of the substrate molecules at the surface, even if PIFE had not yet been observed on that molecule. By plotting the ensemble PIFE intensity histogram for these experiments, we retrieved the number of events with PIFE values ranging from 1 to approximately 2 for all of the substrates of a given sequence (Figure 3B). Repeating the experiment with the 7, 8, and 24 base overhang substrates provided an idea of the distribution of PIFE values versus substrate length. When we examined smPIFE on the Cy3-S19/Y19mer in greater detail, among 228 substrate molecules, 23 exhibited at least two different normalized fluorescence intensities, or PIFE levels (referred hereafter to as $L_{\text{PIFE}}$). These substrate molecules were further analyzed individually and included to construct a subensemble normalized intensity histogram. From this analysis the subensemble normalized intensity histograms shown in Figure 3B were constructed for the different substrates in the presence of 100 nM WT NS3h. Next, we scrutinized smPIFE on the 7, 8, and 24 base overhang Cy3-S19/Y25mer substrates ($c = 7, 8, \text{or } 24$). Again, subensemble normalized intensity histograms were derived for each substrate, where these histograms were constructed on the basis of dozens of individual single-molecule normalized intensity—time trajectories (i.e., 75 molecules for the Cy3-S19/Y25mer, 69 molecules for the Cy3-S19/Y8mer, and only 31 molecules for the Cy3-S19/Y24mer as PIFE occurred less frequently on the 24 base overhang).

We observed a decrease in PIFE as the length of the overhang increased (shift to normalized intensity of 1.0), as was observed in the ensemble PIFE assay. Interestingly, on the 6, 7, and 8 base overhangs, we observed what appeared to be multiple different PIFE distributions. This was most pronounced with the 6 base overhang. In contrast, on the 24 base overhang the major contribution to the subensemble histogram was a normalized intensity of 1.0 for no PIFE; however, we observed some PIFE corresponding to binding of NS3h in close proximity to the junction. This signal would have been obscured in the ensemble PIFE assay by the majority of substrate molecules with no NS3h bound at a given point in time.

**WT NS3h Is Capable of Transitioning between Binding Modes.** A close look at the smPIFE data of individual molecules provides details on the dynamics of NS3h binding to the substrates. Figure 4 summarizes the representative single-molecule normalized intensity—time trajectories, along with the corresponding subensemble normalized intensity histograms for each substrate. The subensemble histograms were rebuilt to correspond to the time regions as of the initial fluorescence enhancement on each molecule, that is, the first time point at which a protein arrived and the concomitant PIFE was recorded for a given DNA substrate, in order to focus on when PIFE was actually recorded. As observed in the individual traces shown in Figure 4B, NS3h was capable of switching between different $L_{\text{PIFE}}$ values, which was observed among all of the substrates applied in the smPIFE assay. If we interpret the different $L_{\text{PIFE}}$ values as different binding positions on the overhang, occasionally NS3h moved toward or away from the ss/ds junction in a stepwise, sequential manner as indicated by the red arrows in Figure 4B; however, we could also occasionally observe NS3h jumping directly from an $L_{\text{PIFE}} = 1$ to the $L_{\text{PIFE}} = 2$ and vice versa (additional traces for individual substrate molecules are provided in Figures S5–S8).

The Gaussian fitting for the Cy3-S19/Y25mer sustains three PIFE distributions centered at normalized intensities of 1, 1.36, and 1.89, representing the Cy3-S19/Y25mer alone and two distinct binding modes derived from NS3h/DNA binary complexes (Figure 4C). The Gaussian fitting to the subensemble histogram for the Cy3-S19/Y19mer yielded three distinct distributions, centered at 1.03, 1.37, and 1.89, nearly identical to those obtained with the Cy3-S19/Y24mer (Figure 4C). For the Cy3-S19/Y8mer, the histogram was best fit by a quadrmodal set of Gaussian distributions, yielding an additional distribution shifted to the lower $L_{\text{PIFE}}$ centered at 1.16, in addition to the other two above-mentioned $L_{\text{PIFE}}$ values (centered at 1.42 and 2.04 for the S19/Y8mer). When an $L_{\text{PIFE}}$ of ~1.4 was the dominant $L_{\text{PIFE}}$ (or the most populated peak) for NS3h on the shorter overhangs (6, 7, and 8 bases), this mode was shifted to the lower $L_{\text{PIFE}}$ of ~1.2 and eventually to a $L_{\text{PIFE}}$ of ~1 as the overhang length extended to 24 bases (Figure 4C). From the smPIFE data we infer that as the length...
Figure 4. NS3h induces different levels of fluorescence enhancement. (A) Structures of the DNA partial duplexes used in the smPIFE assay. The Cy3 dye is attached 5′ to the G shown in green. (B) Representative intensity—time trajectories displaying different fluorescence enhancement levels induced by binding of NS3h to each substrate. The shaded areas highlight the regions where PIFE was observed and that were analyzed for the subensemble histograms. Red arrows indicate examples of sequential transitioning from one induced by binding of NS3h to each substrate. The shaded areas highlight the regions where PIFE was observed and that were analyzed for the initial fluorescence enhancement event to construct a subensemble histogram focusing on when PIFE was actually observed. In the subensemble histogram W501A produced a major $L_{\text{PIFE}}$ at 1.38 on the Cy3-S19/Y6mers (c = 6, 7, 8, and 24) acquired in the presence of 100 nM NS3h WT. The subensemble histograms were fitted to multimodal Gaussian distributions as described under Materials and Methods.

Transitioning between Binding Modes for NS3h W501A is Compromised. Residue W501 in NS3h is known to play an important role in binding to the nucleic acid substrate, acting as a bookend residue stacking with a base at the 3′ end of the nucleic acid strand bound by NS3h. As a result, mutation of the 3′ bookend tryptophan to an alanine leads to poor nucleic acid binding and unwinding.46−49 We performed smPIFE to probe the effect of the W501A mutation, which not surprisingly led to decreased binding of NS3h W501A to DNA and the rapid dissociation of W501A/DNA binary complexes as monitored by smPIFE. As a result, during the course of image acquisition (∼3 min), W501A (at 100 nM) triggered PIFE on only 35% of the Cy3-S19/Y6mer molecules, whereas on the same substrate WT (also at 100 nM) induced fluorescence enhancement on 91% of substrate molecules. Of the 28 molecules that exhibited PIFE with NS3h W501A, we analyzed the traces as of 5 s after the earliest initial fluorescence enhancement to construct the subensemble histogram shown in Figure 5A. The result is a peak centered at a normalized intensity of ∼1 for no PIFE, and a second smaller distribution for PIFE representing binding to the short overhang.

When we looked at the ability of the W501A mutant to transition between different $L_{\text{PIFE}}$ values, the smPIFE data further emphasize the distinction between the mutant and WT. Transitioning by NS3h W501A was observed on only two Cy3-S19/Y6mer substrate molecules (compared to 23 molecules for WT). Instead, PIFE was most frequently observed at a single $L_{\text{PIFE}}$ for W501A on a given substrate molecule (Figure 5B and Figure S9). The Cy3-S19/Y6mers that exhibited PIFE in the presence of W501A were each analyzed individually as of the initial fluorescence enhancement event to construct a subensemble histogram focusing on when PIFE was actually observed. In the subensemble histogram W501A produced a major $L_{\text{PIFE}}$ at 1.38 on the Cy3-S19/Y6mers (c = 6, 7, 8, and 24) acquired in the presence of 100 nM NS3h WT. The subensemble histograms were fitted to multimodal Gaussian distributions as described under Materials and Methods.

DISCUSSION

Although the mechanism of nucleic acid unwinding by HCV NS3h has been extensively studied, the dynamic interactions with the 3′ overhang prior to the melting of the duplex remain elusive. We have employed complementary fluorescence-based approaches to study the movement of the helicase on partially dsDNA substrates. Classic ensemble studies with NS3h did not generate FRET or PIFE signals on ss overhangs shorter than 5 bases. Increasing the length of the ss overhang to 6 bases resulted in a strong PIFE signal, which decreased on the 7−9 base overhangs and was lost as the overhang length extended beyond 10 bases. Overall, the ensemble data show that NS3h does not readily form a stable binary complex with ssDNA overhangs of ≤5 bases, but binds closely to the ss/ds junction on 6−9 base overhangs. Previous structural and biochemical data have suggested an optimal footprint for NS3h of approximately 7−8 bases.50,52,53 Overhangs that are longer than the footprint provide additional opportunities for binding. Both FRET and PIFE data with longer substrates support this notion, and smPIFE traces revealed the existence of discrete binding sites along the overhang.

Multiple PIFE levels observed in smPIFE experiments are indicative of different binding modes of NS3h in close proximity to the ss/ds DNA junction (Figure 6A). The data revealed two commonly shared binding modes of NS3h on the 6, 7, and 8 base overhangs, with a primary mode at a PIFE of
Figure 5. W501A mutant is deficient in transitioning between fluorescence enhancement levels. (A) The subensemble histogram constructed from the normalized intensity-time trajectories for the Cy3-S19/Y28mer acquired in the presence of 100 nM NS3h W501A is shown in black, whereas the subensemble histogram for the Cy3-S19/Y28mer acquired in the presence of 100 nM NS3h WT (from Figure 3B) is shown in blue for reference. (B) Representative intensity-time trajectory for NS3h W501A where binding is usually observed at a single PIFE level. (C) Subensemble histogram constructed from the regions where PIFE was observed of individual normalized intensity-time trajectories for the Cy3-S19/Y28mer acquired in the presence of 100 nM NS3h W501A.

~1.4 and a secondary mode at ~1.9. Interestingly, an additional binding mode with a PIFE of ~1.2 emerged on the 8 base overhang and became the dominant L_PIFE when the overhang length increased to 24 bases. The three PIFE values reflect that NS3h interacts with the Cy3 dye at the ss/ds junction with three different modes within the detection limit of PIFE in our system. As the length of the overhang increases, NS3h is prone to move away from the junction, without necessarily dissociating from the nucleic acid.

Figure 6B translates these findings in a model. On the 6 base overhang, movement of NS3h away from the junction by 1 base will lead to the dissociation of NS3h as we do not observe significant binding to a 5 base overhang. Thus, we attribute the PIFE of 1.4 to the localization of NS3h at the junction, which is applied to all substrates regardless of overhang length. By extension, the PIFE of 1.2 would correspond to the movement of NS3h away from the junction by 1 base, which can be observed on the overhang lengths of ≥7 bases. Under conditions where NS3h is still bound but there is no fluorescence enhancement, a PIFE of 1 would correspond to the movement of NS3h away from the junction by 2 bases or more.

To generate the higher PIFE of 1.9, NS3h would need to move closer to the junction, presumably by causing local melting of the duplex region at the ss/ds junction as previously observed by Levin et al.26 Another possibility is that the increased PIFE of 1.9 is due to the intrinsic photophysics of the Cy3 dye interacting with the protein/DNA differently, yet with no change in position of NS3h on the DNA, for example, an increased interaction between the dye and the surface of the protein (i.e., a pocket on the surface of NS3h). Interestingly, the L_PIFE of 1.9 was drastically reduced between the 6 and 7 base overhangs, even if the population of L_PIFE at 1.4 was not. On the long 24 base overhang, it was essentially nonexistent, implying that the higher L_PIFE of 1.9 depends on the stable localization of NS3h at the ss/ds junction to trigger the interactions between NS3h and the complementary strand of the duplex at the junction.

The PIFE distribution at 1 was consistently observed among all of the substrates tested in the smPIFE experiments, but we ascribe it to two different scenarios: The first scenario would be the dissociation of NS3h from the overhang, and the second scenario would be the binding site of NS3h being distant from the ss/ds junction on longer overhangs. In addition to the aforementioned FRET data, the EMSA demonstrated increased complex formation between NS3h and the 24 base overhang compared to the 6 base overhang, consistent with previous findings that increasing the length of the ss loading region increases binding of NS3h to the overhang.26,51 Consequently, the PIFE distribution at 1 on the 6−8 base overhangs would be due to the dissociation of NS3h from the DNA overhang. In contrast, the increased population with a PIFE of 1 on the 8 and 24 base overhangs is plausibly due to preferential binding of NS3h farther from the ss/ds junction so as to be beyond the short-range sensitivity of the PIFE assay.

Substitution of the aromatic W501 residue with alanine significantly compromised binding affinity of NS3h to the ss DNA overhang. The two commonly observed binding modes observed with WT on the 6 base overhang were still observed with W501A, with the primary binding mode being at the junction (PIFE ~ 1.4), along with the infrequently proposed
tighter binding mode (PIFE $\sim 1.9$). On the short overhangs (6–8 bases), WT NS3h frequently switched between different binding modes, whereas the W501A mutant is severely compromised in this regard. A one-base stepwise transitioning between $L_{\text{PIFE}}$ of $\sim 1.2$, $\sim 1.4$, and $\sim 1.9$ corresponds to a movement toward or away from the junction. However, NS3h WT could also move two steps at once. For the W501A mutant, we only captured the switching between modes on two of the substrate molecules. The compromised switching activity of W501A between $L_{\text{PIFE}}$ may account for the relatively unstable DNA/NS3h binary complexes compared to WT. Hence, the mutant is likely to be more prone to dissociation during the dynamic motion involved in transitioning from one binding mode to another. Diminished switching may also prevent W501A from engaging in tight binding at the ss/ds binding mode to another. Diminished switching may also occur during the dynamic motion involved in transitioning from one binding mode to another.

Taken together, our data demonstrate that smPIFE provides a sensitive tool to study the dynamics of binding of NS3h to the nucleic acid substrate. Owing to the short distance resolution of PIFE, we were able to detect distinct binding modes at adjacent nucleotides in close proximity to the ss/ds junction. Switching between binding modes can occur in the absence of recurring dissociation and association events and provides strong evidence for enzyme sliding in both directions along the overhang. Sliding provides a mechanism that facilitates the interaction between NS3h and the ss/ds junction. Whereas sliding does not require ATP, on its own sliding is not sufficient for efficient unwinding, which is driven by ATP binding and hydrolysis.

**MATERIALS AND METHODS**

**Enzymes and Nucleic Acids.** The coding sequence for HCV NS3h (genotype 1a) corresponding to amino acids 167–631 of NS3 in the expression vector pET21b (Novagen) with a C-terminal hexa-histidine tag was expressed in *Escherichia coli* BL21(DE3) cells. Cultures were grown in LB broth at 37 °C to an OD$_{600}$ of 0.6–0.7 and then induced with 1 mM IPTG for 5 h at 30 °C. NS3h was purified from pelleted cells by a two-step nickel heparin column purification as described elsewhere with the following modifications:19 Cells were resuspended in buffer A (20 mM Tris pH 7.9, 500 mM NaCl, 10 mM imidazole, and 10% glycerol) with 1 mg/mL lysozyme and a protease inhibitor cocktail and disrupted by sonication for nickel affinity chromatography. After the supernatant was loaded on a HiTrap HP Ni Sepharose column (GE Healthcare Life Sciences), NS3h was washed with buffer B (buffer A with 20 mM imidazole) and eluted against a gradient of buffer C (buffer A with 250 mM imidazole). Fractions containing NS3h were pooled and buffer exchanged into buffer D (10 mM sodium phosphate, pH 7.0, and 10% glycerol) using a HiPrep 26/10 desalting column (GE Healthcare Life Sciences). NS3h was then loaded on a HiTrap heparin HP column (GE Healthcare Life Sciences) and eluted against a gradient of buffer E (buffer D with 1 M NaCl). Purified NS3h was then buffer exchanged into 40 mM Tris, pH 7.9, and 500 mM NaCl and again using a HiPrep 26/10 desalting column. NS3h was concentrated, and glycerol was added to a final concentration of 50%. Protein concentration was determined by absorbance at 280 nm on a NanoDrop 2000 (Thermo Scientific) using the calculated extinction coefficient of 48,205 M$^{-1}$ cm$^{-1}$. Mutant enzymes were generated via site-directed mutagenesis and sequencing performed at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada).

All DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). Cy3-S19 ($5'\text{-}GTGTTGAAAATCTCTTAGCA-3'$) was ordered with a Cy3 dye attached to the S' terminus via a phosphoramidite linker and HPLC purified. Sequences complementary to S19 were designed to form partial duplexes, where the complementary strands are denoted “X”mers and the length “a” varies from 5 to 19 nucleotides, with the resulting S' ss overhang on the S19 strand serving as the NS3h loading region. Therefore, the X$^a$mer ($5'\text{-}TGCTAGAGATTTTCCACAC-3'$) when annealed to S19 forms a blunt-ended duplex with no ss overhang, whereas the S19/X$^12$mer has a single base 3' ss overhang on S19. When unpaired bases were introduced at the ss/ds junction, the substrate denoted X$^8$mer was used, with 8 nucleotides complementary to S19 (as with the X$^a$mer) and an additional 2 mismatched nucleotides with respect to S19 at the ss/ds junction. Alternatively, oligonucleotides complementary to S19 but of greater length are denoted “Y”mers. Here, “19” indicates that the Y$^a$mer fully complements S19 with additional “c” (c = 0–24) nucleotides forming 3' ss overhangs in the S19/ Y$^a$mer duplexes. As such, the first 19 nucleotides from the S’ terminus of the Y$^a$mer have the same sequence as the X$^a$mer, and the S19/Y$^a$mer duplexes results in the ss loading region present on the Y$^a$mer rather than the S19 strand. The sequence for the longest Y$^a$mer, the Y$^{24}$ mer, was 5'-TGCTAGAGATTTTCCACACCTCGAAGGCTCTGTGACT-3' (the sequence fully complementary to S19 is underlined). Biotinylated Y$^a$mers (c = 6, 7, 8, or 24) employed in the smPIFE studies were ordered with a biotin attached to the S’ terminus via a phosphoramidite linker and were either HPLC or PAGE purified. S'-Radiolabeling of S19 for the control EMSA experiment was performed with [γ$^32$P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (Fermentas) for 1 h at 37 °C. Labeled DNA was subjected to phenol–chloroform extraction and buffer exchanged using P-30 size exclusion columns (Bio-Rad). To form DNA duplexes, the Cy3-S19 or 32P-S19 was heat-annealed to a 2-fold excess of X$^8$mer rather than the S19 mer. Therefore, the X$^8$mer rather than the S19 strand. The sequence for the longest X$^8$mer, the X$^{24}$mer, was 5'-TGCTAGAGATTTTCCACACCTCGAAGGCTCTGTGACT-3'. The concentration of dye in the labeled protein solution was determined by the absorbance at 650 nm using a NanoDrop 2000 (Thermo Scientific) and an extinction coefficient of 26,205 M$^{-1}$ cm$^{-1}$. Mutant enzymes were generated via site-directed mutagenesis and sequencing performed at the McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada).

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coefficients of 250,000 M$^{-1}$ cm$^{-1}$ for Cy5. The labeling efficiencies were determined by dividing the Cy5 dye concentration by the protein concentration to give Cy5/NS3h.

**Ensemble FRET Measurements.** Ensemble FRET experiments were conducted in 96-well round-bottom black plates (Greiner), and the ratiometric intensity-based FRET efficiency ($E_{\text{FRET}}$) was determined upon irradiation at 520 nm using a SpectraMax M3 plate reader (Molecular Devices). Note that here $E_{\text{FRET}}$ is defined as $I_{\text{Yc}}/(I_{\text{Xc}} + I_{\text{Yc}})$, where $I_{\text{Xc}}$ and $I_{\text{Yc}}$ are the intensities of the samples at 670 and 655 nm, respectively, corresponding to the emission maxima of Cy5 (acceptor) and Cy3 (donor). For FRET between NS3h and the substrate, a 200 nM solution of Cy3-S19/X$^c$mer duplex was incubated with 400 nM NS3h-Cy5 (unless otherwise stated) in NS3h binding buffer (25 mM MOPS, pH 6.0, 10 mM NaCl, 3 mM MgCl$_2$, and 0.1% Tween 20). Plates were incubated for 10 min with shaking before reading.

**Analysis of Cy5 Labeled NS3h with Mass Spectrometry (MS).** Chemically modified peptides were examined as described previously. Briefly, labeled and unlabeled NS3h constructs were subjected to SDS-PAGE. Protein bands were excised, destained, and subjected to in-gel proteolysis using 0.5 μg of trypsin, 0.1 %, and 0.25 μg of AspN, 0.1% Tween 20). Plates were incubated for 10 min with shaking before reading.

**Gel-Based EMSA.** Gel-based binding assays were performed as described elsewhere. Briefly, serial dilutions of NS3h were made in 1.5-fold increments from 12 to 0.09 μM and incubated with 40 nM of either Cy3-S19/Y$^{99}$mer or P-S19/Y$^{99}$mer, where “c” was either 6 or 24, for 1 h at room temperature in NS3h EMSA binding buffer (25 mM MOPS, pH 6.0, and 10 mM NaCl). At the end of the incubation, sucrose was added to 8.33% and the samples were run on a 6% native polyacrylamide gel containing NS3h EMSA binding buffer. The gel was scanned with a Bio-Rad gel imager equipped with a S32 nm laser for detection of the fluorescent substrate. For the radiolabeled substrate the gel was dried, exposed to a phosphorimaging screen, and scanned with a Bio-Rad phosphorimager.

**PIFE Measurements.** Ensemble PIFE experiments were conducted in 96-well round-bottom black plates, and fluorescence intensities at 655 nm were recorded upon irradiation at 520 nm using a SpectraMax M3 plate reader. The concentration of Cy3-S19/Y$^{99}$mer in the assay was 200 nM, with 600 nM unlabeled NS3h added unless otherwise stated. The assay was performed in NS3h binding buffer. Plates were incubated for 10 min with shaking before reading. The normalized average fluorescence intensity was calculated as the average intensity of two wells containing substrate with NS3h divided by the average intensity of two wells containing substrate alone.

The smPIFE measurements were performed using surface-anchored Cy3-S19/Y$^{99}$mers (c = 6, 7, 8, or 24) on the poly(ethylene glycol)-grafted coverslip as previously described. The imaging chamber was constructed and connected to a flow system to allow the delivery of the imaging buffer (25 mM HEPES, pH 7.2, 10 mM NaCl, and the oxygen scavenging components) to the substrate during the imaging acquisition. Prior to the PIFE measurements, the syringe was replaced with imaging buffer containing 100 nM NS3h WT or W501A mutant. Image acquisition and the flow of NS3h began simultaneously into the chamber. Typically, PIFE on Cy3 occurred between 40 s and 1 min after the initiation of NS3h flow at 10 μL per minute, depending on the dead volume of the tubing system.

The imaging chamber containing surface-anchored Cy3-S19/Y$^{99}$mer substrates was placed on a home-built TIRF microscope, illuminated by a 532 CW diode-pumped solid state laser. The fluorescence of substrates was collected by an oil immersion objective (60× Plan Apo N; NA = 1.45; Olympus) and recorded by a back-illuminated electron multiplying charge-coupled device (EM-CCD) detector (Cascade II:512; Roper Scientific) at a frame rate of 10 Hz (i.e., 100 ms/frame). The fluorescence of hundreds of Cy3-S19/Y$^{99}$mer molecules was recorded at room temperature simultaneously as a video for 2.5–3 min using Image-Pro Plus 5.1 software (Media Cybernetics). The video was then processed to yield fluorescence intensity–time trajectories of individual Cy3-S19/Y$^{99}$mer molecules, normalized to their initial intensities in the absence of NS3h using self-written algorithms in IDL and Matlab. The individual normalized fluorescence intensity–time trajectories were replotted in OriginPro, and only the partial trajectory until photobleaching of Cy3 on the substrate (or the end of the acquisition if photobleaching did not occur) was included in the normalized intensity histograms for individual substrate molecules. At each condition, individual Cy3-S19/Y$^{99}$mer substrate traces from a given acquisition were analyzed either starting as of 5 s after the earliest initial fluorescence enhancement time of all the traces (substrate with NS3h present in solution, Figure 3B) or as the partial trajectories as of the onset of the initial fluorescence enhancement on each molecule (time regions where PIFE was observed, Figure 4C) to construct single-molecule normalized intensity histograms. Binding of NS3h relative to the ss/ds junction was assessed on the basis of the fluorescence enhancement levels obtained by fitting the (individual single-molecule trace or subensemble) normalized intensity histograms to either single, bimodal, trimodal, or quadrimodal Gaussian distributions using OriginPro.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.6b00177.

Figures S1–S9, Table S1, and supplementary text for identifying modified cysteines on the Cy5-labeled NS3h (PDF)

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ABBREVIATIONS

HCV, hepatitis C virus; NS3, nonstructural protein 3; NS3h, nonstructural 3 helicase domain; FRET, Förster resonance energy transfer; PIPE, protein-induced fluorescence enhancement; ss, single-stranded; ds, double-stranded; SF2, helicase superfamily 2; E_{HIPFRET}^a, intensity-based FRET efficiency; E_{HIPFRET}^D, internal intensity-based FRET efficiency; EMA, electrophoretic mobility shift assay; TIRF, total internal reflection fluorescence; L_{PIPE}, PIPE levels; I_{DA}, intensity of the acceptor; I_{DD}, intensity of the donor; MS, mass spectrometry; EM-CCD, electron multiplying charge-coupled device; RFU, relative fluorescence units; SD, standard deviation

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