Dynamics of Hepatitis C Virus (HCV) RNA-dependent RNA Polymerase NS5B in Complex with RNA*

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Background: The dynamics associated with RNA binding by the hepatitis C virus (HCV) polymerase remain elusive.

Results: Single molecule experiments reveal changing populations of binary RNA-enzyme complexes.

Conclusion: Rapid enzyme conformational changes facilitate sliding/wrapping of the polymerase along its RNA substrate.

Significance: This study provides novel insight into mechanisms associated with viral replication and its inhibition.

The hepatitis C virus (HCV) non-structural protein 5B (NS5B) is an RNA-dependent RNA polymerase that is essentially required for viral replication. Although previous studies revealed important properties of static NS5B-RNA complexes, the nature and relevance of dynamic interactions have yet to be elucidated. Here, we devised a single molecule Förster Resonance Energy Transfer (SM-FRET) assay to monitor temporal changes upon binding of NS5B to surface immobilized RNA templates. The data show enzyme association-dissociation events that occur within the time resolution of our setup as well as FRET-fluctuations in association with stable binary complexes that extend over prolonged periods of time. Fluctuations are shown to be dependent on the length of the RNA substrate, and enzyme concentration. Mutations in close proximity to the template entrance (K98E, K100E), and in the center of the RNA binding channel (R394E), reduce both the population of RNA-bound enzyme and the fluctuations associated to the binary complex. Similar observations are reported with an allosteric non-nucleoside NS5B inhibitor. Our assay enables for the first time the visualization of association-dissociation events of HCV-NS5B with RNA, and also the direct monitoring of the interaction between HCV NS5B, its RNA template, and finger loop inhibitors. We observe both a remarkably low dissociation rate for wild type HCV NS5B, and a highly dynamic enzyme-RNA binary complex. These results provide a plausible mechanism for formation of a productive binary NS5B-RNA complex, here NS5B slides along the RNA template facilitating positioning of its 3’ terminus at the enzyme active site.

The hepatitis C virus (HCV)6 is recognized as a major human pathogen with ~170 million infected people worldwide (1, 2). Chronic HCV infection is associated with severe liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (3). HCV is a positive strand virus that belongs to the Flaviviridae family (4). It contains a single stranded (ss) 9.6 kb RNA genome, which encodes a polyprotein that is processed into several structural and non-structural proteins (3). The non-structural protein 5B (NS5B), which is a prime target in current drug discovery efforts, shows RNA-dependent RNA polymerase (RdRp) activity (5–9).

The three-dimensional fold of NS5B is comparable with that of many other viral polymerases and resembles a human right hand. “Fingers” and “thumb” subdomains provide important contacts for the nucleic acid substrate, while the “palm” contains the active site (Fig. 1a). Two flexible finger loops (A1 and A2) can bridge fingers and thumb subdomains in a closed conformation (10). Thumb pocket 1 non-nucleoside inhibitors (NNIs) or finger loop NNIs bind to a region of the thumb that is otherwise occupied by the flexible A1 finger loop, which seems to impede productive RNA binding (Fig. 1b) (11–14).

The structure of NS5B with a short 5mer RNA template suggests that the closed conformation may only accommodate ssRNA (15). In this structure, the RNA is located at the template entrance (Fig. 1c). A β-hairpin protrudes in the active site and represents an obstacle for RNA translocation (16, 17). The structure of an engineered HCV NS5B enzyme that lacks this β-hairpin shows that a 6mer RNA primer-template can be accommodated under these conditions (18). The data also

6 The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ss, single stranded; NS5B, non-structural protein 5B; RdRp, RNA-dependent RNA polymerase; NNi, non-nucleoside inhibitor; ds, double stranded; SM-FRET, single molecule Förster resonance energy transfer; TIRF, total internal reflection fluorescence; PEG, polyethylene glycol.
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FIGURE 1. Comparison of different structures of HCV NS5B. In each structure, the following coloring scheme is employed for the domains of NS5B: yellow, thumb subdomain; red, palm subdomain; blue, fingers subdomain. The nucleic acid is colored as follows: green, template strand; magenta, primer strand. The same orientation of the enzyme is conserved across the panels. Crystal structure of HCV (a) apo NS5B (genotype 1b J4 strain, PDB code 3MWV) and (b) NS5B (genotype 1b J4 strain) complexed with a representative NNI Class I thumb binding site inhibitor with an indole core (PDB code 3MWV) (20). The NNI causes a structural reorganization of the thumb subdomain, disrupting critical enzyme-RNA interactions and abrogating RNA synthesis. c. structure of HCV NS5B (genotype 1b J4 strain) bound to a short, ssRNA substrate (PDB code 1NB7) (15), d. HCV NS5B (genotype 2a JFH-1 strain) crystal structure containing a dsRNA substrate (PDB code 4E7A). A regulatory β-hairpin loop located in the fingers subdomain is removed from the NS5B construct to allow for binding of a short dsRNA substrate (18).

reveals significant movements of the thumb relative to the fingers, which creates sufficient room for the double stranded (ds)RNA region that is located at the dsRNA exit (Fig. 1d). The entire RNA binding channel can accommodate ~10 nucleotides with a ~90° bend centered in the vicinity of the active site that separates template entrance from dsRNA exit (17, 19). Structures with RNA substrates that cover the entire footprint of NS5B or structures of ternary complexes that contain both RNA and nucleotide substrates are not available.

HCV RNA synthesis is likely initiated de novo, a process that necessitates a single nucleotide that acts as a primer and an incoming nucleotide substrate that binds to the 3′ terminus of the RNA template (20–27). Biochemical data have shown that the initiation complex composed of a ssRNA template and the bound nucleotides is fragile and frequently dissociates (13). In contrast, the elongation of RNA synthesis is highly processive and the corresponding complexes with dsRNA are stable over protracted periods of time. Binding of the ssRNA template, formation of a productive initiation complex which may require sliding along the RNA template, and the transition to elongation once initiation has commenced, are three different dynamic events of which little is known.

In attempts to visualize the association-dissociation dynamics of HCV-NS5B, the possible sliding and overall interaction between HCV NS5B and its RNA template in binary complexes, and the overall role of mutations and finger loop inhibitors in both dynamic processes described above, we resorted to single molecule Förster Resonance Energy Transfer (SM-FRET) (28–35) studies. We found that binding of the enzyme to RNA resulted in highly dynamic binary complexes giving rise to FRET fluctuations extending to a few seconds. Mutations at positively charged residues that constitute the template entrance and the interior of the RNA binding channel decreased the FRET fluctuations. The presence of a finger loop inhibitor caused similar reductions in FRET fluctuations, while a resistant mutant enzyme preserved the fluctuating FRET trajectories under these conditions (presence of finger loop inhibitor). The combined data suggest conformational changes of HCV NS5B can translate in lateral, random motions along the ssRNA template. These findings provide novel mechanistic insight for HCV RNA synthesis and its inhibition.

EXPERIMENTAL PROCEDURES

Nucleic Acids and Enzymes—DNA and RNA oligonucleotides were purchased from Trilink (San Diego, CA). The coding sequence for HCV NS5B (genotype 1b, BK strain) was cloned into the bacterial expression vector pet21b. The hydrophobic region at the C terminus was removed (Δ21), and a hexa-histidine tag was introduced to facilitate protein purification (20, 36). Mutant enzymes were generated via site-directed mutagenesis using the Stratagene QuikChange kit according to the manufacturer’s protocol. Sequencing was conducted at the Genome Quebec Innovation Center.

Single Molecule Measurements—Fluorescence single molecule experiments were carried out using total internal reflection fluorescence microscopy (TIRFM). The TIRF microscope consisted of an inverted microscope (IX71, Olympus) equipped with a laser-based TIRFM illumination module (IX2-RFAEVA-2, Olympus) coupled to a diode-pumped solid-state green laser (532 nm, CrystaLaser). The beam position was adjusted using the illuminator to attain total internal reflection through an oil-immersion objective (N.A. 1.45, Olympus PlanApo N 60×). Fluorescence emission was collected through the objective and images were captured with an EMCCD camera (CascadeII:512B, Photometrics, Roper Scientific). Emission was chromatically separated using dichroic mirrors (640dxcr, Chroma) with the Donor and Acceptor emission filtered through band pass filters (HQ590/70 M and HQ685/80M, respectively, Chroma) before being captured by the EMCCD camera. The camera was controlled using Image-Pro Plus 5.1 (Media Cybernetics), capturing 8-bit 512×512 pixel images with an exposure time of 200 ms, a conversion gain of 3, and multiplication gain of 4095. Excitation was carried out at a full power setting (25 milliwatt) with a power output of 6.6 milliwatt measured from the objective.

Glass coverslips were functionalized with a mixture of polyethylene glycol (PEG) and biotin-PEG in order to prevent nonspecific adsorption of DNA/RNA hybrids and NS5B proteins. Following streptavidin incubation, 100 pm of annealed DNA:RNA hybrid were immobilized on PEG-coated glass coverslips via biotin-streptavidin interactions. Flow chambers were prepared with a predrilled polycarbonate film with an adhesive
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Ensemble Fluorescence Measurements—Ensemble fluorescence experiments were conducted and apparent FRET efficiencies were determined. Note that apparent FRET here is given by $I_\alpha/(I_\alpha + I_\delta)$, where $I_\alpha$ and $I_\delta$ are the intensities of acceptor and donor, respectively, measured at their peaks. A 200 nm solution of duplex DNA:RNA ($R^{S}_{20}$) hybrid in 10 mm HEPES and 20 mm NaCl at pH 7.3 was excited at 514 nm. Control experiments were conducted on a doubly labeled (3’Cy3, 5’Cy5) 20mer ssRNA, whose sequence is identical to the 3’ overhang in $R^{A}_{20}$ with increasing NS5B ssRNA concentrations.

Role of Dye Functionalization and Base Sequence on the FRET Fluctuations—Single molecule FRET experiments were conducted on $R^{S}_{20}$ (cytidylate terminated overhang with dye tethered to base 20 via a succinimide linker), $R^{DS}_{20}$ (cytidylate terminated overhang with internally labeled deoxycytidine (base 19)), and $R^{A}_{22}$ (adenylate terminated overhang) with 10 nm NS5B. These results were compared with those obtained with $R^{A}_{20}$.

Methodology of Enzymatic Activity Assays—All enzymatic reactions were performed in a buffer containing 40 mm HEPES pH 8.0, 1 mm DTT, 15 mm NaCl, and 0.5 mm EDTA. The concentration of NS5B was 1 µM while the concentration of the RNA template was 500 µM. Nucleotides were present at 100 µM with the exception of ATP, which was present at 100 nM to allow for incorporation of the radiolabeled nucleotide [α-32P]ATP, of which 1 µCi was added. Reactions were initiated with the addition of 1.25 mM MnCl$_2$ and MgCl$_2$ and were incubated at room temperature for 45 min. Reactions were stopped with the addition of 2× volume of formamide. Samples were heat-denatured for 5 min at 95 °C and resolved on a 20% polyacrylamide-7 M urea gel. Visualization of product bands was performed using a phosphorimag (Bio-Rad Molecular Imager FX). For studies involving the inhibitor, concentrations up to 25 µM were tested.

RESULTS

Single Molecule Fluorescence Assays—To study the interaction dynamics of HCV NS5B with its ssRNA template, we designed a FRET assay that relies on nucleic acid substrates containing both acceptor and donor fluorophores. This approach helps to bypass technical difficulties in obtaining homogeneously labeled enzyme at sufficient yields.

The nucleic acid is composed of an 18 base pair DNA:RNA hybrid duplex and a ssRNA overhang that provides the binding site for NS5B (Fig. 2a). We devised RNA templates of varying sequence, length (Fig. 2b), and structure (Fig. 2c), that were labeled with a donor Cy3 fluorophore at the 3’-end unless otherwise stated. Dye functionalization strategies were surveyed to ensure that little or no perturbation to the donor fluorophore resulted from labeling position/chemistry utilized. The various different functionalizations did not introduce any differences in our single molecule results (see Fig. 3 and also text below). An acceptor Cy5 fluorophore was attached at the 3’-end of the complementary DNA strand, at the junction of the DNA:RNA hybrid and the ssRNA template overhang. The DNA strand also bears a biotin at the 3’-end, enabling immobilization of the hybrid duplex onto a polyethylene glycol-coated glass coverslip via biotin-streptavidin interactions (Fig. 2d). Assembled flow chambers allowed delivery of NS5B enzyme to its immobilized substrate.
With our design, we intended to monitor potential FRET fluctuations between the Cy3 dye and the Cy5 dye upon binding of purified HCV NS5B. We additionally expected to follow dynamics (sliding and/or wrapping) of the enzyme along the RNA template by monitoring once again FRET fluctuations over time for binary-RNA complexes.

Single DNA:RNA duplexes were visualized on a CCD camera following excitation of the Cy3 tag with a 532 nm evanescent field generated in a total internal reflection fluorescence (TIRF) microscope setup (34). Fluorescence intensity time trajectories of single DNA:RNA duplexes were acquired for periods of up to 300 s, or until acceptor photobleaching occurred. FRET time trajectories and associated single molecule FRET distributions were calculated for all molecules under a given set of conditions. The collection of individual FRET time trajectories was used next to construct ensemble FRET distributions.

**NS5B Binding to RNA Overhangs of Variable Structure and Length**—We initially monitored the effects of the addition of NS5B to 14, 20, and 26 base-long ssRNA templates that are represented by the 3′ RNA overhang region of the nucleic acid. In the absence of enzyme, we observed decreasing average FRET values for RAn sequences with increasing overhang length n. Such a result is expected, given the increasing number of bases separating the donor and acceptor dyes, and, in turn, the increasing conformational flexibility (37). Experiments recorded with 10 nM NS5B showed a marked increase of the average FRET from 0.40 to 0.50 for R^26n and from 0.50 to 0.75 for R^20n while only a broadening of the FRET distribution was recorded for R^14n with increasing concentrations of NS5B (FRET ~ 0.70) (Fig. 4, a–c). The increase in FRET efficiency shows that binding of NS5B to the RNA overhang reduces the distance between donor and acceptor (see also “Discussion”).

Ensemble fluorescence experiments performed with either the DNA:RNA duplex bearing the ssRNA overhang (R^20n) or with a 20mer ssRNA of identical sequence to the overhang section in R^20n showed similar FRET changes (Fig. 5, a and b and text therein). We therefore rule out that the 18 base pair DNA:RNA duplex segment contributes to NS5B binding. These observations are consistent with the reported narrow binding tunnel associated with the closed conformation of NS5B, which is unable to accommodate a double stranded segment (7, 38, 39).

To confirm the assignment of an increase in FRET to enzyme binding events we performed a single molecule experiment where we started imaging single R^20n strands immediately before enzyme addition and continued monitoring through the arrival of HCV NS5B. As can be observed in Fig. 6a, the FRET value increased, within one data point, from a value of 0.50 before to ca. 0.70 after the arrival of HCV NS5B. This is also reflected in the two peaks recorded for the single molecule FRET distribution and displayed in Fig. 6b. The lower FRET peak resulting from the period before protein arrival, the higher FRET one arising from the period recorded following protein.
arrival and until photobleaching of the acceptor dye. Similar trajectories were recorded for all the molecules imaged, resulting in the ensemble FRET distribution displayed in Fig. 6c.

To determine whether one or multiple proteins are bound to the RNA template we conducted single molecule experiments with increasing NS5B concentrations up to 1 μM. Fig. 4d displays ensemble FRET distribution results for R^A_{26}. At 5 nM NS5B (Fig. 4d, the histogram in cyan), we observed a bimodal distribution arising from two different populations. Of the 255 trajectories analyzed, 8 molecules (~3% of the population) were observed displaying FRET values of 0.50, which is consistent with the FRET values of free substrates. 232 molecules (~91%) were recorded with FRET values of 0.75, which is indicative for a binary NS5B-RNA complex. We further detected rapid (within two consecutive data points) increases in FRET values extending over tens of seconds. Fig. 7 displays a representative intensity trajectory and associated FRET trajectory, and Fig. 7g displays the associated single molecule FRET distribution histogram.

At 10 nM NS5B concentrations, a single population of molecules was observed with high FRET values stable over time and typically centered at ~0.75 that we assign to the dominant presence of a binary NS5B-RNA complex involving a single protein bound to the 3’ RNA overhang. Under these conditions most molecules display a narrow FRET distribution. The single molecule intensity-time trajectories further display anticorrelated donor and acceptor fluorescence intensity fluctuations of short duration, ~200 ms, our frame acquisition rate, see Fig. 7b for a representative intensity trajectory and associated FRET trajectory. Fig. 7g displays the associated single molecule FRET distribution histogram.

At 100 nM or higher NS5B concentrations the recorded FRET distributions became increasingly broad (Fig. 7g). Molecules showed much ampler, longer lasting anticorrelated donor-acceptor intensity fluctuations than those at low NS5B concentrations (~10 nM) and associated large FRET fluctuations extending over tens of seconds. Fig. 7c displays a representative fluorescence and FRET intensity trajectory, and Fig. 7g displays the associated single molecule FRET distribution histogram. Notably, in the case of 50 nM or higher NS5B concentration, a transition may be observed from the regime characterized by a stable FRET of ~0.75 and involving a single protein bound to the 3’ RNA overhang, to a highly fluctuating regime with appreciable anticorrelated donor and acceptor fluctuations extending over seconds. We assign this transition to the binding of a second NS5B protein, and the ensuing highly fluctuating FRET trajectories to the presence of a ternary complex involving two proteins bound to the 3’ RNA overhang. Upon flushing the chamber with protein free solution the distribution reverts within a few minutes to the narrow distribution centered at 0.75 characteristic of the binary complex, and ultimately to the distribution centered at 0.50, acquired with no protein.

Ensemble FRET distributions recorded for R^A_{26} and increasing concentrations of NS5B yielded similar results as obtained with R^A_{20}, yet increasing the length of the ssRNA template gave
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rise to more complex FRET behavior over time (Fig. 8). Starting with a single state and narrow FRET distribution recorded with no protein (Fig. 7d), two states were observed for RA26 at low NS5B concentration \( \approx 10 \text{ nM} \) arising from 2 different non-interconverting populations. A single state was observed with 10 nM NS5B, characterized by fast donor-acceptor anticorrelated fluorescence intensity fluctuations (Fig. 7e). We assign this population to the binary protein-RNA complex formed by a single protein bound to the 3’ RNA overhang. Prominent FRET fluctuations were obtained with 100 nM or higher NS5B as was also observed with RA20 (Fig. 7f). Given the NS5B footprint of \( \approx 10 \) nucleotides, it is reasonable to assume that RA26 can accommodate at least two enzyme molecules. The motion of a second bound protein along the template arguably contributes to the slow FRET fluctuations.

From the analysis of the FRET fluctuations recorded with NS5B and the RA20 and RA26 constructs we conclude that both a specific and tight complex at low protein concentration and a highly dynamic complex at 100 nM or higher protein concentrations are observed. Following binding of one protein, very rapid (200 ms or faster) dynamic processes occur at the 3’ RNA overhang of either RA20 or RA26 (Fig. 7). Brownian motion of the

FIGURE 6. FRET trajectory recording the arrival of HCV NS5B and its binding. a, FRET time trajectory for a tethered DNA:RNA duplexes (RA20). Recording started under a constant flow (10 \( \mu \text{L/minute} \)) of oxygen scavenger buffer before enzyme addition, and continued through the arrival of a 10 nM HCV NS5B solution in oxygen scavenger buffer flowed in at the same flow rate. b, corresponding single molecule FRET distribution histogram for the same molecule. c, ensemble single molecule FRET distribution histogram for all the molecules imaged following the arrival of HCV NS5B. For all molecules the initial FRET was 0.50 and increased to 0.70 after the arrival of the enzyme.

FIGURE 7. Representative single molecule fluorescence intensity time trajectories and associated FRET distributions upon NS5B binding to 3’ RNA overhangs. Fluorescence intensity time trajectories for Cy5 (red) and Cy3 (green) tethered to DNA:RNA duplexes with 3’ RNA overhangs 20 (RA20) and 26 (RA26) bases long, respectively. Trajectories were acquired with 0, 10, and 100 nM NS5B (panels a, b, and c, respectively for RA20, and panels d, e, and f, respectively, for RA26). Also shown to the right is a cartoon illustrating binding of one or multiple NS5B proteins. Data points where the Cy5 intensity is zero (calculated FRET = 0) are due to reversible photobleaching of the Cy5 dye. Panels g and h display the corresponding single molecule FRET distribution histograms acquired with RA20 and RA26, respectively.
free 3’ RNA overhang occurs within microseconds and is averaged out at our data acquisition rate; thus, no FRET fluctuations are observed in the absence of protein (Fig. 7, a and d) (37). We interpret the FRET fluctuations recorded in the presence of 10 nM NS5B as arising from sliding/wrapping of the protein along the RNA template. In turn, we assign the slow fluctuations recorded at high protein concentration to the motion of a second bound protein along the template.

To shed further light on the nature of the dynamic process taking place at the 3’-end RNA overhang, we next designed a 52 base overhang cytidylate terminated sequence with an internal Cy3 tethered to the phosphate backbone between bases 25 and 26 (RA<sub>52</sub>). This sequence provides for twice the RNA template footprint compared with RA<sub>26</sub>, yet the acceptor and donor dyes in both sequences are equally spaced. Similar FRET distributions and FRET trajectories were recorded for RA<sub>52</sub> and RA<sub>26</sub> in the presence of NS5B (see Fig. 9). We conclude that formation of highly dynamic binary complexes are not limited to positioning of the protein at or close to the 3’-end of the RNA template but are also observed when it binds along the RNA template. The observed FRET fluctuations in the presence of NS5B are thus related to the length of the ssRNA separating the donor and acceptor dyes, but not to the position of the Cy3 dye on the 3’ RNA overhang (middle versus terminal).

No differences in single molecule FRET distributions were found when different dye functionalization strategies were employed on the 20 base long 3’ RNA overhang (see Fig. 3 for the functionalization and Fig. 10 for the single molecule results). Enzymatic assays conducted on DNA:RNA duplexes bearing a 3’ RNA overhang lacking secondary structure showed full replication product in the presence of nucleotide triphosphates and Mg<sup>2+</sup> for the various different labeling schemes employed (Fig. 11). Hence, the different dye labeling strategies do not interfere with the enzymatic activity of NS5B nor do they influence the recorded fluorescence intensity-time trajectories. We note however that an adenylate terminated RNA overhang showed both reduced NS5B enzymatic activity.
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![Figure 11](image_url)

**FIGURE 11.** Effect of the dye functionalization and base sequence on the activity of WT NS5B. De novo RNA synthesis by NS5B using varying template lengths and fluorophore positioning (see also Fig. 2). Each reaction includes a control in the absence of metal ions. Activity after hybridization to the DNA:RNA duplex used in single molecule experiments was tested using the RA^20 (including RA^31) and RA^31 templates (lanes 4 and 8). Lanes 2, 4, 10, 12, 14, and 16 show full-length products in addition to shorter products representing pausing sites. Lanes 6 and 8 show a truncated product.

(Fig. 11, lane 14) and lower binding affinity reflected in the SMS ensemble histogram (Fig. 10d). The above observations are in line with the requirement of one cytidylate at the 3’-end of the template strand for initiation of enzyme activity.

To assess whether the proposed sliding of NS5B can extend past secondary structures we also monitored FRET changes upon NS5B addition to a DNA:RNA duplex bearing a 3’-RNA overhang segment which is able to form a 6-base stem hairpin (RA^20). The hairpin was designed to bring an internally located Cy3 donor dye in close proximity to the acceptor Cy5 dye at the 5’ end of the complementary DNA strand (Fig. 2c), giving rise to FRET values of ca. 1 for the DNA:RNA duplex. Addition of NS5B did not result in a change in FRET, indicating that the protein is unable to unwind the RNA hairpin structure (Fig. 5c). Enzymatic activity studies further show a truncated replication product consistent with the protein inability to unwind and fully replicate the RNA overhang in this construct (Fig. 11, lanes 6 and 8).

**NS5B Mutations along the RNA Binding Channel Lead to Reduced Binding and Increased Complex Dissociation**—We next employed our single molecule FRET assay to study the effects of mutations at specific amino acid residues along the binding channel of NS5B. Substitutions of basic amino acids that are implicated in RNA binding (10, 11) with glutamic acid resulted in reductions or loss of enzymatic activity (40). Here we focused on residues that constitute the template entrance (Lys-98, Lys-100), and the center of the RNA binding channel (Arg-394). We acquired single molecule intensity-time trajectories, calculated FRET time trajectories and FRET distributions for the DNA:RA^20 duplex with increasing concentrations of the various mutant NS5B enzymes: K98E, K100E, K98E-K100E, and R394E (Fig. 12 and Fig. 13).

Similar to our observations with WT NS5B, the ensemble FRET distributions recorded with increasing concentrations of mutant enzymes showed a narrow peak appearing at FRET = 0.75. A significant increase in the equilibrium dissociation constant ($K_D$) for the mutant enzymes compared with that of WT NS5B is appreciated from the amount of protein required to achieve the binary NS5B-RNA complex with characteristic FRET of 0.75. Whereas binding of a single enzyme (FRET = 0.75) is complete at 10 nM WT NS5B, concentrations of ~10 nM of K98E, 10 nM of K100E, 10 nM of K98E-K100E and 500 nM of R394E only resulted in ~50% binding of one enzyme to DNA: RA^20 duplex. Under the above conditions a bimodal distribution with peaks at FRET = 0.75 (one protein) and FRET = 0.50 (no protein) was recorded. The lower affinity of the various mutants correlates with their lower RdRp activity in comparison to WT NS5B (40). The mutations we studied reduced the stability of the binary NS5B-RNA complex, which in turn compromises the polymerization reaction.

We inspected the individual FRET-time trajectories in order to detect the association or dissociation of enzymes over time, easily recognized by the rapid (within one data point) FRET increase from 0.50 to 0.75, or equivalent decrease, respectively (see also Fig. 6). Representative trajectories are shown in Fig. 13. Upon analysis of the trajectories, pie-charts were also constructed illustrating the percentile of single DNA:RA^20 duplexes that remain either free of enzyme or bound to enzyme during the course of the imaging experiment, as well as the percentile of duplexes undergoing association/dissociation. We also explored the correlation between the average FRET value (<FRET>) and the FRET standard deviation ($\sigma_{\text{FRET}}$) characterizing the FRET trajectories of individual molecules (Fig. 13, right panel). Here, small values of $\sigma_{\text{FRET}}$ are associated with static samples, where no association/dissociation or sliding/ wrapping and concomitant FRET fluctuations are observed in individual RNA templates (see for example the correlation for DNA:RA^20 duplexes acquired with no NS5B in Fig. 13a). Large values of $\sigma_{\text{FRET}}$ correspond to FRET fluctuations arising from e.g. binding/dissociation and/or sliding/wrapping over time (Fig. 13, b–f).

DNA:RA^20 duplexes imaged with 10 nM K100E in solution had either one or no mutant enzyme bound, displaying <FRET> of ~0.75 and ~0.50, respectively (Fig. 13b, right...
Only a small fraction of the duplexes, ca. 4%, underwent enzyme association or dissociation while imaging. Two well-defined species that do not interconvert over time are dominant. The $\sigma_{\text{FRET}}$ values characteristic to each species are small, reflecting mostly shot noise contribution to the trajectory but no dynamics (association-dissociation) related to the FRET fluctuations (Fig. 13b). For comparison, all the trajectories recorded with 10 nM WT NS5B had high FRET values characteristic of the binary enzyme-RNA complex (Fig. 13f). They were also characterized by larger values of $\sigma_{\text{FRET}}$, consistent with a more dynamic protein-RNA complex. The fact that for K100E neither enzyme association nor dissociation events were observed from trajectories acquired over up to 300 s would indicate that both the dissociation rate constant ($k_{\text{off}}$) and the apparent association rate constant ($k_{\text{on,app}}$) are smaller than $3 \times 10^{-3}$ s$^{-1}$ for this enzyme. Considering that the concentration of K100E used in our experiments is 10 nM, we may further state that the bimolecular association rate constant $k_{\text{on}} = k_{\text{on,app}}/[\text{K100E}]$ is significantly smaller than the diffusion controlled rate constant. Increasing the enzyme concentration present in our imaging studies resulted in a larger number of binary K100E-RNA complexes; however, we were not able to detect an increasing number of enzyme binding events.

Experiments conducted with 10 nM K98E-K100E gave similar results to those obtained with K100E. Two species are present and with exception of a few molecules, they do not interconvert over time are dominant. The $\sigma_{\text{FRET}}$ values characteristic to each species are small, reflecting mostly shot noise contribution to the trajectory but no dynamics (association-dissociation) related to the FRET fluctuations (Fig. 13b). For comparison, all the trajectories recorded with 10 nM WT NS5B had high FRET values characteristic of the binary enzyme-RNA complex (Fig. 13f). They were also characterized by larger values of $\sigma_{\text{FRET}}$, consistent with a more dynamic binary complex, similar to results obtained for WT NS5B. Molecules with large $\sigma_{\text{FRET}}$ values and
values intermediate between 0.50 and 0.75 are thus observed (Fig. 13).

**NS5B Benzimidazole-based Inhibitor: Decreased Binding Correlates with Reduced Activity**—Benzimidazole-based NNIs, referred to as thumb site 1 inhibitors, bind to a pocket between the thumb subdomain and the N-terminal finger loop (Fig. 1). Upon binding, the finger loop is likely to be displaced and adopts a disengaged flexible conformation (41). These inhibitors block RNA synthesis specifically at the level of initiation; presumably by affecting formation of a competent binary NS5B-RNA complex. Here, we used a prototype inhibitor, i.e. compound C (11, 41), to study whether the mechanism of action involves changes in the dynamic interaction between NS5B and its ssRNA template.

We conducted single molecule FRET studies on DNA:R^20 duplexes with increasing concentrations of compound C and 100 nM WT NS5B (Fig. 14). An analysis of the FRET trajectories showed two well defined non-interconverting populations with characteristic FRET values of ~0.50 (no NS5B bound) and ~0.75 (enzyme-bound population). The associated $\sigma_{\text{FRET}}$ versus $\langle \text{FRET} \rangle$ and pie chart plots for trajectories acquired with 100 nM NS5B and 3 $\mu$M compound C are shown in Fig. 14, c and f, respectively. Within the monitoring time window (up to 300s), protein association or dissociation events are rarely observed. Ensemble FRET distributions acquired under increasing concentrations of the inhibitor show a growth of the population with no NS5B bound in detriment of the enzyme-bound population (Fig. 14a). At 10 $\mu$M compound C any enzyme binding is almost completely inhibited and the ensemble FRET distribution, $\sigma_{\text{FRET}}$ versus $\langle \text{FRET} \rangle$ and pie chart plots resemble those obtained with no protein (see Fig. 14, a, d, and g, respectively.

Pro495 is part of thumb site 1 and provides critical contacts to compound C. Substitution of this residue with alanine is associated with resistance to this class of NNIs (11). We thus explored next the interaction dynamics of P495A with RA^20 and compared the results to those acquired with WT NS5B (Fig. 14). Ensemble FRET distributions recorded with 10 $\mu$M compound C and 100 nM P495A suggests that this mutation neutralizes the effects of the inhibitor as the enzyme-bound population characterized by FRET values of ~0.75 is partially recovered (in the absence of compound C we observed no substantial differences between results with P495A and WT NS5B). Importantly, intensity time trajectories reveal that at a concentration of 10 $\mu$M of compound C, the P495A mutant enzymes rapidly associate and dissociate from the ssRNA template. A large fraction of the single molecules underwent rapid FRET increase from 0.50 to 0.75 and equivalent decrease during the observation period. A representative trajectory is displayed in Fig. 14i. The rapid dynamics are also underscored by the large dispersion in the plot of $\sigma_{\text{FRET}}$ versus $\langle \text{FRET} \rangle$ (Fig. 14e). The pie chart in Fig. 14h illustrates that ~36% of recorded

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**FIGURE 13.** Mutations on NS5B and associated FRET fluctuations. FRET time trajectories of DNA:R^20 duplex with (a) no protein, (b) 10 nM K100E, (c) 10 nM K98E-K100E, (d) 10 nM R394E, (e) 10 nM K98E, and (f) 10 nM WT NS5B. Also shown to the right are the plots of standard deviations of FRET distribution ($\sigma_{\text{FRET}}$) versus average FRET ($\langle \text{FRET} \rangle$) for individual molecules (here the $\sigma_{\text{FRET}}$ was calculated upon removal of reversible photobleaching events from the trajectories), and a pie chart illustrating the percentile of molecules showing low FRET (~0.50, enzyme free, green sector), high FRET (~0.75, bound to enzyme, blue sector), or enzyme association/dissociation events, as reflected by FRET transitions from low to high/high to low, respectively (orange sector).
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FIGURE 14. Effect of a benzimidazole-based inhibitor. Series of FRET distributions for DNA-RNA duplex obtained with increasing concentration of compound C and (a) 100 nM WT NS5B and (b) 100 nM P495A mutant. Panels c, d, and e display the standard deviations of FRET distribution (\(\langle FRET \rangle\)) for individual molecules of R\(^{20}\) with 3 µM compound C and 100 nM NS5B, 10 µM compound C and 100 nM NS5B, and 10 µM compound C and 100 nM P495A mutant, respectively. Panels f, g, and h, display the corresponding pie charts illustrating the percentile of molecules showing low FRET (\(\leq 0.50\), enzyme free, green sector), high FRET (\(>0.75\), bound to enzyme, blue sector), or enzyme association/dissociation events, as reflected by FRET transitions from low to high/high to low, respectively (orange sector). Panel i displays a representative FRET trajectory acquired with R\(^{20}\) 10 µM compound C and 100 nM P495A. The arrows illustrate the association of proteins over time.

DISCUSSION

Previous structural and biochemical data have suggested that the HCV NS5B enzyme maximizes contacts with a bound ssRNA template to form the most stable complexes. Protein footprinting experiments with short model RNA templates revealed interactions across the entire binding channel including template entrance and dsRNA exit (40). Moreover, the crystal structure of a binary complex with a short 5mer RNA shows that the 3' terminus of the template is not precisely positioned at the active site to allow de novo initiation of RNA synthesis (15). In this work, we developed single molecule FRET assays to study the dynamic behavior of binary HCV NS5B-RNA complexes in real time. Key to our assay is the ability to work with unlabeled enzymes. This enables us to rapidly monitor and analyze constructs with substitution at various different residues, under various conditions, including the presence of NNIs.

We show that binding of NS5B to model RNA templates that are labeled with donor and acceptor fluorophores gives rise to an overall FRET increase, where FRET fluctuations extending from hundreds of milliseconds to seconds characterize the newly formed enzyme-RNA complex. We note that not all binding events monitored may reflect binding of NS5B in conformation that supports polymerase activity. Our results may not distinguish an active from an inactive enzyme.

We consider three scenarios that may help explain the observed increase in FRET upon enzyme binding. Firstly, binding of NS5B can cause a compression of the nucleic acid. Secondly, the geometry of the nucleic acid binding channel with its 90° bend will shorten the distance between donor and acceptor provided that the RNA occupies the entire channel from entry to exit. These related interpretations are consistent with our findings that such shift of the average FRET signal is reduced if the length of the template is significantly smaller or larger than the optimal 20 mer. A plausible third explanation is that the binding of HCV NS5B induces an enhancement of Cy3 emission intensity via protein induced fluorescence enhancement (PIFE), also a distance dependent phenomenon (42–44). Emission enhancement of Cy3 may give rise in turn to enhanced FRET when Cy5 is in close proximity and may thus account for our observations. We have seen that while Cy5 emission is not affected by binding of HCV NS5B, Cy3 does indeed undergo an increase in emission intensity consistent with PIPE. Regardless, the precise position of the NS5B enzyme would for the above reasons also affect the FRET between donor and acceptor. Thus, the fluctuations of the FRET signal at the single molecule level suggest that the polymerase can slide along its RNA substrate.

An alternative explanation to sliding is that fluctuations partly arise from wrapping and releasing an otherwise dangling end of RNA. Wrapping dynamics are expected to be extremely fast, and would be averaged out in our 100 ms data point acquisition rate.

We rarely observed enzyme dissociation during a period of up to 300 s, which confirms that binary complexes are sufficiently stable. This is in stark contrast with other polymerase enzymes such as HIV-RT, where dissociation events occur within a few seconds under similar conditions (42).

Substitution at basic amino acid residues, that constitute the putative template entry (Lys-98, Lys-100), and the center of the RNA binding channel (Arg-394), by glutamic acid resulted in severe reductions in substrate affinity and binary complex dynamics, as well as loss of enzymatic activity. Single molecule trajectories with NS5B R394E show sharp increases in dissociation events, which are not seen with mutant enzymes K98E, K100E, K98E-K100E, or WT NS5B. This is somewhat surprising given that the structure of NS5B in complex with a dsRNA substrate shows the side chain of Arg-394 in contact with the primer strand, while our experiments were conducted with ssRNA templates. The data therefore suggest that Arg-394 plays a dual role in RNA binding. Residues Arg-394 and Arg-386 are located on the primer grip helix of the thumb subdomain that undergoes structural rearrangements when comparing the closed conformation with the open structure that accommodates dsRNA (18). In the open structure, Arg-394 is in...
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contact with the phosphate backbone at primer positions 0 and −1. By contrast, our data provide strong evidence to show that Arg-394 can also interact with ssRNA. Under these circumstances, the distance between the primer grip helix and the bound ssRNA template must be shorter. Hence, the enzyme could adopt an intermediate structure between the open conformation seen with dsRNA and the closed conformation that is described for the apo enzyme.

It has previously been suggested that finger-loop inhibitors form either unproductive complexes or interfere with the conformational changes required to adopt a more open conformation (11–14). Our data show that a prototype compound of this class reduces the shift in average FRET values (reduced binding) and FRET fluctuations (reduced sliding-wrapping dynamics) in a dose-dependent manner. These findings support the notion that the enzyme is locked in a closed conformation that also diminishes binding of ssRNA. In our proposed model, increases in FRET fluctuations can be ascribed to enzyme sliding relative to the bound ssRNA. This could be accomplished via rapid changes between open and closed conformations that are essential for the sliding movement of HCV NS5B. The transition to an open conformation is tantamount to loosening the grip on the bound nucleic acid, which increases the mobility of the bound nucleic acid. By contrast, the transition from an open to a closed structure tightens the grip and prevents dissociation of the binary complex. A periodic repetition of these events can ultimately lead to enzyme sliding that is driven by Brownian motion. This type of motion could also be relevant for the precise positioning of the 3′-end of the template at the polymerase active site during the process of de novo RNA synthesis initiation.

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REFERENCES

1. Shepard, C. W., Finelli, L., and Alter M. J. (2005) Global epidemiology of hepatitis C virus infection. Lancet Infectious Diseases 5, 558–567
2. Sy, T., and Jamal, M. M. (2006) Epidemiology of hepatitis C virus (HCV) infection. Int. J. Med. Sci. 3, 41–46
3. Reed, K. E., and Rice, C. M. (2000) Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. 242, 55–84
4. Inchauspe, G., Zebedee, S., Lee, D. H., Sugitani, M., Nasoff, M., and Prince, A. M. (1991) Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. Proc. Natl. Acad. Sci. U.S.A. 88, 10292–10296
5. Behrens, S. E., Tomei, L., and De Francesco, R. (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. 15, 12–22
6. Ferrari, E., Wright-Minogue, J., Fang, J. W. S., Baroudy, B. M., Lau, J. Y. N., and Hong, Z. (1999) Characterization of Soluble Hepatitis C Virus RNA-Dependent RNA Polymerase Expressed in Escherichia coli. J. Virol. 73, 1649–1654
7. Lohmann, V., Körner, F., Herian, U., and Bartenschlager, R. (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J. Virol. 71, 8416–8428
8. Yamashita, T., Kaneko, S., Shirota, Y., Qin, W., Nomura, T., Kobayashi, K., and Murakami, S. (1998) RNA-dependent RNA Polymerase Activity of the Soluble Recombinant Hepatitis C Virus NS5B Protein Truncated at the C-terminal Region. J. Biol. Chem. 273, 15479–15486
9. Zhong, W., Gutshall, L. L., and Del Vecchio, A. M. (1998) Identification and Characterization of an RNA-Dependent RNA Polymerase Activity within the Nonstructural Protein 5B Region of Bovine Viral Diarrhea Virus. J. Virol. 72, 9365–9369
10. Lesburg, C. A., Cable, M. B., Ferrari, E., Hong, Z., Mannarino, A. F., and Weber, P. C. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6, 937–943
11. Kukolj, G., McGibbon, G. A., McKercher, G., Marquis, M., LeFèbvre, S., Thauvette, L., Gauthier, J., Goulet, S., Poupant, M.-A., and Beaulieu, P. L. (2005) Binding Site Characterization and Resistance to a Class of Non-nucleoside Inhibitors of the Hepatitis C Virus NS5B Polymerase. J. Biol. Chem. 280, 39260–39267
12. Tomei, L., Altamura, S., Bartholomew, L., Biroccio, A., Ceccacci, A., Pacini, L., Narjes, F., Gennari, N., Biscocchi, M., Incitti, L., Orsatti, L., Harper, S., Stansfeld, I., Rowley, M., De Francesco, R., and Migliaccio, G. (2003) Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 77, 13225–13231.
13. Liu, Y., Jiang, W. W., Pratt, J., Rockway, T., Harris, K., Vasavanonda, S., Tripathi, R., Pithawalla, R., and Kati, W. M. (2006) Mechanistic Study of HCV Polymerase Inhibitors at Individual Steps of the Polymerization Reaction. Biochemistry 45, 11312–11323
14. Di Marco, S., Volpari, C., Tomei, L., Altamura, S., Harper, S., Narjes, F., Koch, U., Rowley, M., De Francesco, R., Migliaccio, G., and Carfi, A. (2005) Interdomain Communication in Hepatitis C Virus Polymerase Abolished by Small Molecule Inhibitors Bound to a Novel Allosteric Site. J. Biol. Chem. 280, 29765–29770
15. O’Farrell, D., Trowbridge, R., Rowlands, D., and Jäger, J. (2003) Substrate Complexes of Hepatitis C Virus RNA Polymerase (HC-14): Structural Evidence for Nucleotide Import and De-novo Initiation. J. Mol. Biol. 326, 1025–1035
16. Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., and Miyano, M. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure 7, 1417–1426
17. Bressanelli, S., Tomei, L., Roussel, A., Incitti, L., Vitale, R. L., Matieu, M., De Francesco, R., and Rey, F. A. (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. 96, 13034–13039
18. Mosley, R. T., Edwards, T. E., Murakami, E., Lam, A. M., Grice, R. L., Du, J., Sofia, M. J., Furman, P. A., and Otto, M. I. (2012) Structure of Hepatitis C Virus Polymerase in Complex with Primer-Template RNA. J. Virol. 86, 6503–6511
19. Kim, Y.-C., Russell, W. K., Ranjith-Kumar, C. T., Thomson, M., Russell, D. H., and Kao, C. C. (2005) Functional Analysis of RNA Binding by the Hepatitis C Virus RNA-dependent RNA Polymerase. J. Biol. Chem. 280, 38011–38019
20. Kao, C. C., Del Vecchio, A. M., and Zhong, W. (1999) De Novo Initiation of RNA Synthesis by a Recombinant Flaviridae RNA-dependent RNA Polymerase. Virology 253, 1–7
21. Kao, C. C., Singh, P., and Ecker, D. J. (2001) De Novo Initiation of Viral RNA-Dependent RNA Synthesis. Virology 287, 251–260
22. Kim, M.-J., Zhong, W., Hong, Z., and Kao, C. C. (2000) Template Nucleotide Moieties Required for De Novo Initiation of RNA Synthesis by a Recombinant Viral RNA-Dependent RNA Polymerase. J. Virol. 74, 10312–10322
23. Luo, G., Hamatake, R. K., Mathis, D. M., Racela, J., Rigat, K. L., Lemm, J., and Colombo, R. J. (2000) De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J. Virol. 74, 851–863
24. Ranjith-Kumar, C. T., Gutshall, L., Kim, M. J., Sarisky, R. T., and Kao, C. C. (2002) Requirements for de novo initiation of RNA synthesis by recombinant flaviviral RNA-dependent RNA polymerases. J. Virol. 76, 12526–12536
25. Ranjith-Kumar, C. T., Kim, Y. C., Gutshall, L., Silverman, C., Khandekar, S., Sarisky, R. T., and Kao, C. C. (2002) Mechanism of de novo initiation by the hepatitis C virus RNA-dependent RNA polymerase: role of divergent
metals. J. Virol. 76, 12513–12525
26. Sun, X. L., Johnson, R. B., Hockman, M. A., and Wang, Q. M. (2000) De novo RNA synthesis catalyzed by HCV RNA-dependent RNA polymerase. Biochim. Biophys. Res. Commun. 268, 798–803
27. Zhong, W., Uss, A. S., Ferrari, E., Lau, J. Y. N., and Hong, Z. (2000) De Novo Initiation of RNA Synthesis by Hepatitis C Virus Nonstructural Protein 5B Polymerase. J. Virol. 74, 2017–2022
28. Abbondanzieri, E. A., Bokinsky, G., Rausch, J. W., Zhang, J. X., Le Grice, S. F. J., and Zhuang, X. (2008) Dynamic binding orientations direct activity of HIV reverse transcriptase. Nature 453, 184–189
29. Liu, S., Abbondanzieri, E. A., Rausch, J. W., Le Grice, S. F. J., and Zhuang, X. (2008) Dynamic binding orientations direct activity of HIV reverse transcriptase. Nature 453, 184–189
30. Hass, T., Enderle, T., Ogletree, D. F., Chemla, D. S., Selvin, P. R., and Weiss, S. (1996) Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. Proc. Natl. Acad. Sci. U.S.A. 93, 6264–6268
31. Moerner, W. E., and Fromm, D. P. (2003) Methods of single-molecule fluorescence spectroscopy and microscopy. Review Science Instruments 74, 3597–3619
32. Weiss, S. (1999) Fluorescence Spectroscopy of Single Biomolecules. Science 283, 1676–1683
33. Joo, C., Balci, H., Ishitsuka, Y., Buranachai, C., and Ha, T. (2008) Advances in Single-Molecule Fluorescence Methods for Molecular Biology. Annu. Rev. Biochem. 77, 51–76
34. Roy, R., Hohng, S., and Ha, T. (2008) A practical guide to single-molecule FRET. Nature Methods 5, 507–516
35. Ha, T., Kozlov, A. G., and Lohman, T. M. (2012) Single-Molecule Views of Protein Movement on Single-Stranded DNA. Annu. Rev. Biophys. 41, 295–319
36. Deval, J., Powdrill, M. H., D’Abramo, C. M., Cellai, L., and Götte, M. (2007) Pyrophosphorolytic Excision of Nonobligate Chain Terminators by Hepatitis C Virus NS5B Polymerase. Antimicrob. Agents Chemother. 51, 2920–2928
37. Murphy, M. C., Rasnik, I., Cheng, W., Lohman, T. M., and Ha, T. (2004) Probing Single-Stranded DNA Conformational Flexibility Using Fluorescence Spectroscopy. Biophys. J. 86, 2530–2537
38. Ferrari, E., He, Z., Palermo, R. E., and Huang, H.-C. (2008) Hepatitis C Virus NS5B Polymerase Exhibits Distinct Nucleotide Requirements for Initiation and Elongation. J. Biol. Chem. 283, 33893–33901
39. Ranjith-Kumar, C. T., and Kao, C. C. (2006) Recombinant viral RdRps can initiate RNA synthesis from circular templates. RNA 12, 303–312
40. Deval, J., D’Abramo, C. M., Zhao, Z., McCormick, S., Coutsinos, D., Hess, S., Kvaratskhelia, M., and Götte, M. (2007) High Resolution Footprinting of the Hepatitis C Virus Polymerase NS5B in Complex with RNA. J. Biol. Chem. 282, 16907–16916
41. LaPlante, S. R., Gillard, J. R., Jakalian, A., Aubry, N., Coulombe, R., Brochu, C., Tsantrizos, Y. S., Poirier, M., Kukolj, G., and Beaulieu, P. L. (2010) Importance of Ligand Bioactive Conformation in the Discovery of Potent Indole-Diamide Inhibitors of the Hepatitis C Virus NS5B. J. Am. Chem. Soc. 132, 15204–15212
42. Marko, R. A., Liu, H.-W., Ablenas, C. J., Ehteshami, M., Götte, M., and Costa, G. (2013) Binding Kinetics and Affinities of Heterodimeric versus Homodimeric HIV-1 Reverse Transcriptase on DNA–DNA Substrates at the Single-Molecule Level. J. Phys. Chem. B 117, 4560–4567
43. Hwang, H., Kim, H., and Myong, S. (2011) Protein induced fluorescence enhancement as a single molecule assay with short distance sensitivity. Proc. Natl. Acad. Sci. U.S.A. 108, 7414–7418
44. Luo, G., Wang, M., Konigsberg, W. H., and Xie, X. S. (2007) Single-molecule and ensemble fluorescence assays for a functionally important conformational change in T7 DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 104, 12610–12615