

Multiple Full-length NS3 Molecules are Required for Optimal Unwinding of Oligonucleotide DNA in vitro

Alan J. Tackett¹, Yingfeng Chen¹, Craig E. Cameron², and Kevin D. Raney^{1,*}

¹Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205

²Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802

Running title: Optimal DNA Unwinding by NS3 Requires Excess Enzyme

*author to whom correspondence should be addressed: Tel.: (501) 686-5244, Fax: (501) 686-8169, email: raneykevind@uams.edu

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Non-structural protein 3 (NS3) from the hepatitis C virus is a 3'-to-5' helicase classified in helicase superfamily 2. The optimally active form of this helicase remains uncertain. We have used unwinding assays in the presence of a protein trap to investigate the first cycle of unwinding by full-length NS3. When enzyme was in excess of substrate, NS3 (500 nM) unwound > 80% of a DNA substrate containing a 15 nucleotide overhang and a 30 base pair duplex (45:30mer)(1 nM). This result indicated that the active form of NS3 that was bound to the DNA prior to initiation of the reaction was capable of processive DNA unwinding. Unwinding with varying ratios of NS3 to 45:30mer allowed us to investigate the active form of NS3 during the first unwinding cycle. When the substrate concentration slightly exceeded that of the enzyme, little or no unwinding was observed, indicating that if a monomeric form of the protein is active, then it exhibits very low processivity. Binding of NS3 to the 45:30mer was measured by electrophoretic mobility shift assays resulting in a K_D value of 2.7 ± 0.4 nM. Binding to individual regions of the substrate was investigated by measuring the K_D value for a 15mer oligonucleotide as well as a 30mer duplex. NS3 bound tightly to the 15mer ($K_D = 1.3 \pm 0.2$ nM) and, surprisingly, fairly tightly to the double-stranded 30mer ($K_D = 11.3 \pm 1.3$ nM). However, NS3 was not able to

rapidly unwind a blunt-end duplex. Thus, under conditions of optimal unwinding, the 45:30mer is initially saturated with enzyme, including the duplex region. The unwinding data are discussed in terms of a model whereby multiple molecules of NS3 bound to the ssDNA portion of the substrate are required for optimal unwinding.

Helicases are enzymes that provide single-stranded (ss) nucleic acid intermediates for processes such as replication, recombination, and repair of nucleic acids (1-4). These enzymes are proposed to use the energy gained from ATP hydrolysis to separate double-stranded (ds) nucleic acid. The unwinding mechanism of a helicase may reflect the oligomeric state of the enzyme. Helicases such as and *E. Coli* DnaB (5), bacteriophage T4 gp41 (6), bacteriophage T7 gp4 (7), have been shown to exist as hexamers. These hexameric helicases are proposed to operate through a DNA exclusion mechanism in which the hexamer encircles one strand of a duplex and excludes the complementary strand during translocation along the DNA (3,8-10). It has also been determined that some hexameric helicases such as DnaB may drive branch migration by acting as a molecular pump (11,12). Most helicases are believed to translocate with a directional bias along ssDNA. Dda helicase from bacteriophage T4 was proposed to use force to remove proteins bound to DNA based upon its ability to displace streptavidin from biotin-labeled oligonucleotides in a directionally biased manner (10,13). The ability for Dda to displace proteins

from DNA increases when multiple molecules of Dda are involved in the reaction (14).

The active form of many helicases still remains unresolved. Dda was shown to be capable of functioning as a monomer during unwinding of dsDNA (15). PcrA helicase from *Bacillus stearothermophilus* has been proposed to function as a monomer based on x-ray crystallographic studies (16-18). Biochemical studies of the translocation activity of PcrA on ssDNA also support PcrA's function as a monomer (19). Dda and PcrA are proposed to utilize an "inchworm-like" mechanism in which a monomeric helicase has two DNA binding sites that allow a cycle of unwinding and translocation along a nucleic acid lattice. Biochemical and biophysical evidence has been presented supporting a dimeric form of the UvrD helicase (20,21).

Non-structural protein 3 (NS3) from the hepatitis C virus contains 3'-to-5' helicase activity in the C-terminal domain as well as serine protease activity in the N-terminal domain (22). In work with the helicase domain of NS3 (NS3h), Preugschat *et al.* (23) mixed NS3h mutants lacking strand-separating activity in steady-state unwinding assays with wild type NS3h and observed no dominant negative phenotype that would be expected if NS3h requires oligomerization to function. This negative result was proposed to illustrate a monomeric form of the helicase domain of NS3 and supported an inchworm-like mechanism as suggested from the x-ray crystal structure of NS3h bound to a dU₈ oligonucleotide (24). In contrast, Levin and Patel (25) reported that the helicase domain of NS3 is active as an oligomer. Oligomerization of NS3h was indicated by protein-protein cross-linking and by unwinding assays in which an ATPase deficient mutant form of NS3h was mixed with wt NS3h under conditions of excess enzyme, which revealed that 13.5 molecules of NS3h were required for unwinding a 33 bp substrate. Levin and Patel (25) proposed a model of unwinding that utilizes a cycle of transient protein-protein interactions that result in exchange of NS3h molecules through dissociation during translocation and unwinding. Most recently, the Patel lab has proposed a model for unwinding by NS3h that does not require protein-protein interactions but

does rely on functional cooperativity between helicase monomers (26).

We have investigated the active form of full-length NS3 during the first unwinding cycle with a series of unwinding experiments in the presence of a protein trap and varying ratios of NS3 to DNA substrate. A protein trap allowed the investigation of the actively bound NS3 during the first unwinding cycle by removing NS3 that is not initially bound to the DNA substrate and sequestering NS3 that dissociates from the DNA substrate. Optimal unwinding was observed only under conditions that favor binding of multiple NS3 molecules to the single stranded region of the substrate. The results are qualitatively similar to those reported by the Patel lab for the NS3 helicase domain (26).

EXPERIMENTAL PROCEDURES

Materials - Na₄EDTA, SDS, BSA, BME, MOPS, NaCl, acrylamide, bis-acrylamide, MgCl₂, KOH, ATP, formamide, xylene cyanole, bromophenol blue, Hepes, urea, and glycerol were from Fisher. [γ -³²P]ATP was purchased from PerkinElmer Life Sciences. Poly(U) was purchased from Amersham Pharmacia Biotech. DNA oligonucleotides were from Integrated DNA Technologies. Sephadex G-25 was from Sigma. T4 polynucleotide kinase was obtained from New England Biolabs. Recombinant full-length NS3 was expressed and purified as previously described (27).

Unwinding substrate - The primary substrate used to monitor NS3 unwinding was a 45:30mer partial duplex that contained a T₁₅ ss overhang (45:30mer):

3'-
TTTTTTTTTTTTTTTCATCATGCAGGACAGTC
GGATCGCAGTCAG-5'

5'-

GTAGTACGTCCTGTCAGCCTAGCGTCAGTC-
3'

A 60:30mer was used in some experiments that was identical to the 45:30mer except that the 3'-overhang contained 15 additional thymidine residues. Each DNA oligonucleotide was purified by preparative gel electrophoresis (13). Crude oligonucleotide was suspended in formamide and resolved on a 20% polyacrylamide / 7 M urea vertical slab gel (Hofer Scientific Instruments). Oligonucleotide was visualized by UV shadowing and excised from the gel. The purified oligonucleotide was electroeluted from the gel slice using a Schleicher & Schuell

ELUTRAP apparatus. Purified oligonucleotides were then desalted on a C₁₈ Sep-Pak column (Waters) and lyophilized by SpeedVac (Savant). The lyophilized oligonucleotides were suspended in 10 mM Hepes (pH 7.5) + 1 mM EDTA. Oligonucleotide concentration was determined by measuring absorbance at 260 nm in 0.2 M KOH and applying calculated extinction coefficients (28). To create the partial duplex unwinding substrate, a 1:2 ratio of T45mer to 30mer was heated at 95 °C for 10 min and allowed to cool to room temperature. The mixture of 45:30mer partial duplex was resolved on a native 20% polyacrylamide gel, electroeluted, desalted, and quantified as described above.

Purified 45mer was 5' radiolabeled with [γ -³²P]ATP by T4 polynucleotide kinase. The labeling reaction proceeded for 1 hr at 37 °C, followed by heat denaturation of the enzyme at 70 °C for 10 min. One equivalent of unlabeled 30mer was added to the 5' radiolabeled 45mer. The excess [γ -³²P]ATP was removed by passage through two, 1 mL sephadex G-25 spin columns. The 5' radiolabeled 45:30mer was heated to 95 °C for 10 min and allowed to cool to room temperature. This stock solution of radiolabeled 45:30mer was used to spike a more concentrated stock solution of gel purified 45:30mer to create the substrate for DNA unwinding experiments.

Unwinding assays - NS3 unwinding experiments in figures 1-4 were performed by using a KinTek rapid chemical quench-flow instrument maintained at 37 °C with a circulating water bath. All concentrations listed are after mixing. NS3 unwinding buffer consisted of 50 mM MOPS-K⁺ (pH 7.0), 50 μ M EDTA, 0.1 mg/mL BSA, and 10 mM NaCl. For excess enzyme experiments, the DNA 45:30mer (1 nM) was incubated with NS3 (500 nM) prior to initiating the unwinding reaction with 5 mM ATP and 10 mM MgCl₂. For experiments performed with a protein trap, 5 - 75 μ M poly(U) (nt) was incubated with ATP and MgCl₂ unless otherwise stated. After rapid mixing of the NS3-45:30mer solution with the ATP-MgCl₂-poly(U) solution, the reaction was quenched with 200 mM EDTA + 0.7 % SDS. To prevent re-annealing of the ssDNA products, a DNA trapping strand was added to the vial in which the quenched solution

was collected. The DNA trapping strand was a 30mer that was complementary to the 30mer in the 45:30mer substrate and was added in 30-fold excess of substrate. A 25 μ L aliquot of the quenched reaction mixture was added to 5 μ L of loading buffer (30 % glycerol, 0.1 % bromophenol blue, 0.1 % xylene cyanole), and electrophoresed on a 20 % polyacrylamide gel. The amount of free 45mer and 45:30mer was quantified by using a Molecular Dynamics Phosphorimager and ImageQuant software (13,27,29,30). Unwinding experiments with 25 nM or 100 nM 45:30mer and NS3 were performed as above except that 3 μ M DNA trapping strand was incubated with the ATP-MgCl₂-poly(U) solution in order to prevent re-formation of the substrate during the reaction. The addition of 30mer DNA trapping strand to the reaction will affect the observed rate for unwinding during multiple cycle conditions in the absence of protein trap by serving a binding site for NS3, but it should not affect the first cycle of unwinding by the initially bound NS3 with or without protein trap (15). Unwinding data were fit to Scheme 1 by the program Scientist (MicroMath) (27). This method of data analysis utilizes differential equations to describe each of the species, and provides the best fit to the data in accordance to the model. Scheme 1 describes the steps involved in product formation which accounts for the "lag" during the rapid phase of strand separation as well as the slow conversion of nonproductively bound NS3 to productive NS3 (31).

Electrophoretic mobility shift assays -

Electrophoretic mobility shift assays (EMSAs) were used to monitor binding between NS3 and various components of the 45:30mer DNA substrate. The EMSA binding buffer consisted of 50 mM MOPS-K⁺ (pH 7.0), 50 μ M EDTA, 0.1 mg/mL BSA, and 10 mM NaCl. Varying concentrations of NS3 were incubated with 0.1 nM of 5' radiolabeled T₁₅, 30:30mer duplex (identical to duplex portion of 45:30mer), or 45:30mer DNA in binding buffer for 5 min at 37 °C. For stoichiometric binding assays, DNA concentration was increased to more than 10-fold above the K_D as determined from the EMSA. 100 nM 45:30mer or T₁₅ was incubated with varying concentrations of NS3 in binding buffer for 5 min at 37 °C. Following the incubation, glycerol was added to 5 %, and the samples were resolved on a 4% polyacrylamide gel at 200 V using a protean II xi

system (BioRad). The amount of DNA bound was determined with a Molecular Dynamics Phosphorimager and ImageQuant software. The amount of DNA bound was quantified from the loss of the band representing the free DNA. The binding data at 0.1 nM DNA was fit to a hyperbola to determine K_D values (KaleidaGraph software).

Fluorescence Polarization with fluorescein labeled DNA - Fluorescence polarization was used to monitor the interaction of NS3 with the DNA substrate. The fluorescence polarization buffer consisted of 50 mM MOPS- K^+ (pH 7.0), 50 μ M EDTA, 0.1 mg/mL BSA, and 10 mM NaCl. A 3'-fluorescein labeled T_{15} (F- T_{15}) and 3'-fluorescein labeled 30mer (F-30mer) were purchased from Integrated DNA Technologies and purified by preparative gel electrophoresis. The F-30mer was identical to the 30mer in the 45:30mer DNA substrate. Duplexes were prepared by mixing F-30mer with a complementary 30mer or T45mer. The blunt-end F-30:30mer and T45:F-30mer were purified by non-denaturing preparative gel electrophoresis as described above. Varying concentrations of NS3 were incubated with 0.1 nM F- T_{15} , F30:30mer, or T45:F-30mer in fluorescence polarization buffer for 3 min at 37 °C in a final volume of 1 mL per sample. Fluorescence polarization was monitored on a Beacon Fluorescence Polarization Instrument (PanVera). Fluorescence polarization buffer was used as the blank. The change in millipolarization (mP) units was determined using a sample of 0.1 nM fluorescein-labeled DNA as the zero point. After reading each sample, 10 mM $MgCl_2$ and 1 mM ATP- γ -S (Sigma) were added to each sample. Following a 3 min incubation at 37 °C, the fluorescence polarization of each sample was again obtained. Fluorescence polarization data was fit a hyperbola to obtain K_D values (KaleidaGraph software).

Fluorescence titration assays measuring intrinsic protein fluorescence - NS3 was titrated with polyU and the change in protein fluorescence was measured by using an SLM Amnico luminescence spectrometer. Samples were excited at 280 nm with bandpass of 4nm and emission was monitored at 340nm with

bandpass 8 nm. The instrument was maintained at 25 °C with a circulating water bath. The titration buffer contained 50 mM MOPS, pH 7.0, 50 μ M EDTA, 10 mM NaCl, and 2 mM BME. NS3 concentration was 100 nM. Correction for dilution and inner filter effects were applied. Prior to the addition of poly(U), NS3 was allowed to equilibrate for about 45 min because the intrinsic fluorescence of the sample exhibited a gradual reduction over this time (32). The titration data was fit to quadratic equation by using KaleidaGraph3.5 software.

RESULTS

Unwinding under excess enzyme conditions - The ability of full-length NS3 to unwind partial duplex DNA was monitored under excess enzyme conditions. NS3 (500 nM) was incubated with 45:30mer (1 nM) partial DNA duplex prior to initiating unwinding with 5 mM ATP and 10 mM $MgCl_2$ at 37 °C. Under these conditions of low DNA concentration, re-annealing of ssDNA products to reform substrate during the reaction is very slow and does not affect the observed rates (27,29). To prevent re-annealing of ssDNA products after the reaction has been quenched with 200 mM EDTA and 0.7% SDS, a 30-fold excess of 30mer DNA trapping strand that is complementary to the 30mer in the 45:30mer substrate was placed in the receiving vial for the KinTek rapid chemical quench-flow apparatus. The formation of ssDNA product was observed by 5' radiolabeling the T45mer of the substrate. Free T45mer unwinding product was separated from 45:30mer unwinding substrate on a 20% polyacrylamide gel, and visualized by using a phosphorimager (Fig. 1A). Under these conditions, product formation consisted of a fast phase of rapid unwinding followed by a second slower phase (Fig. 1). The majority of ssDNA is formed during the fast phase, which is characterized by a lag in the initial portion of the unwinding curve. The lag is due to the fact that ssDNA product is not observed until the helicase unwinds most of the duplex. More than one step is required to do this, so a lag appears in the early portion of the time course for DNA unwinding (33). The unwinding data was fit to Scheme 1 by using the program Scientist (27,31). The steps in Scheme 1 are the minimal number required to fit the unwinding data for the 30 bp substrate. Two species for DNA unwinding are shown, ES_1 and ES_2 , that

correspond to productively bound enzyme and a kinetic intermediate, respectively. The intermediate is believed to represent a partially unwound substrate that occurs along the pathway to fully unwound DNA. The lag phase in product formation is described by the formation of the ES_2 intermediate prior to complete unwinding. A slower phase is observed after the lag phase which is accounted for by the conversion of a nonproductive ES complex (ES_{NP}) to a productive complex (27,31,33). All rates and amplitudes for each phase are listed in Table 1.

To investigate NS3 unwinding under single-cycle conditions, a protein trap, poly (U), was introduced into the reaction to sequester unbound NS3. NS3 (500 nM) and 45:30mer (1 nM) were incubated and the unwinding reaction was initiated with 5 mM ATP, 10 mM $MgCl_2$, and 75 μM in nt poly(U) protein trap. In the presence of the poly(U) protein trap, NS3 unwound the 45:30mer with a similar rate and amplitude in the lag phase as were observed in the absence of the protein trap (Fig. 1, Table 1). This result indicates that the form of NS3 that is initially bound prior to addition of ATP is the processive species that can separate > 80 % of the 30 bp substrate prior to dissociation from the DNA. The unwinding data in the presence of protein trap, like the data collected in the absence of protein trap, could be best fit by a lag phase followed by rapid product formation followed by a slower phase. All data obtained from unwinding in the presence of protein trap was fit to Scheme 1. The efficiency of the poly(U) protein trap was investigated by incubating 75 μM poly(U) with 1 nM 45:30mer and then initiating the reaction by adding 500 nM NS3, ATP (5 mM) and $MgCl_2$ (10 mM). Very little product formed under these conditions indicating that the protein trap was effective (Fig. 1).

Unwinding with varying ratios of NS3 and 45:30mer - In the presence of excess enzyme, the active form of enzyme cannot be determined. However, under pre-steady state conditions, in which the substrate is in excess of the enzyme, then the amplitude of the burst phase can provide information about the active species of enzyme. Dda helicase was recently shown to be capable of functioning as a monomer when

unwinding short oligonucleotides under pre-steady state conditions (15). The first cycle of unwinding by NS3 was monitored at varying ratios of NS3 to 45:30mer in order to investigate the active form of NS3. The amount of 45:30mer DNA was increased to 100 nM in order to conduct experiments in the presence of excess substrate. To prevent re-annealing of the ssDNA products during the reaction, 3 μM 30mer DNA trapping strand was included in the reaction. The presence of the DNA trapping strand does not affect the first cycle of the reaction with or without protein trap (Fig. 1) because the NS3 is incubated with substrate prior to unwinding; however, including the 30mer trapping strand in the reaction will influence unwinding rates after the first cycle in the absence of protein trap (15). Under these conditions, the amplitude of the first phase of unwinding reflects the quantity of enzyme capable unwinding the substrate completely.

Unwinding of 100 nM 45:30mer by NS3 was measured by gel electrophoresis (Fig. 2A). Initially, 100 nM 45:30mer was incubated with 1000 nM NS3 and the reaction was initiated by addition of 5 mM ATP, 10 mM $MgCl_2$ and 3 μM 30mer DNA trapping strand, with or without 5 μM poly(U) (Fig. 2B). The data collected was fit to Scheme 1. Under single-cycle conditions with the poly(U) protein trap, less than half of the substrate was unwound as determined by the summation of the amplitudes for each phase (Fig. 2B, Table 1). The low amplitude suggests that multiple NS3 molecules are involved in the active unwinding complex because a 10:1 ratio of NS3 to 45:30mer was not sufficient to provide optimal activity. In the absence of poly(U) protein trap, 77% of the substrate was unwound (Fig. 2B, Table 1). When NS3 was lowered to 100 nM and 45:30mer was kept at 100 nM, only ~ 4 % of the substrate was unwound in the presence of the poly(U) protein trap (Fig. 2C, Table 1). Under the same conditions in the absence of poly(U) protein trap, ~ 9 % of the substrate was unwound after 60 s (Fig. 2C, Table 1). The data in Fig. 2 reveal that a large excess of NS3 to 45:30mer was required for optimal unwinding activity.

To more thoroughly investigate the active form of NS3, the single-cycle experiments with poly(U) protein trap were performed with varying ratios of NS3 to DNA (Fig. 3). 50 nM, 100 nM, 500 nM, 1000 nM, or 2000 nM NS3 was incubated with 100 nM 45:30mer and the reaction was initiated by

addition of 5 mM ATP, 10 mM MgCl₂, 3 μM 30mer DNA trapping strand, and 5 μM poly(U) (Fig. 3). Under conditions where NS3 concentration was below that of the substrate (50 nM NS3, 100 nM 45:30mer) essentially no product was formed (Fig. 3, Table 1). As the concentration of NS3 was raised up to 2000 nM, the amplitude for the first cycle of product formation was increased to a level that was similar to the amplitude observed under optimal conditions in Fig. 1. Thus, a ratio of 20 NS3 molecules to 1 substrate molecule was necessary for optimal unwinding.

Experiments were performed at lower concentration of substrate in order that NS3 concentration could be in large excess of substrate, while maintaining substrate concentration well above the K_D value for NS3 binding to DNA. Full-length NS3 is not highly soluble under conditions in which unwinding is optimal. High concentrations of full length NS3 are possible at very high concentration of NaCl (1 M), but unwinding is greatly reduced in high salt (Tacket and Raney, unpublished observations). The solubility/activity limitations prevented us from raising the NS3 concentration above 2 μM, therefore the substrate concentration was lowered to 25 nM. At this concentration, the substrate was more than 10-fold greater than the equilibrium dissociation constant, which is useful for interpretation of pre-steady state experiments (see below). Substrate (25 nM 45:30mer) was incubated with 10 nM, 25 nM, 125 nM, 250 nM, 500 nM, and 750 nM NS3 and the reaction was initiated by addition of 5 mM ATP, 10 mM MgCl₂, 3 μM 30mer DNA trap, and 5 μM poly(U) (Fig. 4). As observed in Figure 3, pre-steady state conditions in which substrate was in excess of enzyme (25 nM 45:30mer, 10 nM NS3) resulted in essentially no product formation. Conditions favoring binding of multiple NS3 molecules to a single substrate (25 nM 45:30mer, 750 nM NS3) resulted in significant unwinding. A ratio of 30 molecules of NS3 per 45:30mer molecule produced an amplitude of ~ 70 % product formation, which is similar to that observed in Figure 1. The data in Figures 3 and 4 were fit to Scheme 1. All of the rates and amplitudes for Figures 3 and 4 are listed in Table 1. Values not

listed were not defined due to insufficient product formation.

Electrophoretic mobility shift assays - The requirement for multiple NS3 molecules for optimal unwinding suggested that multiple molecules were bound per substrate molecule. To investigate this possibility, electrophoretic mobility shift assays (EMSAs) were performed with T₁₅ ssDNA, 30mer duplex (identical to the duplex portion of 45:30mer), and 45:30mer partial duplex (Fig. 5). Radiolabeled DNA (0.1 nM) was incubated with varying NS3 at 37 °C, then analyzed by PAGE (Fig. 5A). The amount of T₁₅, 30mer duplex, and 45:30mer bound was fit to a hyperbola resulting in K_D values of 1.3 ± 0.2 nM, 11.3 ± 1.3 nM, and 2.7 ± 0.4 nM respectively (Fig. 5B, 5C). Binding of NS3 to the T₁₅, 30mer duplex, and 45:30mer resulted in formation of a species that did not migrate into the 4 % polyacrylamide gel, but was retained in the well (Fig. 5A). This suggests that multiple NS3 molecules bind to each type of DNA investigated leading to charge neutralization of the DNA.

Binding to the duplex region was weaker by ~ ten-fold than binding to the single-stranded regions. However, the K_D value indicated that the duplex region should be completely bound under the optimal unwinding conditions in Figure 3 and Figure 4. Binding was investigated further by using fluorescence polarization of fluorescein-labeled DNA. Equilibrium dissociation constants were measured by titrating DNA with NS3 followed by incubation for 3 min at 37 °C. The resulting K_D values were 4.7 ± 1.2 nM, 15.7 ± 2.9 nM, and 5.2 ± 0.52 nM for the 15mer, 30:30mer, and 45:30mer, respectively. Hence, the values were similar to those observed in the gel shift assays. Binding of some helicases to DNA is modulated by the binding and hydrolysis of ATP. We added a slowly hydrolysable ATP analog, ATP-γ-S, to each of the samples in Fig. 6A and measured the fluorescence polarization. Interestingly, binding was reduced for each oligonucleotide, but more so for the 30:30mer DNA than for the 15mer or the 45:30mer. The mechanistic interpretation of this result remains to be determined, but it may support a model whereby NS3 binds to different forms of DNA as a function of ATP binding and hydrolysis, which facilitates unwinding. A recent report suggests that ATP binding and hydrolysis mediates affinity of NS3 helicase domain for DNA (34).

To investigate the stoichiometry of the active NS3 complex, EMSAs were performed as described above using 100 nM 45:30mer and varying concentrations of NS3 (Fig. 7). This concentration of DNA is well above the K_D value measured for all three DNA strands (T15, 30mer duplex, and 45:30mer). The stoichiometric binding revealed that 90% of the DNA was shifted when the concentration of NS3 was in excess of the DNA by five-fold (lane in Figure 7 marked with 0.5 μ M NS3). This suggests that multiple NS3 molecules can bind along the 45:30mer because much more protein is needed than expected to shift all of the DNA. The free 45:30mer ultimately was mobility-shifted to a species that did not enter the gel (Fig. 7A). The complex between NS3 and DNA that did not enter the gel was formed under conditions in which unwinding was optimal (ratio of NS3 to DNA of \sim 20:1). Thus, the active species for initiation of DNA unwinding likely contains multiple NS3 molecules that bind to the single-stranded and duplex regions of the substrate. However, NS3 was not able to unwind the blunt end 30mer duplex rapidly (Tackett and Raney, data not shown).

DISCUSSION

Understanding the mechanism for helicase activity requires knowledge of the active form of the enzyme. The active form of NS3 remains uncertain. Utilizing the C-terminal helicase domain of NS3 (NS3h), Levin and Patel (25) provided evidence for an active oligomeric form of the helicase domain; however, Preugschat *et al.* (23) concluded that the functional form of the helicase was a monomer. Additionally, Kim *et al.* (24) suggested a monomeric form of NS3h based on the x-ray crystal structure with bound dU₈ oligonucleotide. Very recently, the Patel lab has proposed a model for unwinding that invokes functional cooperativity for unwinding by NS3 helicase domain (26). In this model, multiple molecules of NS3h are shown to bind to the single stranded region of partially duplex substrates. Monomeric forms of NS3h are proposed to be non-processive, thereby requiring multiple molecules for processive DNA unwinding. We recently reported DNA

unwinding experiments under pre-steady state conditions, which indicated that Dda helicase is able to unwind short oligonucleotides as a monomer (15). Here we have applied a similar approach to the full-length form of NS3.

In order to investigate the active form of NS3, we first compared unwinding in the presence and absence of a protein trap to determine whether the enzyme could unwind 30 base pairs in a single binding event (Fig. 1). When protein was in great excess over substrate, unwinding was similar in the presence and absence of the protein trap (Fig. 1, Table 1). Product formation displayed two distinct phases as previously reported for other helicases like bacteriophage T4 Dda, *E. coli* UvrD, and bacteriophage T7 gp4 (30,33,35,36). The unwinding data was fit to Scheme 1 by using the program Scientist (27). This model takes into account the multi-step strand separation for the initial fast phase as well as the conversion of “nonproductive” to “productive” NS3 for the slower phase. The results under conditions of a large excess of enzyme over substrate illustrate that the NS3 species that is initially bound to DNA can unwind \sim 85% of the 45:30mer prior to dissociation.

It is interesting to compare the activity of this particular form of NS3 with that reported by others. The fastest rate obtained from fitting the data to the two-step scheme was $2.0 \pm 0.1 \text{ s}^{-1}$ when NS3 was 1 μ M and substrate was 100 nM (Table 1). This rate corresponds to 30 bp s^{-1} at 37 °C. Others have reported unwinding of an 18 base pair substrate with a rate constant of $2.2 \pm 0.7 \text{ min}^{-1}$ which corresponds to 0.66 base pairs s^{-1} (37). The form of NS3 believed to be biologically relevant contains the NS4a peptide bound to the protease domain of NS3. A report of unwinding by NS3-4a indicated that an 18 base pair substrate was unwound at a rate of $2.5 \pm 0.9 \text{ min}^{-1}$ which corresponds to 0.75 base pairs s^{-1} . The recent report from the Patel lab provided a rate for unwinding by NS3h of 2.2 bp s^{-1} at 22 °C making a direct comparison with our data difficult (26). Thus, full length NS3 reported here unwinds DNA somewhat faster at 37 °C than other forms of the enzyme reported thus far. The processivity of the full-length protein appears greater than the helicase domain. More than 80% of a 30 bp substrate was unwound by full length NS3 under optimal conditions (Fig. 1) compared to <20 % for a similar substrate in the case of NS3h (26).

Under pre-steady state conditions the concentration of substrate is in excess to that of enzyme so that product formed in the first unwinding cycle will reflect the concentration of active enzyme. Thus, varying the enzyme concentration relative to the substrate concentration should reveal the amount of NS3 that is required for optimal activity. Initially, the amount of NS3 (100 nM) was equivalent to 45:30mer (100 nM) resulting in only ~ 4% of the substrate being unwound (Fig. 2C, Table 1). This illustrates that NS3 cannot effectively unwind a 30 bp substrate at a 1:1 ratio in the presence of protein trap. When we lowered the NS3 (50 nM) below the 45:30mer (100 nM) concentration, essentially no product was observed (Fig. 3). The experiments in Fig. 2B and 2C suggest that the most active species of NS3 is formed under conditions when enzyme is in large excess to DNA allowing multiple enzyme molecules to bind per substrate molecule. This strongly suggests that monomeric NS3 is not highly processive *in vitro*, as suggested for NS3h (26).

The 15mer single-stranded region of the 45:30mer should be able to bind one and perhaps two molecules of NS3 based on the crystal structure of the enzyme (24) and the binding site size (26,38), assuming that the full-length protein behaves similarly to the helicase domain. The unwinding data suggested that far more than two-fold excess NS3 was needed to unwind the substrate, leading us to investigate the distribution of NS3 molecules along the 45:30mer by using electrophoretic mobility shift assays (EMSAs) (Fig. 5). We observed that NS3 interacted with the ssDNA overhang and 30mer duplex region of the 45:30mer. This interaction promoted formation of a large molecular mass species that ultimately was retained in the wells of the gel (Fig. 5). Importantly, the K_D values for the ss overhang (1.3 ± 0.2 nM) and 30mer duplex portion of 45:30mer (11.3 ± 1.3 nM) revealed that NS3 was bound to single-stranded and duplex regions of the 45:30mer under optimal unwinding conditions (Figs. 3,4). Binding assays using fluorescence polarization provided similar K_D values as the gel shift assay (Fig. 6). The absolute values for binding to single-stranded DNA are similar to those reported by Levin and

Patel (38), however, duplex binding reported here for full-length NS3 is stronger than reported for NS3h. Binding of NS3 to DNA was reduced in the presence of ATP- γ -S (Fig. 6B), as was observed for NS3h (38).

Binding to the 45:30mer was further investigated under conditions in which the DNA concentration was raised well above the K_D value. Multiple species were observed indicating that multiple molecules bound to the 45:30mer (Fig. 7A). Based on the number of species observed on the gel, and the quantity of NS3 required to saturate binding, as many as five molecules of NS3 bind to the 45:30mer under conditions in which unwinding is optimal. It is possible that NS3 melted the 45:30mer simply in an ATP-independent manner and therefore all of the observed binding might have been to single-stranded DNA rather than duplex. However, we measured DNA unwinding under these conditions in the absence of ATP and observed no unwinding (Tackett and Raney, unpublished observations). Thus, NS3 binding to the ssDNA region, or the ss/ds DNA junction appears to be required for optimal unwinding to occur.

It is possible that some or even most of the recombinant NS3 protein is inactive. To determine the amount of NS3 that was bound to the 45:30mer, we quantitated the bands in the lane in which ~ one-half of the DNA was bound in Fig. 7 (marked as 0.25 μ M NS3). The fraction of DNA in each band correlates to the quantity of DNA in that band (Fig. 7C). The number of NS3 molecules bound for each species was estimated from the binding site size of NS3 (~ 8 - 11 nucleotides from (38)) and from the number of DNA species on the gel. As shown in Figure 7C, the quantity of bound NS3 could be calculated by multiplying the quantity of DNA by the estimated number of NS3 molecules bound. The results of this analysis indicate that ~ 195 nM out of a total of 250 nM NS3 was bound under these conditions. Hence, greater than 75% of the NS3 was able to bind to DNA, suggesting that the majority of the protein is active. To further explore the quantity of active protein, a fluorescence titration was performed under conditions where NS3 (100 nM) was above the equilibrium dissociation constant. Based on the binding site size of 8 - 11 nt, ~ 1000 nM poly(U) would be expected to saturate binding of 100 nM NS3 if all of the protein were active. Our results indicate that ~ 90% of the protein was bound at 1000 nM poly(U) (Fig. 8). If a substantial

fraction of NS3 were inactive, we would observe saturation at a much lower concentration of poly(U). Therefore, the data are not consistent with a large quantity of inactive NS3. We conclude that at least 75 % of protein in our preparation is capable of binding to RNA or DNA based on the EMSA data and the fluorescence titration.

Based on the model provided by the Patel lab (26), binding of more than two molecules of NS3 to the ssDNA might increase the quantity of product formed. This suggests that increasing the length of the single-stranded region of the substrate might lead to enhanced unwinding by allowing more molecules of NS3 to bind. We prepared a 60:30mer substrate and analyzed unwinding under the same conditions as for the 45:30mer. Similar to the 45:30mer, high concentrations of NS3 are required for optimal unwinding of the 60:30mer (Fig. 9). However, higher amplitudes for unwinding were observed in the case of the 60:30mer when compared to the 45:30mer (Fig. 9B). These data are qualitatively consistent with the model previously proposed by the Patel lab (26), in which multiple molecules of NS3 bound to the single stranded region of the substrate are required for optimal unwinding.

We further investigated unwinding by using a substrate containing 15 nucleotides of ssDNA adjacent to 15 base pairs (30:15mer). Similar amplitudes are observed with the 30:15mer compared to the 45:30mer, but the overall requirement for multiple helicase molecules for efficient unwinding remains (Fig. 10). For example, 25 nM NS3 incubated with 25 nM 30:15mer produced essentially no product under single turnover conditions. Only when the enzyme concentration was raised well above substrate concentration was significant product formation observed. Thus, multiple molecules are required for optimal unwinding even with a short duplex. These data were fit to a single exponential which does not include a lag phase for unwinding. Unzipping of 15 base pairs can be fit with a single step for unwinding whereas unzipping of 30 base pairs can be fit with two steps. The appearance of or lack of a lag phase must be interpreted with caution, because a slow step prior to unwinding can greatly change the interpretation of the number

of steps needed to unwind the duplex (39). A thorough description of the kinetic analysis of helicase stepping has been provided by Lucius *et al.*, 2003 (40). The Patel lab reported a step size of ~ 9 bp for NS3h (26), assuming that several bp spontaneously melt before the helicase reaches the very end of the oligonucleotide (26,39). A recent report from the Pyle lab suggested that full-length NS3 unwound RNA with a large step size of ~ 18 base pairs and functioned as a dimer. Our current data are insufficient to compare with the data from the Patel and Pyle labs in regards to the step size, however the amplitude that we observe is not consistent with a simple, dimeric form of NS3 as the active species. It is possible that multiple molecules are bound to one another in some form of oligomeric structure, but the substrate might be bound by a “functional dimer” that is responsible for unwinding.

Our lab has recently presented a model for Dda helicase that is similar to the model for NS3h. Dda can displace streptavidin from biotin labeled oligonucleotides. The monomeric form of Dda displays streptavidin displacement activity, but activity is enhanced significantly when multiple molecules of Dda are bound to the oligonucleotide (14). Interaction between helicase molecules is proposed to play a role in the enhanced activity in the case of Dda. Protein-protein interactions are not proposed to be necessary (but are not excluded) in the model for DNA unwinding by NS3h. We have found that the full-length NS3 helicase interacts with itself much more strongly than NS3h, therefore some differences exist between the two forms of the protein (C. Lichti, Y. Chen, and K. Raney, unpublished observations). A substrate saturation model for optimal NS3 unwinding activity is proposed in Figure 11 in which all of the DNA is bound in the presence of excess NS3, including the dsDNA region. At any given time during the unwinding process, a monomer, dimer or oligomer might contribute to the unwinding process, so that multiple enzyme molecules are involved along the reaction pathway. NS3 bound to the ssDNA or ss/dsDNA junction serves as the ATP hydrolysis “motor” pulling the DNA. Multiple molecules can enhance processivity and reduce reannealing that can occur during unwinding (26). We do not have evidence that the molecules bound to the duplex region play an active role in the unwinding process at this time. However, binding to the duplex removes protein from solution that might otherwise

bind to the ssDNA, thereby contributing indirectly to the requirement for excess NS3 to be present for efficient unwinding. We are currently investigating whether protein-protein interactions between NS3 monomers are required for optimal unwinding activity.

The significance of the substrate saturation model for NS3 to the biological role of NS3 remains uncertain, however, numerous studies point to this mechanism as being biologically relevant. The positive strand of HCV RNA is translated into a polyprotein that is processed by proteolysis, giving rise to one copy of NS3 per polyprotein. Numerous protein-protein interactions are known to occur between HCV proteins. NS3 can stimulate the activity of the NS5b polymerase and can interact directly with the polymerase, which might alter NS3's biochemical properties (41). NS4A is known to bind tightly to NS3, and has been shown to alter the binding properties of NS3 (37). Importantly, NS4A contains a membrane spanning region that likely anchors NS3/4A to a cellular membrane along the endoplasmic reticulum. Thus, NS3/4A is likely to be bound to other non-structural proteins as well as the membrane,

which means that nucleic acid is likely to be translocated through the HCV protein complex, rather than the protein moving along the nucleic acid. Additionally, we and others have found that NS3 interacts tightly with itself in vitro (42, Tackett, C. Lichti, Y. Chen, K. D. Raney, unpublished observations). Hence, it is possible that an array or filament of membrane-bound NS3/4a molecules is found in cells. It is not known whether sufficient NS3/4A molecules exist in cells to saturate HCV RNA. However, recent studies in other RNA viruses indicate that sufficient RNA binding proteins exist to saturate most of the RNA. The Ahlquist lab has shown that RNA replication of the Brome Mosaic Virus occurs in invaginated membrane pockets referred to as "spherules" that are formed by hundreds of membrane-bound helicases-like proteins (43). Similar sites of replication referred to as membraneous webs have been observed for HCV replication by electron microscopy (44,45). Multiple roles for NS3 are likely in HCV replication. Saturation of the RNA may protect the RNA from nuclease activity or stabilize specific RNA structures or remove secondary structure for processing; all of which might require multiple copies of NS3 per RNA strand.

REFERENCES

1. Hall, M. C. and Matson, S. W. (1999) *Mol. Microbiol.* **35**, 867-877
2. Lohman, T. M. and Bjornson, K. P. (1996) *Annu. Rev. Biochem.* **65**, 169-214
3. Patel, S. S. and Picha, K. M. (2000) *Annu. Rev. Biochem.* **69**, 651-97
4. Soultanas, P. and Wigley, D. B. (2000) *Curr. Opin. Struct. Biol.* **10**, 124-128
5. Bujalowski, W., Klonowska, M. M., and Jezewska, M. J. (1994) *J. Biol. Chem.* **269**, 31350-31358
6. Dong, F., Gogol, E. P., and von Hippel, P. H. (1995) *J. Biol. Chem.* **270**, 7462-73
7. Patel, S. S. and Hingorani, M. M. (1993) *J. Biol. Chem.* **268**, 10668-10675
8. Egelman, E. H., Yu, X., Wild, R., Hingorani, M. M., and Patel, S. S. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3869-73
9. Jezewska, M. J., Surendran, R., Bujalowska, D., and Bujalowska, W. (1998) *J. Biol. Chem.* **273**, 10515-10529
10. Morris, P. D. and Raney, K. D. (1999) *Biochemistry* **38**, 5164-5171
11. Bujalowski, W. (2003) *Trends Biochem. Sci.* **28**, 116-118
12. Kaplan, D. L. and O'Donnell, M. (2002) *Mol. Cell* **10**, 647-657
13. Morris, P. D., Tackett, A. J., Babb, K., Nanduri, B., Chick, C., Scott, J., and Raney, K. D. (2001) *J. Biol. Chem.* **276**, 19691-8
14. Byrd, A. B. and Raney, K. D. (2004) *Nat. Struct. Biol.* **11**, 531-538
15. Nanduri, B., Byrd, A. K., Eoff, R. L., Tackett, A. J., and Raney, K. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14722-7

16. Bird, L. E., Brannigan, J. A., Subramanya, H. S., and Wigley, D. B. (1998) *Nucleic Acids Res.* **26**, 2686-2693
17. Soutanas, P., Dillingham, M. S., Wiley, P., Webb, M. R., and Wigley, D. B. (2000) *EMBO J.* **19**, 3799-3810
18. Velankar, S. S., Soutanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) *Cell* **97**, 75-84
19. Dillingham, M. S., Wigley, D. B., and Webb, M. R. (2002) *Biochemistry* **41**, 643-51
20. Maluf, N. K. and Lohman, T. M. (2003) *J. Mol. Biol.* **325**, 889-912
21. Maluf, N. K., Fischer, C. J., and Lohman, T. M. (2003) *J. Mol. Biol.* **325**, 913-35
22. Neddermann, P., Tomei, L., Steinkuhler, C., Gallinari, P., Tramontano, A., and De Francesco, R. (1997) *Biol. Chem.* **378**, 469-476
23. Preugschat, F., Danger, D. P., Carter, L. H. 3., Davis, R. G., and Porter, D. J. (2000) *Biochemistry* **39**, 5174-83
24. Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, C., and Caron, P. R. (1998) *Structure* 89-100
25. Levin, M. K. and Patel, S. S. (1999) *J. Biol. Chem.* **274**, 31839-46
26. Levin, M. K., Wang, Y. H., and Patel, S. S. (2004) *J. Biol. Chem.* **279**, 26005-26012
27. Tackett, A. J., Wei, L., Cameron, C. E., and Raney, K. D. (2001) *Nucleic Acids Res.* **29**, 565-72
28. Carroll, S. S., Benseler, F., and Olsen, D. B. (1996) *Meth. Enzymol.* **275**, 365-82
29. Amaratunga, M. and Lohman, T. M. (1993) *Biochemistry* **32**, 6815-6820
30. Tackett, A. J., Morris, P. D., Dennis, R., Goodwin, T. E., and Raney, K. D. (2001) *Biochemistry* **40**, 543-8
31. Cheng, W., Hsieh, J., Brendza, K. M., and Lohman, T. M. (2001) *J. Mol. Biol.* **310**, 327-50
32. Lam, A. M., Keeney, D., Eckert, P. Q., and Frick, D. N. (2003) *J. Virol.* **77**, 3950-3961
33. Ali, J. A. and Lohman, T. M. (1997) *Science* **275**, 377-380
34. Levin, M. K., Gurjar, M. M., and Patel, S. S. (2003) *J. Biol. Chem.* **278**, 23311-6
35. Ahnert, P., Picha, K. M., and Patel, S. S. (2000) *EMBO J.* **19**, 3418-3427
36. Ali, J. A., Maluf, N. K., and Lohman, T. M. (1999) *J. Mol. Biol.* **293**, 815-34
37. Pang, P. S., Jankowsky, E., Planet, P. J., and Pyle, A. M. (2002) *EMBO J.* **21**, 1168-76
38. Levin, M. K. and Patel, S. S. (2002) *J. Biol. Chem.* **277**, 29377-85
39. Galletto, R., Jezewska, M. J., and Bujalowski, W. (2004) *J. Mol. Biol.* **343**, 83-99
40. Lucius, A. L., Maluf, N. K., Fischer, C. J., and Lohman, T. M. (2003) *Biophys. J.* **85**, 2224-39
41. Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K. D., and McCarthy, J. E. (2002) *J. Biol. Chem.* **277**, 45670-9
42. Sali, D. L., Ingram, R., Wendel, M., Gupta, D., McNemar, C., Tsarbopoulos, A., Chen, J. W., Hong, Z., Chase, R., Risano, C., Zhang, R., Yao, N., Kwong, A. D., Ramanathan, L., Le, H. V., and Weber, P. C. (1998) *Biochemistry* **37**, 3392-3401
43. Schwartz, M., Chen, J., Lee, W. M., Janda, M., and Ahlquist, P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 11263-11268
44. Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H. E., Bienz, K., and Moradpour, D. (2003) *J. Virol.* **77**, 5487-5492
45. Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M. R., Pedrazzini, E., Bonatti, S., and Migliaccio, G. (2002) *Virology* **293**, 31-43

FIGURE LEGENDS

Fig. 1. Unwinding of 45:30mer partial duplex (45:30mer) with NS3 in the presence or absence of protein trap under excess enzyme conditions. *A*, A representative gel showing unwinding of 1 nM 45:30mer with 500 nM NS3 without a protein trap. Unwinding products were resolved on a 20% polyacrylamide gel, and visualized by using a phosphorimager. The blank (*B*) shows the 45:30mer prior to unwinding, and the heated sample (*H*) shows the DNA trapping strand efficiency. *B*, Unwinding of 1 nM 45:30mer with 500 nM NS3 in the absence of a protein trap (circles), and in the presence of 5 μ M poly(U) protein trap (triangles). Unwinding at 37 °C was initiated by mixing 1 nM 45:30mer + 500 nM NS3 with 5 mM ATP + 10 mM MgCl₂ ± 5 μ M poly(U). The reaction was quenched with 200 mM EDTA, 0.7% SDS, and a 30mer DNA trapping strand (30 nM) that is complementary to the 30mer in the substrate. The protein trapping efficiency of poly(U) was determined by incubating 5 μ M poly(U) with 1 nM 45:30mer prior to adding 500 nM NS3, after which 5 mM ATP + 10 mM MgCl₂ was added (squares). *C*, Expanded view of the lag phase from panel *B*. The unwinding data was fit to Scheme 1 by using the program Scientist (MicroMath), and the unwinding rates and amplitudes are listed in Table 1.

Fig. 2. Unwinding with different ratios of NS3 to 45:30mer in the presence or absence of a protein trap. *A*, A representative gel showing unwinding of 100 nM 45:30mer with 1000 nM NS3 in the presence of protein trap. Unwinding products were resolved on a 20% polyacrylamide gel. The blank sample (*B*) shows the 45:30mer prior to unwinding, and the heated sample (*H*) shows the DNA trapping strand efficiency. *B*, Unwinding of 100 nM 45:30mer with 1000 nM NS3 in the absence of protein trap (circles), or in the presence of 75 μ M poly(U) protein trap (triangles). Unwinding was initiated by rapidly mixing 100 nM 45:30mer + 1000 nM NS3 with 5 mM ATP + 10 mM MgCl₂ ± 5 μ M poly(U) + 3 μ M 30mer DNA trapping strand that is complementary to the 30mer in the partial duplex. The unwinding reaction was performed at 37 °C, and the reaction was quenched with 200 mM EDTA + 0.7% SDS. The protein trapping efficiency of poly(U) was determined by incubating 75 μ M poly(U) with 100 nM 45:30mer prior to adding 2000 nM NS3, after which 5 mM ATP + 10 mM MgCl₂ was added (squares). *C*, Unwinding of 100 nM 45:30mer with 100 nM NS3 in the absence of protein trap (circles), or in the presence of 75 μ M poly(U) protein trap (triangles). Unwinding was performed as described for panel *B*. The unwinding data in panels *B* and *C* were fit to Scheme 1 by using Scientist (MicroMath), and the unwinding rates and amplitudes are listed in Table 1.

Fig. 3. Unwinding of 100 nM 45:30mer with varying NS3. *A*, Unwinding of 100 nM 45:30mer with 50 nM NS3 (filled circles), 100 nM NS3 (open triangles), 500 nM NS3 (squares), 1000 nM NS3 (open circles), and 2000 nM NS3 (filled triangles). Unwinding at 37 °C was initiated by mixing 100 nM 45:30mer + NS3 with 5 mM ATP + 10 mM MgCl₂ + 5 μ M poly(U) + 3 μ M 30mer DNA trapping strand that is complementary to the 30mer in the partial duplex. The reaction was quenched with 200 mM EDTA + 0.7% SDS. *B*, Expanded view of the initial lag phase from panel *A*. The unwinding data was fit to Scheme 1 with Scientist (MicroMath), and the unwinding rates and amplitudes are listed in Table 1.

Fig. 4. Unwinding of 25 nM 45:30mer with varying NS3. *A*, The following concentrations of NS3 were used: 10 nM NS3 (filled circles), 25 nM NS3 (filled triangles), 125 nM NS3 (filled squares), 250 nM NS3 (open circles), 500 nM (open triangles), and 750 nM NS3 (diamonds). Unwinding at 37 °C was initiated by mixing 25 nM 45:30mer + NS3 with 5 mM ATP + 10 mM MgCl₂ + 75 μ M poly(U) + 3 μ M excess 30mer DNA trapping strand that is complementary to the 30mer in the partial duplex. The reaction was quenched with 200 mM EDTA + 0.7% SDS. A control experiment was performed in which 75 μ M poly(U) was incubated with 25 nM 45:30mer and then 750 nM NS3 was added, after which the reaction was initiated with 5 mM ATP, 10 mM MgCl₂, and 3 μ M 30mer DNA trapping strand (open squares). *B*, Expanded view of the initial

lag phase from panel A. The unwinding data was fit to Scheme 1 with Scientist (MicroMath), and the unwinding rates and amplitudes are listed in Table 1.

Fig. 5. Binding of NS3 to T₁₅ DNA, 30:30mer DNA, or 45:30mer DNA as measured by gel shift assay. *A*, Phosphorimages of 4% acrylamide gels that show binding of NS3 to radiolabeled T₁₅, 30:30mer, and 45:30mer DNA. Increasing concentrations of NS3 promoted formation of a species that did not enter the 4% acrylamide gel. *B*, The concentration of T₁₅ DNA (circles) or 45:30mer DNA (squares) bound by NS3 was fit to a hyperbola, and K_D values of 1.3 ± 0.2 nM for T₁₅ DNA and 2.7 ± 0.4 nM for 45:30mer DNA were determined. *C*, The amount of 30:30mer DNA bound by NS3 was also fit to a hyperbola, and a K_D value of 11.3 ± 1.3 nM was determined.

Fig. 6. Binding of NS3 to T₁₅ DNA, 30:30mer DNA, or 45:30mer DNA as measured by fluorescence polarization. Binding was followed by monitoring changes in fluorescence polarization of a 3'-fluorescein-labeled T₁₅ DNA (F-T₁₅), or 3'-fluorescein-labeled 30mer annealed to a 30mer (F-30:30mer) or 45mer (T45:F-30mer). *A*, Binding of NS3 to DNA in fluorescence polarization buffer. NS3 binding to F-T₁₅ (filled circles), F30:30mer (open circles), and T45:F-30mer (squares) resulted in K_D values of 4.7 ± 1.2 nM, 15.7 ± 2.9 nM, and 5.2 ± 0.52 nM respectively. *B*, Binding of NS3 to DNA in fluorescence polarization buffer + 1 mM ATP-γ-S + 10 mM MgCl₂. NS3 binding to F-T₁₅ (filled circles), F30:30mer (open circles), and T45:F-30mer (squares) resulted in K_D values of 12 ± 1 nM, 221 ± 88 nM, and 22.7 ± 6.8 nM respectively. Data was fit to a hyperbola to obtain K_D values using Kaleidagraph (Synergy Software) and standard errors are reported.

Fig. 7. Binding of NS3 at high concentration of DNA. *A*, Phosphorimage of a 4% acrylamide gel showing binding of NS3 to radiolabeled 100 nM 45:30mer. Increasing concentrations of NS3 produced at least four intermediates, and a complex that was retained in the well. *B*, The concentration of bound 45:30mer (circles) is plotted versus NS3 concentration. *C*, The lane corresponding to 0.25 μM NS3 from Fig. 7A is shown in which the individual bands were quantified by using Imagequant software. The fraction of the radioactivity in each band was measured and related to the concentration of DNA in the band based on 100 nM total DNA. The fastest migrating band corresponds to free DNA while the slower running bands correspond to bound DNA containing one, two, three, four, or five molecules of NS3. The concentration of each DNA band was multiplied by the number of NS3 molecules that are proposed to bind to the substrate. The resulting concentration of NS3 that was bound to the DNA was 195 nM, which was 78% of the total NS3 added to the binding reaction mixture.

Fig. 8. Binding to ssRNA by fluorescence titration of NS3 with poly(U). Excitation of NS3 (100 nM) fluorescence was at 280 nm and emission was 340 nm. The concentrations of poly(U) increased from 40 nM to 15 μM nt. Data were fit to the quadratic equation.

Fig. 9. Unwinding of 25 nM 60:30mer with various NS3. *A*, Unwinding was initiated by mixing 25 nM T60:30mer + NS3 with 5 mM ATP, 10 mM MgCl₂, 5 μM poly(U), and 3 μM excess 30mer DNA trapping strand that is complementary to the 30mer in the partial duplex. The following concentrations of NS3 were used: 25 nM NS3 (filled circles), 125 nM NS3 (filled squares), 250 nM NS3 (filled diamonds), 500 nM NS3 (filled triangles), and 750 nM NS3 (open triangles). The open circles are a control experiment to test the effectiveness of the poly(U) trap. The reaction was quenched with 200 mM EDTA + 0.7% SDS. The data were fit to Scheme I using the program Scientist (Micromath). *B*, The fraction of DNA (25nM) unwound for the

60:30mer (open squares) at varying NS3 concentrations are compared with fraction unwound for the 45:30mer (filled circles).

Fig. 10. Unwinding of 25 nM 30:15mer with various NS3. Unwinding was initiated by mixing 25 nM T30:15mer + NS3 with 5 mM ATP, 10 mM MgCl₂, 5 μM poly(U), and 3 μM excess 15mer DNA trapping strand that is complementary to the 15mer in the partial duplex. The following concentrations of NS3 were used: 25 nM NS3 (filled triangles), 125 nM NS3 (filled circles), 250 nM NS3 (filled squares), 500 nM NS3 (filled diamonds). The reaction was quenched with 200 mM EDTA + 0.7% SDS. This experiment was performed at 25 °C rather than 37 °C because significant melting of the 15 bp substrate occurred at the higher temperature. The data were fit to the equation for a single exponential, $A*(1-\exp(-k*t))$, using the program Kaleidagraph. Rates and amplitudes for the fits were $0.35 \pm 0.15 \text{ s}^{-1}$ and $8.3 \pm 0.7 \text{ nM}$ for 125 nM NS3; $0.15 \pm 0.03 \text{ s}^{-1}$ and $15.8 \pm 0.7 \text{ nM}$ for 250 nM NS3; and $0.43 \pm 0.16 \text{ s}^{-1}$ and $16.8 \pm 1.0 \text{ nM}$ for 500 nM NS3.

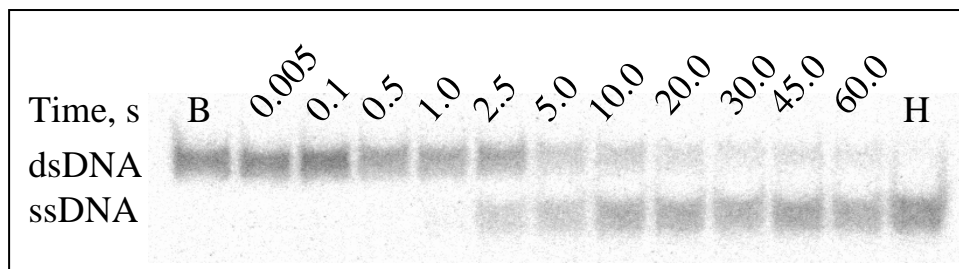
Fig. 11. Substrate saturation model for optimal DNA unwinding by NS3. Multiple molecules of NS3 shown bound to the 60:30mer substrate. NS3 binds to the single-stranded (in white) and double-stranded DNA (in grey) portion of the substrate under optimal unwinding conditions in vitro. NS3 bound to the ssDNA portion hydrolyses ATP to serve as the motor component that pulls ssDNA through the multi-protein complex. NS3 binds relatively weakly to the dsDNA, especially in the presence of ATP, allowing the DNA to slide through the binding site. The molecules are shown bound to one another along the DNA, however, it is not known whether protein-protein interactions play a role in unwinding.

Table 1. Unwinding rates and amplitudes for NS3 unwinding of 45:30^a.

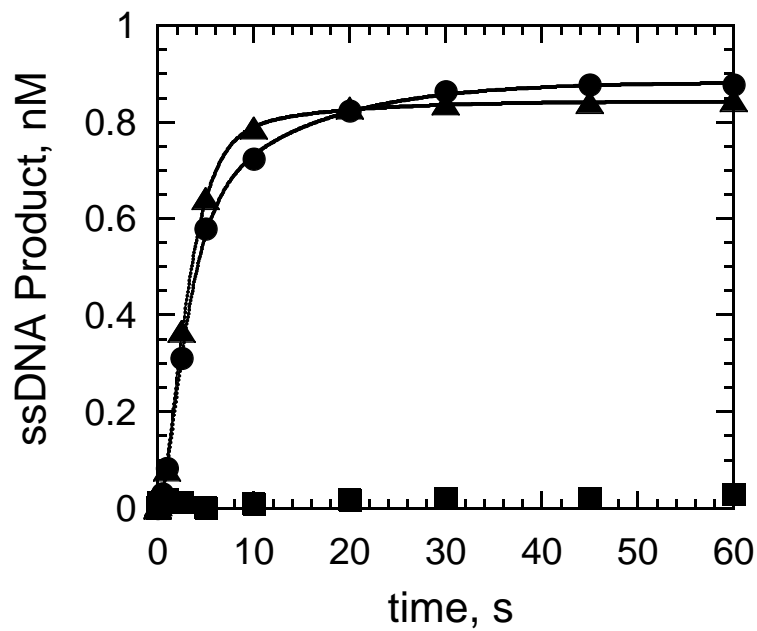
NS3 (μM)	45:30 (nM)	5 μM poly(U)	k_U, s^{-1} fast phase	Amplitude (nM) fast phase	k_{NP}, s^{-1} slow phase	Amplitude (nM) slow phase
0.5	1	-	0.66 ± 0.01	0.62 ± 0.01	0.086 ± 0.004	0.26 ± 0.01
0.5	1	+	0.62 ± 0.01	0.77 ± 0.01	0.087 ± 0.017	0.074 ± 0.01
0.05	100	+			-	-
0.1	100	-	0.62 ± 0.04	3.9 ± 0.2	0.037 ± 0.004	5.3 ± 0.2
0.1	100	+	1.3 ± 0.1	3.5 ± 0.2	-	-
0.5	100	+	0.77 ± 0.03	16 ± 0.4	0.040 ± 0.006	10 ± 0.4
1.0	100	-	1.40 ± 0.05	46 ± 1.2	0.080 ± 0.006	31 ± 1.0
1.0	100	+	2.0 ± 0.1	25 ± 1.2	0.18 ± 0.10	19 ± 1.1
2.0	100	+	1.30 ± 0.03	52 ± 0.8	0.060 ± 0.005	27 ± 0.7
0.01	25	+	1.71 ± 0.05	1.1 ± 0.05	-	-
0.025	25	+	1.41 ± 0.03	1.6 ± 0.2	-	-
0.125	25	+	1.10 ± 0.08	4.3 ± 0.2	-	-
0.25	25	+	1.70 ± 0.10	6.1 ± 0.2	-	-
0.5	25	+	1.01 ± 0.04	11 ± 0.3	0.047 ± 0.02	5.1 ± 0.3
0.75	25	+	0.90 ± 0.02	12 ± 0.2	0.084 ± 0.01	5.6 ± 0.2

^aRates and amplitudes were obtained by fitting data for unwinding to scheme 1 with Scientist (MicroMath). Errors are standard errors for the best fit of the data.

A



B



C

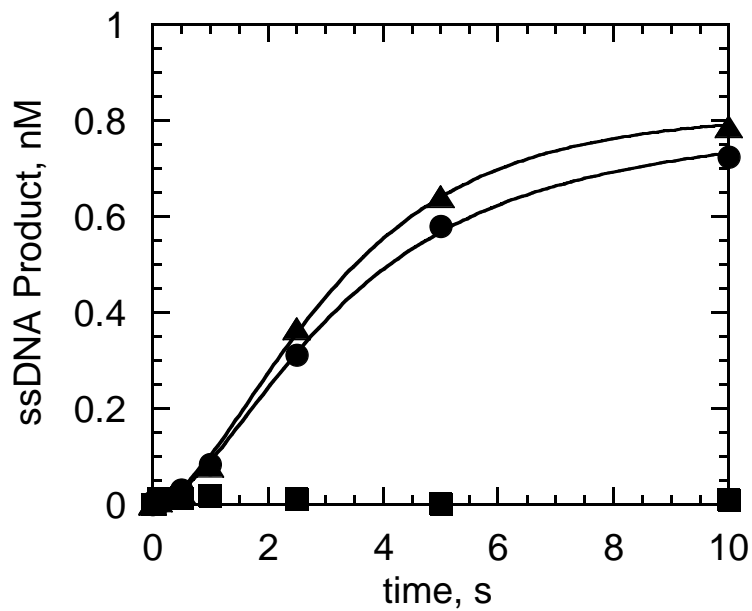
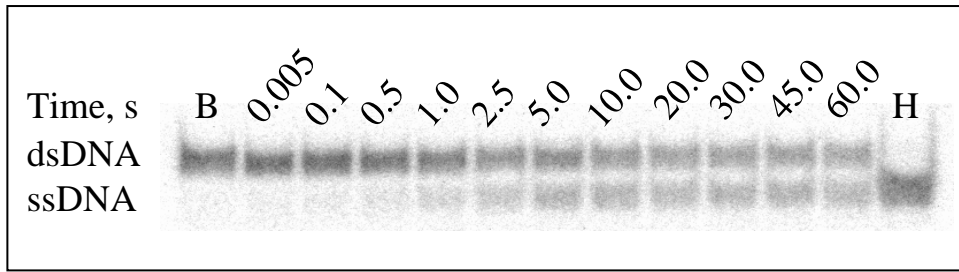
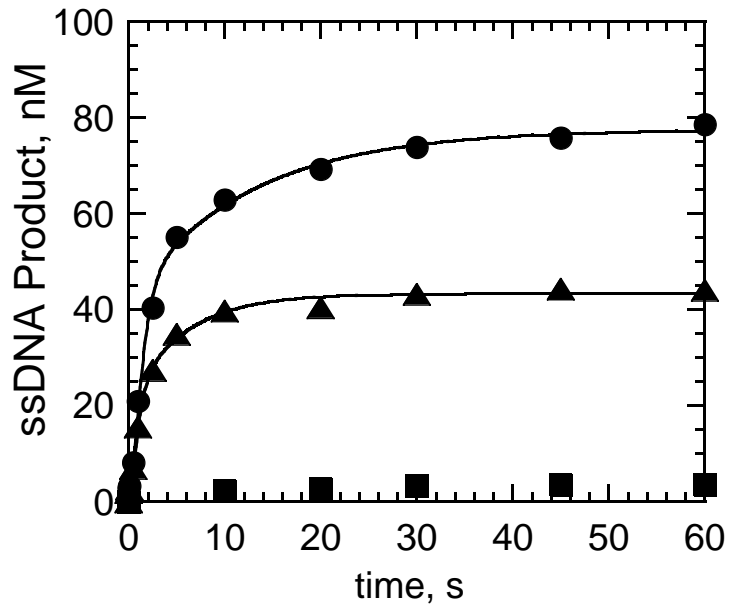


Figure 2

A



B



C

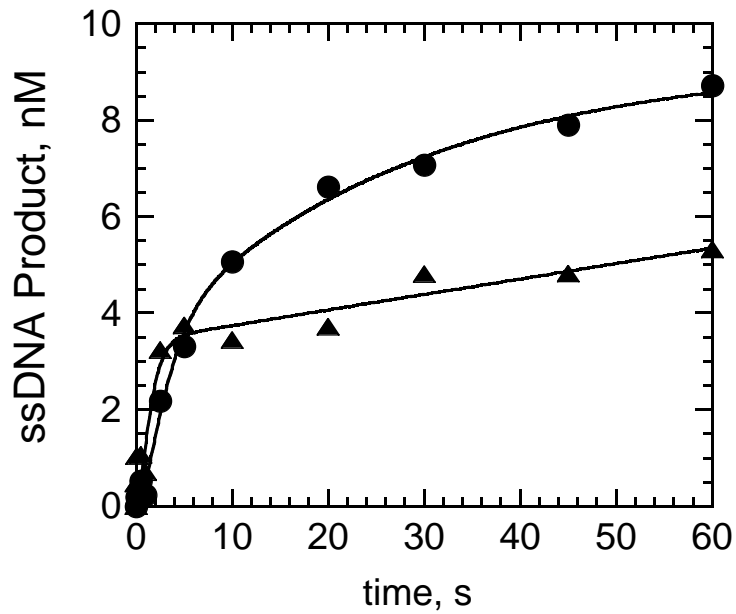


Figure 3

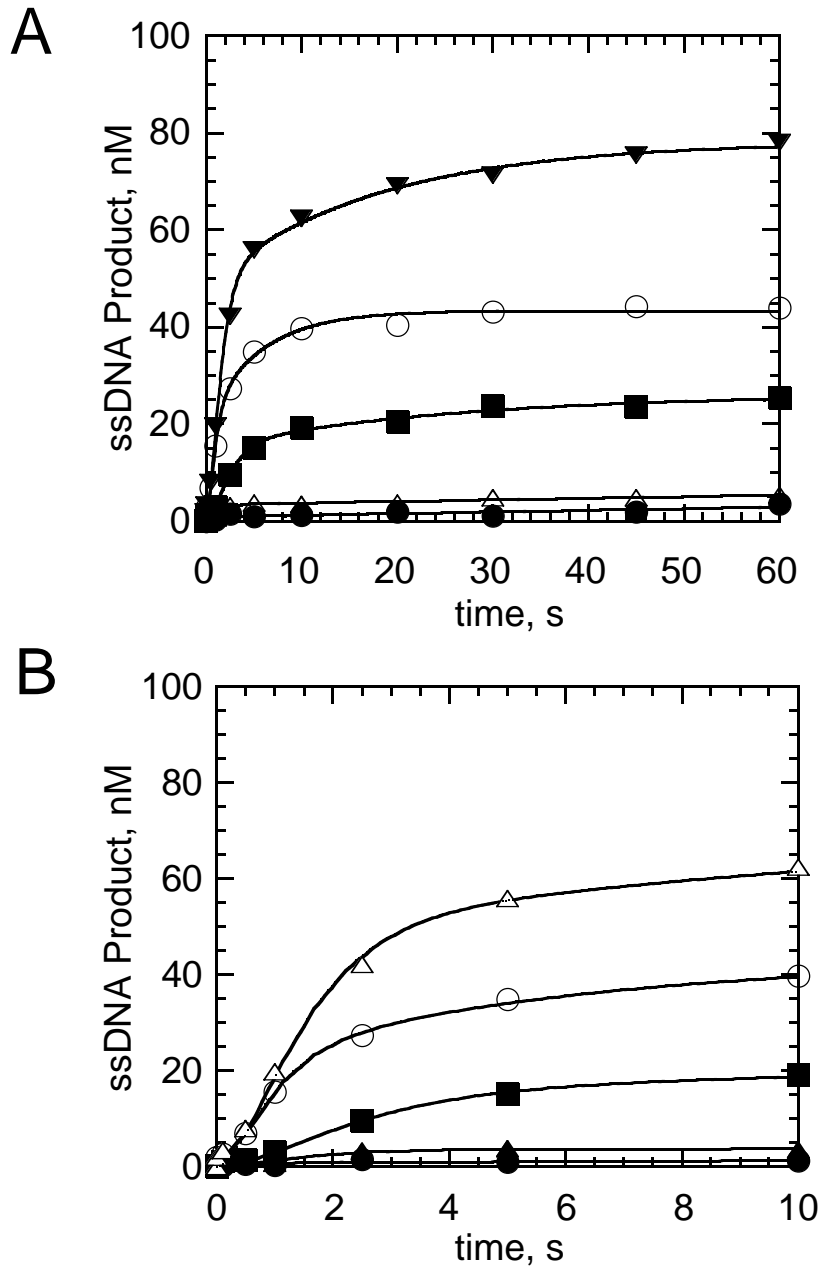
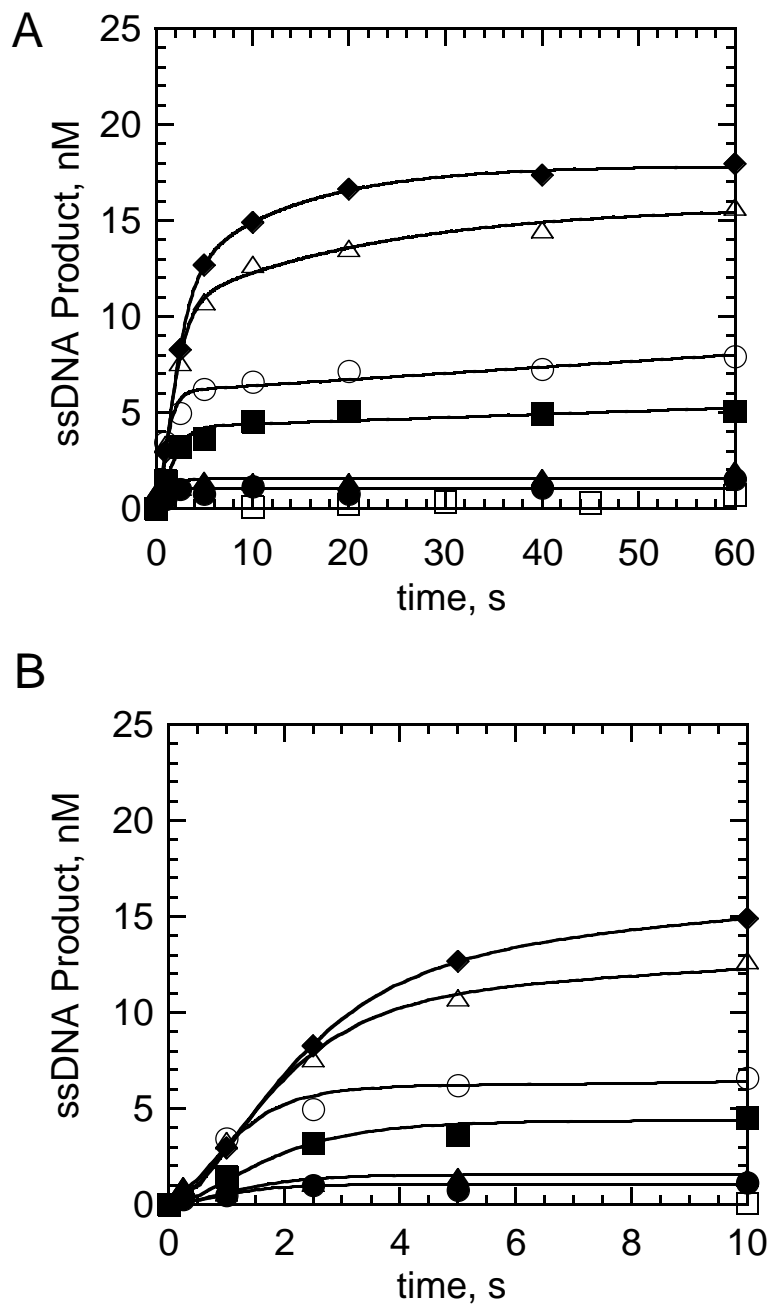
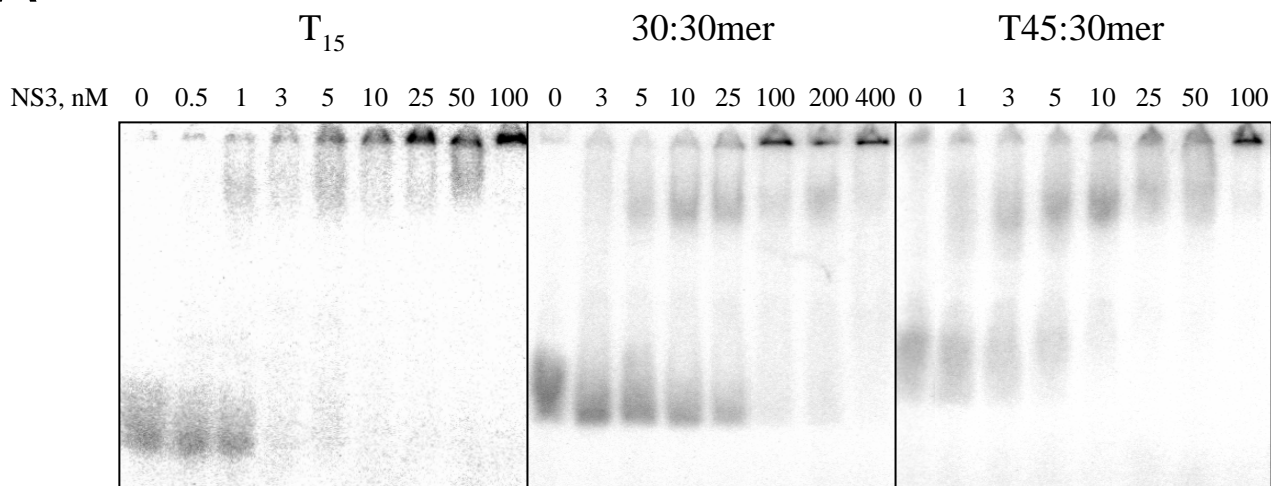


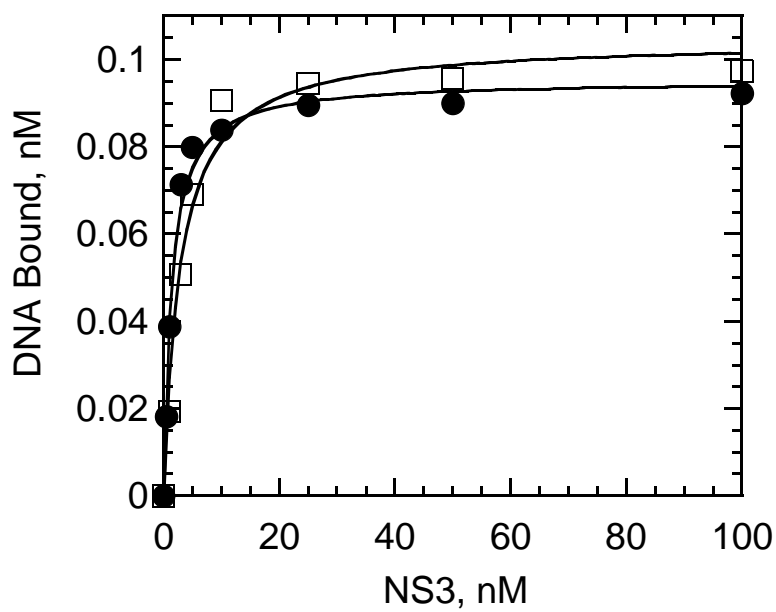
Figure 4



A



B



C

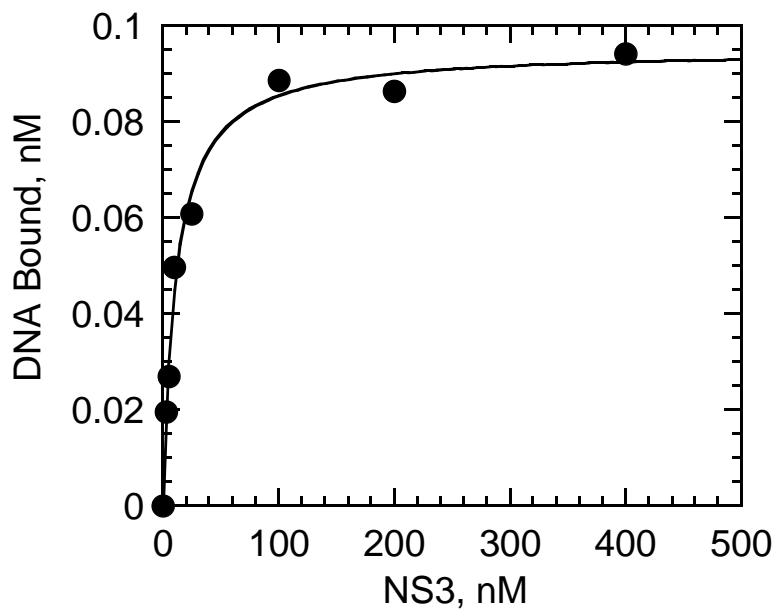


Figure 6

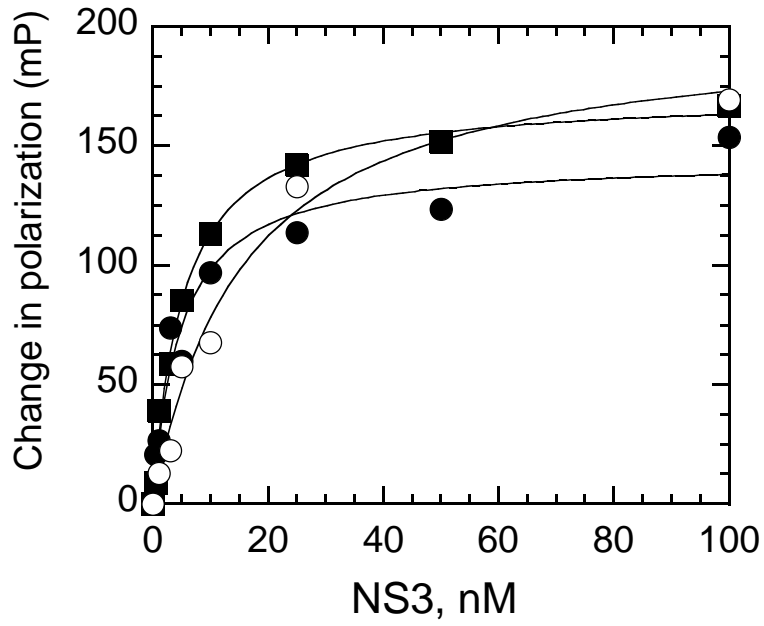
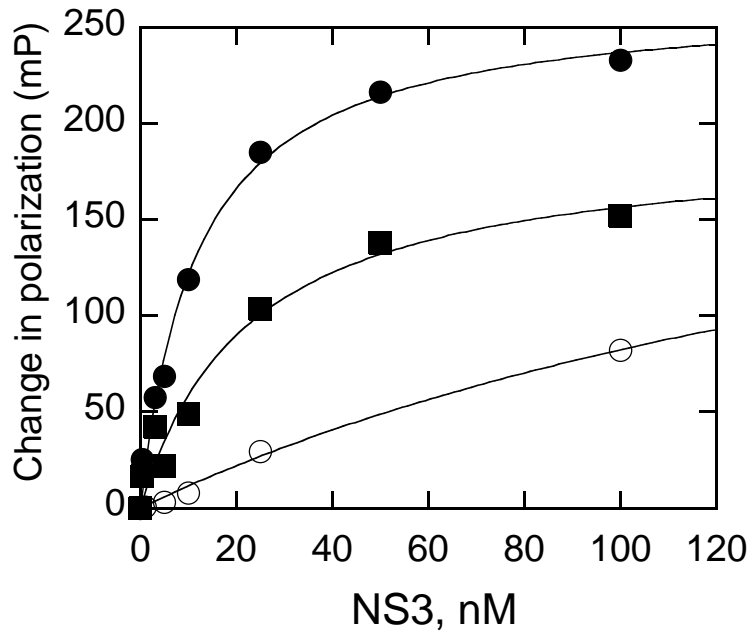
A**B**

Figure 7

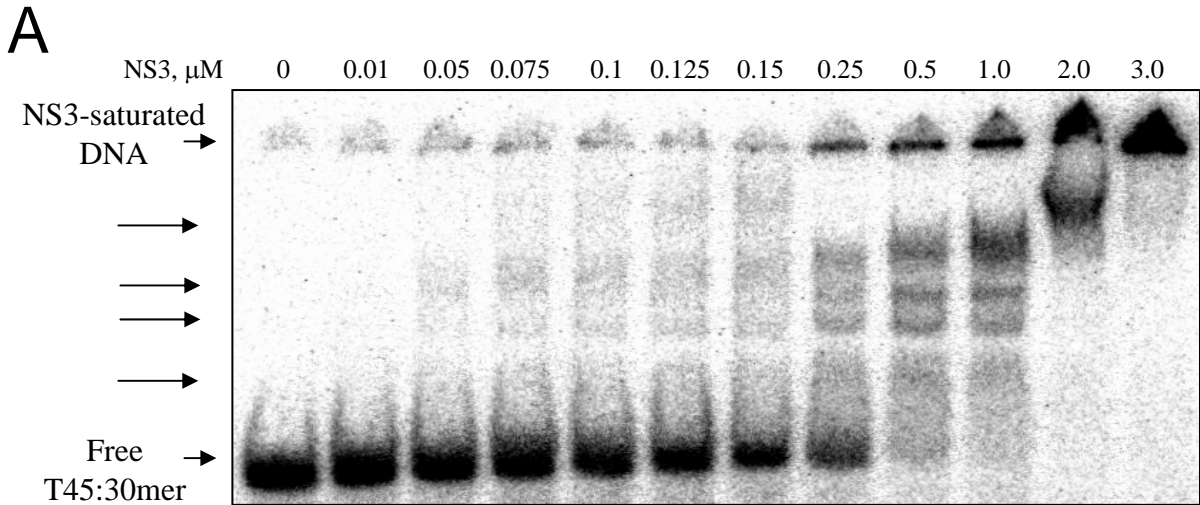
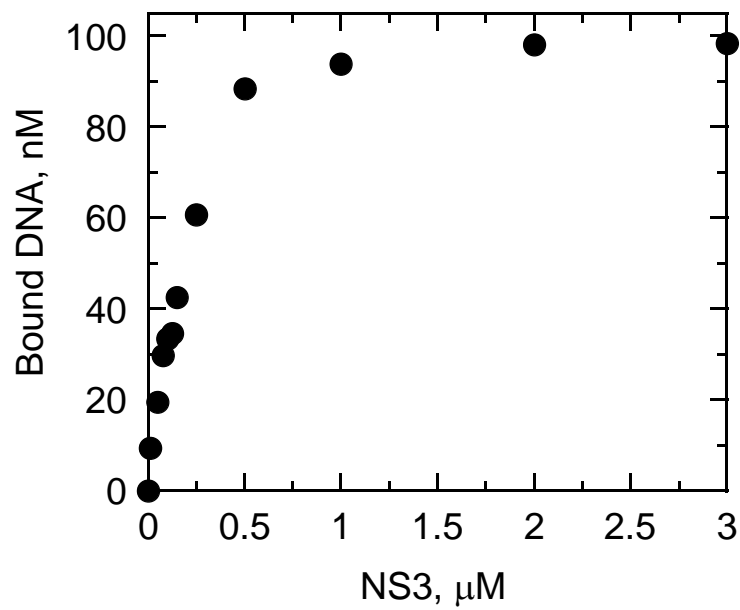
**B**

Figure 7C

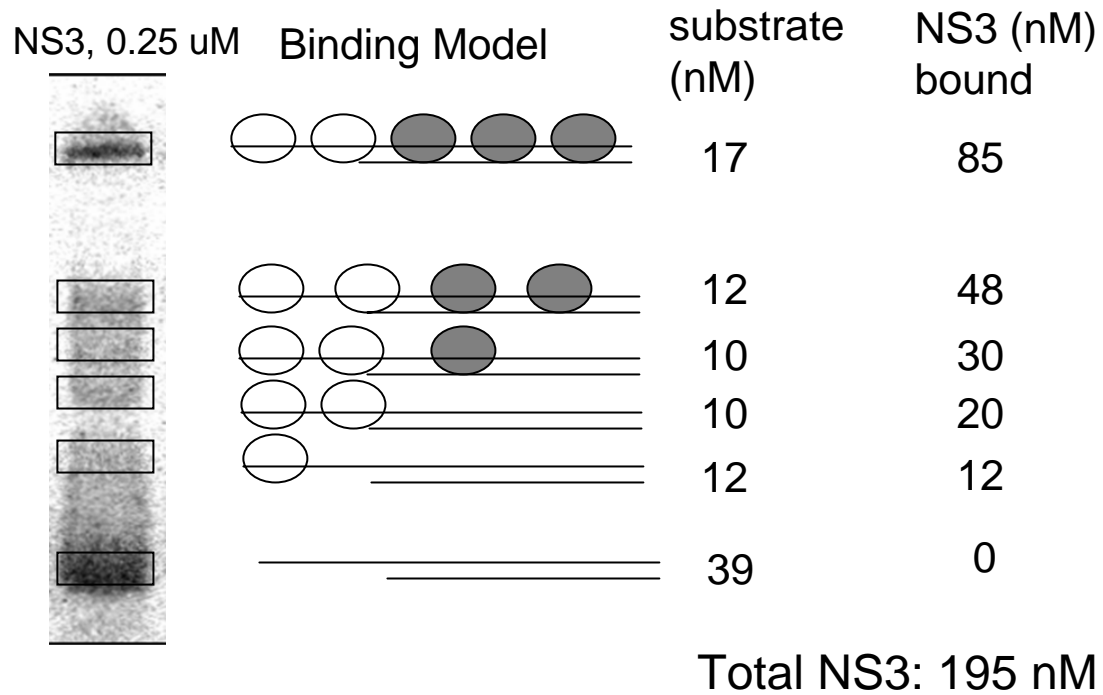
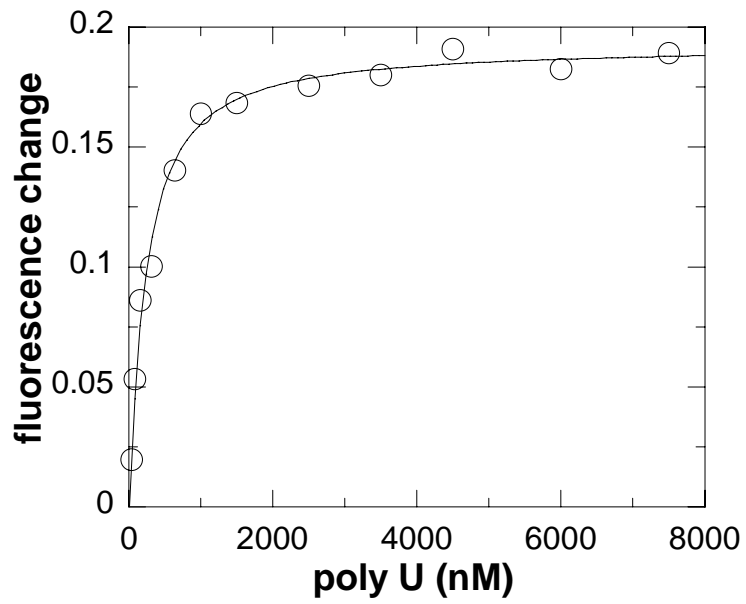
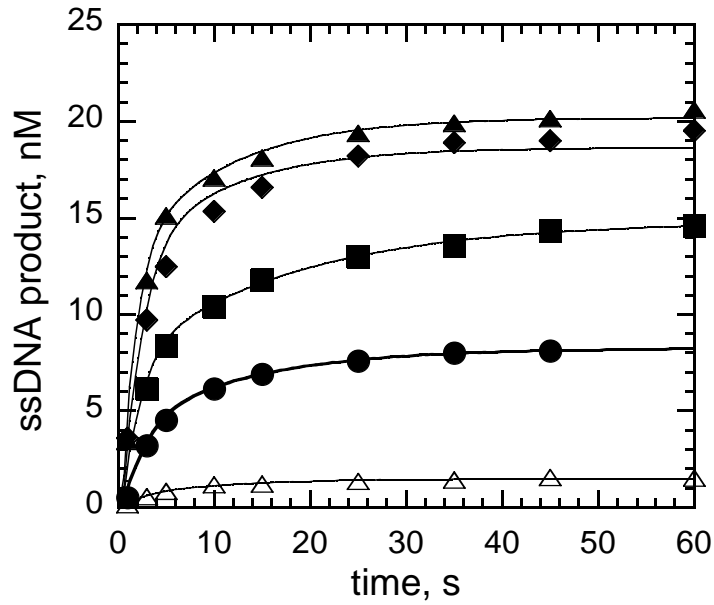


Figure 8



A



B

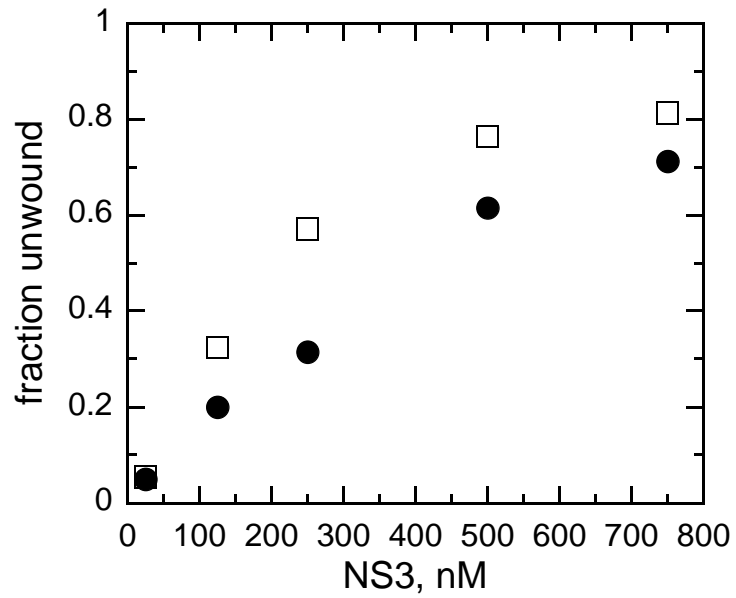


Figure 10

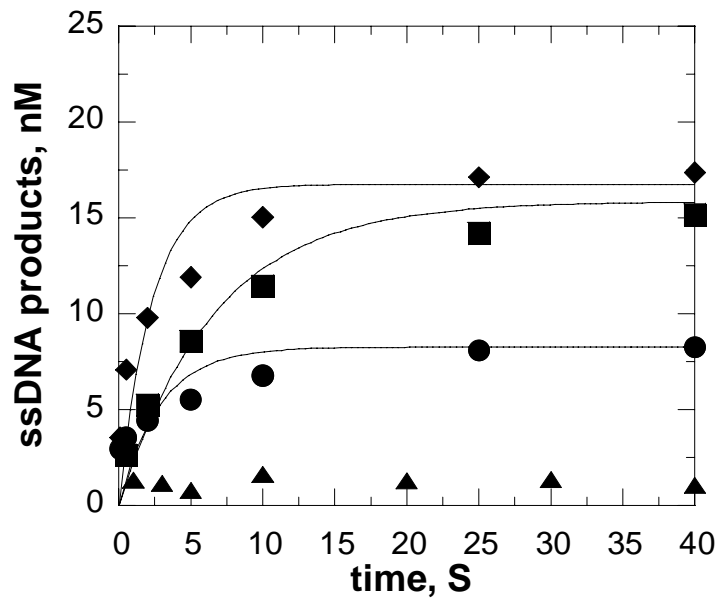
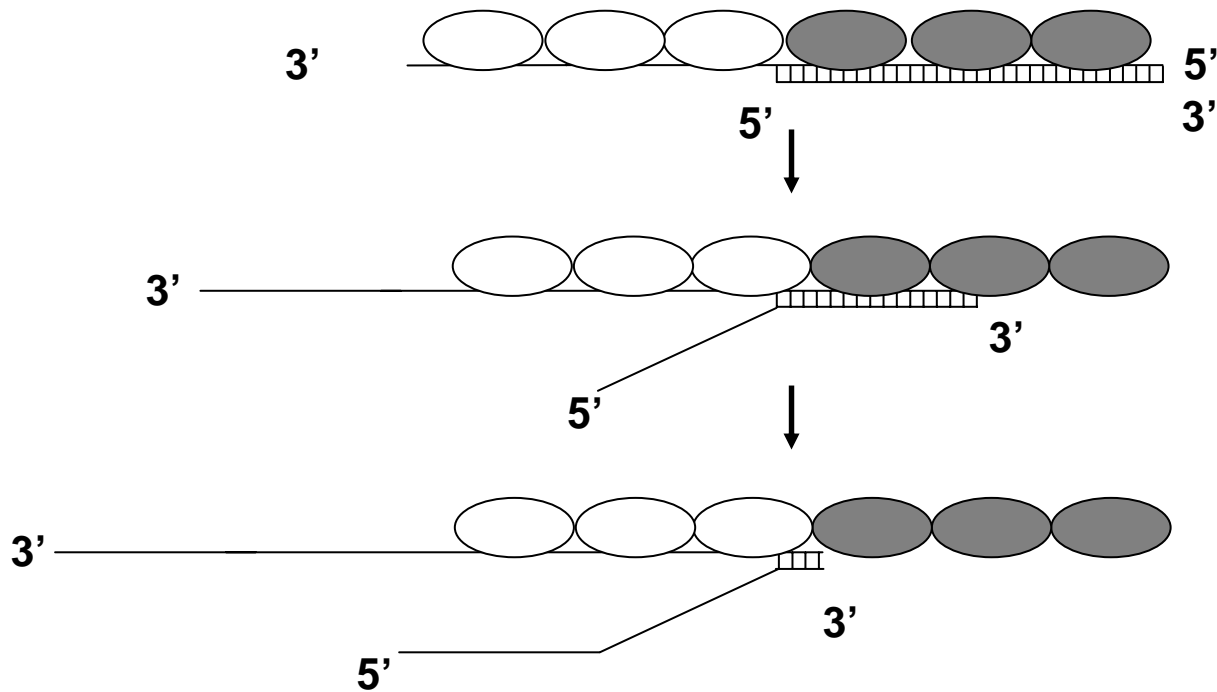
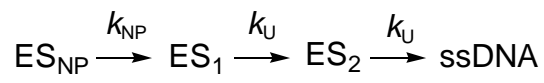


Figure 11





Scheme 1