Probing the Relationship between RNA-Stimulated ATPase and Helicase Activities of HCV NS3 Using 2′-*O*-Methyl RNA Substrates

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ABSTRACT: The hepatitis C virus (HCV) NS3 protein contains an amino terminal protease (NS3 aa. 1−180) and a carboxyl terminal RNA helicase (NS3 aa. 181−631). NS3 functions as a heterodimer of NS3 and NS4A (NS3/4A). NS3 helicase, a nucleic acid stimulated ATPase, can unwind RNA, DNA, and RNA:DNA duplexes, provided that at least one strand of the duplex contains a single-stranded 3′ overhang (this strand of the duplex is referred to as the 3′ strand). We have used 2′-*O*-methyl RNA (MeRNA) substrates to study the mechanism of NS3 helicase activity and to probe the relationship between its helicase and RNA-stimulated ATPase activities. NS3/4A did not unwind double-stranded (ds) MeRNA. NS3/4A unwinds hybrid RNA:MeRNA duplex containing MeRNA as the 5′ strand but not hybrid duplex containing MeRNA as the 3′ strand. The helicase activity of NS3/4A was 50% inhibited by 40 nM single-stranded (ss) RNA but only 35% inhibited by 320 nM ss MeRNA. Double-stranded RNA was 17 times as effective as double-stranded MeRNA in inhibiting NS3/4A helicase activity, while the apparent affinity of NS3/4A for ds MeRNA differed from ds RNA by only 2.4-fold. However ss MeRNA stimulated NS3/4A ATPase activity similar to ss RNA. These results indicate that the helicase mechanism involves 3′ to 5′ procession of the NS3 helicase along the 3′ strand and only weak association of the enzyme with the displaced 5′ strand. Further, our findings show that maximum stimulation of NS3 ATPase activity by ss nucleic acid is not directly related to procession of the helicase along the 3′ strand.

Hepatitis C virus (HCV),[1] a positive strand RNA virus, is a member of the genus *Flaviviridae* and is the major cause of parenterally transmitted and community acquired non-A, non-B hepatitis (*1*−*5*). Approximately 1% of the world's population is affected by the disease (*6*, *7*), and since over 50% of these individuals develop a chronic HCV infection, which is associated with the development of liver cirrhosis and hepatocellular carcinoma (*8*, *9*), there is an acute need to develop effective antiviral therapies to cure HCV associated hepatitis.

The genome of HCV contains 5′ and 3′ untranslated regions, which flank a single open reading frame encoding a polyprotein of approximately 3010 residues (*10*−*13*). The HCV polyprotein is processed by host and viral proteases into 10 mature proteins, including 6 nonstructural proteins: NS2; NS3; NS4A; NS4B; NS5A; NS5B (*14*−*19*). The N terminal portion of NS3 (NS3 residues 1−180) contains a chymotrypsin-like catalytic site and processes the nonstructural proteins downstream of the NS2−NS3 junction (*20*). The NS3−NS4A junction is cleaved in cis and yields a tightly associated heterodimer (NS3/4A) of NS3 and the 54 residue NS4A protein (*21*). The NS4A protein binds to the amino terminal portion of the NS3 protease domain and is a cofactor of NS3 protease activity (*22*−*24*).

The carboxyl terminal domain of NS3 (NS3 residues 181−631) contains all of the consensus sequences of a DEXH box RNA helicase/ATPase (*25*−*27*). We recently published the structure of this protein. Its three domains form a triangular molecule, which contains a deep groove separating its flexibly linked second domain from its closely packed N and C terminal domains (*28*). HCV NS3 possesses NTPase dependent ds RNA, ds DNA, and DNA/RNA hybrid duplex unwinding activity, but requires the presence of a 3′ single stranded region in the helicase substrate for unwinding to occur (*29*, *30*). Although it shows marginal preference for ATP as its substrate, HCV NS3 can also use other NTPs and dNTPs, and possesses both nucleic acid stimulated and nucleic acid independent NTPase activity (*25*). The NTPase activity of NS3 is stimulated by both ss DNA and ss RNA, with polyU and polydU being the preferred polynucleotides (*25*, *29*). The relative stimulation of HCV NS3 ATPase activity by polydeoxynucleotides is greater than that observed for related Pestivirus and Flavivirus enzymes (*25*). Because of the clinical significance of HCV, it would be beneficial to detect any unique features of its NS3 helicase. In the study presented here, we examine 2′-*O*-methyl RNA (MeRNA) as a helicase substrate for NS3/4A and as nucleic acid cofactor

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; ds, double stranded; dNTP, deoxynucleotide 5′-triphosphate; DTT, dithiothreitol; ds, double stranded; EDTA, ethylenediaminetetraacetic acid; HCV, hepatitis C virus; LSC, liquid scintillation counting; MeRNA, 2′-*O*-methyl RNA; Me-uridylate, oligo form of 2′-*O*-methyl uridylate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NS, nonstructural protein; NTP, nucleotide 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; polydU, poly(deoxyuridylate); polyU, poly(uridylate); SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda*; ss, single stranded; TBE, 100 mM Tris/pH 8.3/90 mM borate/1 mM EDTA; TE, 10 mM TrisCl/pH 7.7/1 mM EDTA; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; U_{40}, 40 base oligo form of uridylate.

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in its ATPase stimulation, to probe the mechanism of NS3/4A helicase activity and to study the relationship between its helicase and RNA-stimulated ATPase activities.

EXPERIMENTAL PROCEDURES

Materials. ATP was obtained from Sigma (St. Louis, MO). PolyU was obtained from Pharmacia (Piscataway, NJ). [γ^{32}P]-ATP was obtained from Amersham Corp. (Arlington Heights, IL). Purified RNA and 2′-*O*-methyl RNA (MeRNA) oligos were obtained from CyberSyn (Lenni, PA). PEI Cellulose-F TLC sheets were obtained from EM Science (Gibbstown, NJ). RNase block was obtained from Stratagene (La Jolla, CA). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA).

HCV NS3 Helicase Constructs. Residues are numbered according to their position in the NS3 protein. NS3/4A (H/1a) was cloned in a baculovirus vector, expressed in Sf9 cells, and purified as previously described (*21*). NS3/4A, the product of cis cleavage, is a heterodimer consisting of NS3 residues 1−631 (containing an amino terminal extension consisting of the seven residual residues of an engineered thrombin cleavage site) and the 54 residue NS4A protein, which is bound tightly to the amino terminal portion of the protease domain of NS3 (*21*, *31*). Purified NS3 Helicase (H/1a), consisting of NS3 residues 181−631, was prepared as previously described (*28*).

Helicase Substrates and Nucleic Acid Oligos. The substrates for the helicase assay were based on the design of Lee and Hurwitz (*21*, *32*). In helicase substrates containing one blunt end and one 3′ overhang, the strand contributing the 3′ single strand is referred to as the 3′ strand, and its complementary strand is referred to as the 5′ strand. The base sequence is identical in the duplex region of each substrate and contains 21 base pairs.

Substrate E: Strand

5′CUAGAGGAUCCCCGGGCGAGCUCCUAUAAGUGAGUCGU3′ E1
3′UAGCUUGCACAUAAGGAUCUCCUAGGGGCCCGCUCG5′ E2

Substrates A, B, C and D:

5′CUAGAGGAUCCCCGGGCGAGCUCCUAUAAGUGAGUCGU3′ E1
3′GAUCUCCUAGGGGCCCGCUCG5′ A2

Substrates F and G:

5′CUAGAGGAUCCCCGGGCGAGCUUUUUUUUUUUUUUUUU3′ F1
3′GAUCUCCUAGGGGCCCGCUCG5′ A2

SS RNA and MeRNA for ATPase Stimulation:

5′UUUUUUUUUUUUUUUUUCUAGAGGAUCCCCGGGCGAGC3′ F2

RNA and MeRNA versions of substrate E were prepared. Both strands of substrates A and F were composed of RNA. Both strands of substrates D and G were composed of MeRNA. Substrates B and C were RNA:MeRNA duplexes. Substrate B contained an RNA 3′ strand. Substrate C contained a MeRNA 3′ strand. The substrates were labeled by phosphorylating the 5′ terminus of the 38 base E1 strand (in the case of substrates E, A, B, and D) or the 5′ terminus of the 21 base A2 strand (in the case of substrates C, F, and G) with T4 polynucleotide kinase and [γ^{32}P]ATP (*33*). For annealing, each labeled strand was desalted on a Biorad Biospin column equilibrated with TE and then mixed with a 1.5 molar ratio of the complementary strand in TE. The mixture was heated to 94 °C in a heating block, and the strands were allowed to anneal as the mixture cooled to 23°C over 4 h. The concentration of duplex helicase substrate stocks was calculated from the concentration of the labeled strand, with an estimate of 85% oligo recovery from the BioSpin column.

Helicase Assay. Helicase activity measurement was based on published methods (*34*). The indicated concentration of NS3/4A was assayed in a 20 μL volume containing 0.1 M sodium MOPS buffer, pH 7.2, 0.4 U/μL RNase Block, 4 mM ATP, 2.5 mM $MgCl_2$, 2.4 mM DTT, and 0.18 mg/mL BSA. After incubation at 37 °C for 1 h, the activity was quenched with 4 μL of 0.5 M EDTA and 30 μL of 0.125 M TrisCl, pH 6.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue, and 10% β-mercaptoethanol. The samples were fractionated by electrophoresis on 15% polyacrylamide gels run at 30 mA per gel a 4 °C in Tris/glycine/SDS. The dried gels were autoradiographed, and ^{32}P in the bands corresponding to ss RNA or ds RNA was measured by LSC.

ATPase Assay. ATPase activity was determined by measuring orthophosphate generation using TLC (*35*). In this assay 60 nM NS3/4A was incubated at 25 °C with the indicated concentrations of nucleic acid in 100 μL of 0.1 M sodium MOPS buffer, pH 7.2, 0.2 mg/mL BSA, 0.2 mM DTT, 2.5 mM $MgCl_2$, and 3 mM Mg−ATP (containing [γ^{32}P]ATP). Aliquots were quenched at selected times with one volume of 0.5 M EDTA and spotted on PEI-cellulose TLC sheets, which were then developed with 0.375 M potassium phosphate at pH 3.5. The dried sheets were autoradiographed, and ^{32}P in the regions corresponding to ATP or orthophosphate was measured by LSC.

Gel Shift Assay. The indicated concentrations of NS3/4A or NS3 Helicase were incubated at 22 °C with 1.2 nM of 5′ ^{32}P-labeled RNA or MeRNA oligos in 50 μL of 0.1 M sodium MOPS buffer, pH 7.2, 2.5 mM $MgCl_2$, 0.2 mM DTT, and 90 μg/mL BSA for 1 h. A 10 μL volume of 50% glycerol was then added, and each sample was fractionated by 8% or 15% nondenaturing PAGE in 1 X TBE at 30 mA per gel at 4 °C (*36*). Free RNA or MeRNA was detected by autoradiography, and ^{32}P in the corresponding gel region was measured by LSC.

General Methods. Quantitative UV absorption measurements of ATP and RNA were made in 20 mM sodium phosphate, pH 7.0, and 0.1 M NaCl. The concentration of ATP was determined using an ϵ_{259} of 15.4×10^4 M^{-1} cm^{-1}. The concentrations of polyU and U_{40} were determined using a base ϵ_{260} of 9.35×10^3 M^{-1} cm^{-1}. Estimates of the concentration of strand E1, strand E2, strand A2, and strand F1 were based on calculated ϵ_{260} (M^{-1} cm^{-1}) of 3.85×10^5, 3.65×10^5, 1.97×10^5 and 3.47×10^5, respectively. The protein concentrations of NS3/4A and NS3 helicase were routinely determined by the method of Bradford (*37*) and were correlated to values determined by amino acid analysis.

RESULTS

MeRNA as a Helicase Substrate. The suitability of ds RNA species as helicase substrates was assessed by assaying the unwinding of a fixed concentration of substrate with increasing concentrations of NS3/4A. In Figure 1, the unwinding of RNA substrate E by NS3/4A showed a linear response to enzyme addition up to a concentration of 32 nM, but

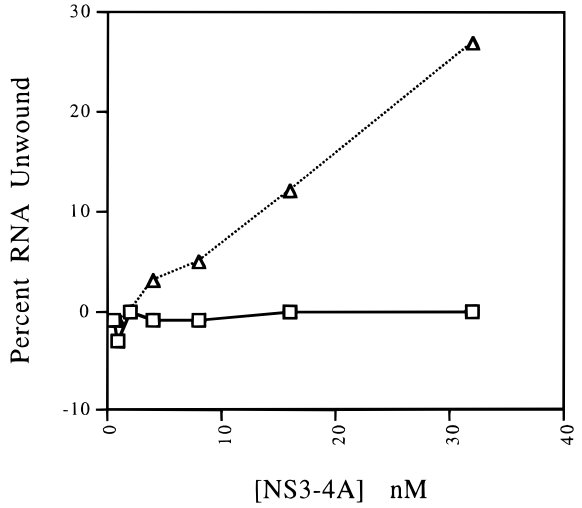

FIGURE 1: Relative duplex nucleic acid unwinding activity of NS3/4A with RNA or MeRNA substrates. The helicase activity of the indicated concentrations of NS3/4A were assayed as described in Experimental Procedures with 3 nM RNA substrate E (△), or 3 nM MeRNA substrate E (□). The percent of the substrate which was unwound after a 1 h incubation with NS3/4A at 37 °C is plotted versus NS3/4A concentration.

MeRNA substrate E was not unwound at any helicase concentration tested.

RNA and MeRNA Helicase Substrates with a 3′ Uridylate Single Strand. To study whether the RNA/MeRNA substrate specificity of NS3/4A might be explained by affinity differences for the single-stranded region of the substrate's 3′ strand, duplex helicase substrates were constructed which contained one blunt end and a single 17 base 3′ overhang consisting of uridylate. The substrates consisted entirely of RNA or entirely of MeRNA (substrates F and G, respectively, in Experimental Procedures) and contained identical base sequences. We posited that the preference of NS3/4A for single-stranded oligos of uridylate for stimulation of its ATPase activity (*25*, *29*) might overcome any affinity related effects precluding the unwinding of MeRNA substrates. The results in Figure 2 indicate that only the RNA substrate containing the 3′ uridylate single-stranded region was unwound by NS3/4A. The MeRNA substrate containing the 3′ Me-uridylate single-stranded region was not unwound by NS3/4A. The Me-uridylate sequence in the single-stranded 3′ overhang did not improve the suitability of MeRNA as a helicase substrate.

Strand Orientation and Helicase Substrate Selectivity. To determine the basis for the selectivity of NS3/4A for RNA over MeRNA in the helicase assay, four helicase substrates were constructed. Each of these substrates consisted of strand E1 annealed to strand A2. All of the substrates contained one blunt end and a single 17 base 3′ overhang, which was contributed by strand E1 (the 3′ strand). The entire base sequence of all four substrates was identical. Substrates A and D consisted of RNA and MeRNA versions of this substrate, respectively. Substrates B and C were hybrid versions of the substrate: substrate B contained MeRNA in the 5′ strand; substrate C contained MeRNA in the 3′ strand. The helicase activity of NS3/4A with each of these substrates is summarized in Figure 3. Substrates A and B were unwound by NS3/4A to a similar extent, but no measurable proportion of substrates C or D were unwound, even by NS3/4A

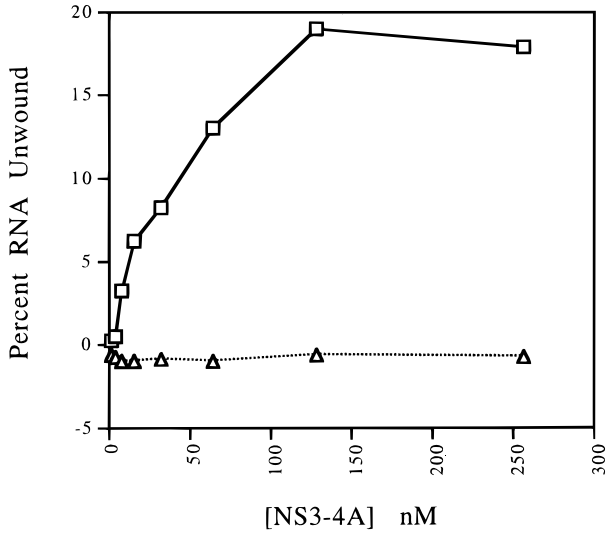

FIGURE 2: Effect of 17 base uridylic acid ss region on unwinding of ds MeRNA by NS3/4A. Both substrates F and G consist of a blunt end 21 base duplex of strands F1 and A2 with a single 17 base 3′ ss uridylic acid region contributed by strand F1 (see Experimental Procedures). The substrates contain an identical base sequence. Substrate F consists of RNA and substrate G consists of MeRNA. The helicase activity was assayed as described in Experimental Procedures, with the indicated concentrations of NS3/4A and either 2.5 nM of RNA substrate (□) or 2.5 nM of MeRNA substrate (△). The percent of substrate which was unwound after a 1 h incubation with NS3/4A at 37 °C is plotted versus NS3/4A concentration.

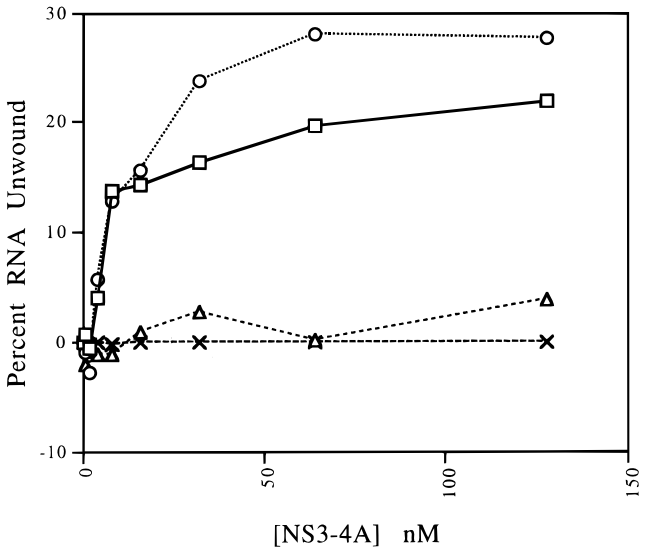

FIGURE 3: Substrate strand specificity of NS3/4A helicase activity. The helicase activity of NS3/4A was assayed with 4 substrates (A−D), consisting of a duplex of strands E1 and A2, containing one blunt end and one 17 base 3′ ss overhang contributed by strand E1 (the 3′ strand). The base sequences of all four substrates were identical. The helicase activity was assayed as described in Experimental Procedures, with the indicated concentrations of NS3/4A and 2.5 nM of either substrate A, consisting of RNA in both strands (□); substrate B, a hybrid substrate containing an RNA 3′ strand (○); substrate C, a hybrid substrate containing a MeRNA 3′ strand (×); or substrate D, consisting of MeRNA in both strands (△). The percent of substrate which was unwound after a 1 h incubation with NS3/4A at 37 °C is plotted versus NS3/4A concentration.

concentrations as high as 130 nM. Helicase substrate containing MeRNA only in the 5′ strand is unwound by NS3/4A as efficiently as the homogeneous RNA substrate.

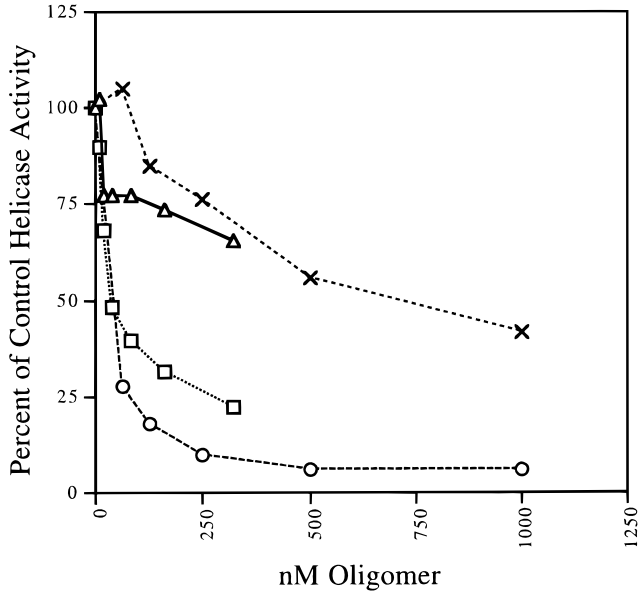

FIGURE 4: Inhibition of NS3/4A helicase activity by RNA or MeRNA. The helicase activity of 32 nM NS3/4A was assayed as described in Experimental Procedures, with 5 nM RNA substrate E, plus or minus the indicated concentration of either RNA strand E1 (□) or MeRNA strand E1 (△) or with 2.5 nM substrate E, plus or minus the indicated concentration of unlabeled RNA substrate E (○) or unlabeled MeRNA substrate E (×). Strand E1 is a heterogeneous 38 base strand, containing the same sequence as the 5′ ^{32}P-labeled strand of substrate E (Experimental Procedures). Note that base pairing of strand E1 with its complementary strand would prevent reannealing of the labeled substrate E strand and so would only be expected to increase the final percentage of unwound substrate by interaction with product RNA strands. The percent of control helicase activity is plotted versus the concentration of RNA oligomer.

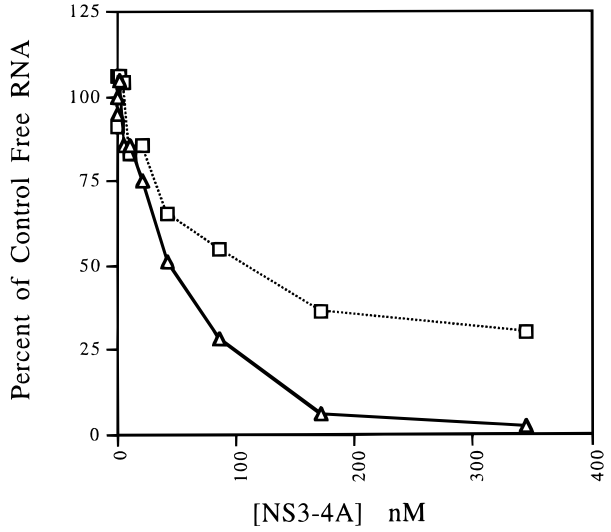

FIGURE 5: Equilibrium binding of RNA substrate E and MeRNA substrate E by NS3/4A. The indicated concentrations of NS3/4A were incubated with 1.2 nM 5′ ^{32}P-labeled substrate E (△), or 1.2 nM 5′ ^{32}P labeled MeRNA substrate E (□) at pH 7.2 and 22 °C and fractionated by nondenaturing PAGE as described in Experimental Procedures. The percent of the control free RNA (sample incubated with carrier protein minus NS3/4A) is plotted versus the concentration of NS3/4A in the sample.

However, substrates containing a MeRNA 3′ strand are not unwound by NS3/4A. This demonstrates the importance of the 3′ strand in RNA unwinding by HCV NS3, consistent with an RNA unwinding mechanism in which the helicase only engages the 3′ strand.

MeRNA as an Inhibitor of Helicase Activity. The helicase assay results with MeRNA substrates indicate that MeRNA might serve as a stable model for NS3 helicase inhibitor design. To determine whether ss MeRNA would bind at the helicase active site with affinity comparable to ss RNA and inhibit ds RNA unwinding, NS3/4A was assayed with substrate E in the presence of increasing concentrations of either RNA strand E1 or MeRNA strand E1. RNA strand E1 is a 38 base strand and has a heterogeneous base composition (see Experimental Procedures). The results in Figure 4 show that ss MeRNA is a much less effective inhibitor of NS3/4A helicase activity than ss RNA. MeRNA containing a 21 base pair duplex region was also a poor inhibitor of NS3/4A helicase activity relative to its RNA counterpart. In this experiment, 2.5 nM of RNA substrate was diluted with either cold RNA substrate or cold MeRNA substrate in the helicase assay. Cold RNA substrate was more than 17 times as effective as cold MeRNA substrate in inhibiting the unwinding of labeled RNA substrate by NS3/4A (Figure 4).

Binding Affinities of NS3/4A and NS3 Helicase for MeRNA. We have found that double-stranded helicase substrates containing a MeRNA 3′ strand are not unwound by NS3/4A and that neither ss MeRNA nor ds MeRNA competes with RNA substrate at the helicase active site as effectively as ss RNA or ds RNA. We wished to determine whether this is due to a difference in the binding affinities of RNA and MeRNA for NS3. The equilibrium binding affinities of NS3 for the nucleic acid species were compared by gel shift assay. Because of the time necessary for the separation of free RNA from protein-bound RNA using this method, the NS3 protein concentrations yielding 50% binding are only approximations of the dissociation constant (K_d) and probably indicate an upper limit for this value. The method is useful however for comparing the apparent equilibrium binding affinities of nucleic acid ligands for NS3 protein. The apparent K_ds of NS3/4A and ds RNA and ds MeRNA were 45 and 110 nM, respectively (Figure 5). The apparent K_ds of NS3 Helicase and ss RNA and ss MeRNA were 32 and 107 nM, respectively (Figure 6). These small differences between the affinities of MeRNA and RNA species for NS3/4A and NS3 helicase cannot account for the poor productive binding of MeRNA by helicase in the steady state, either as helicase substrates or as inhibitors of helicase activity.

Stimulation of NS3/4A ATPase Activity by MeRNA. It is useful to determine whether there is a correlation between the preference of NS3/4A for RNA over MeRNA as the 3′ strand of the helicase substrate and the relative stimulation of its ATPase activity by RNA, or MeRNA. To determine this, the ATPase activity of NS3/4A was measured with or without polyU, RNA, or MeRNA. Comparisons of the ATPase stimulation by RNA and MeRNA oligos are listed in Table 1 and show that MeRNA stimulates NS3/4A ATPase activity as well or better than RNA. The effects of MeRNA in the helicase substrate 3′ strand (Figures 2 and 3), and as a single-stranded inhibitor of NS3/4A helicase activity (Figure 4), do not correlate with its stimulation of ATPase activity (Table 1).

The RNA or MeRNA base compositions had a dramatic effect on stimulation of NS3/4A ATPase activity. Uridylate or Me-uridylate was much more potent in stimulating

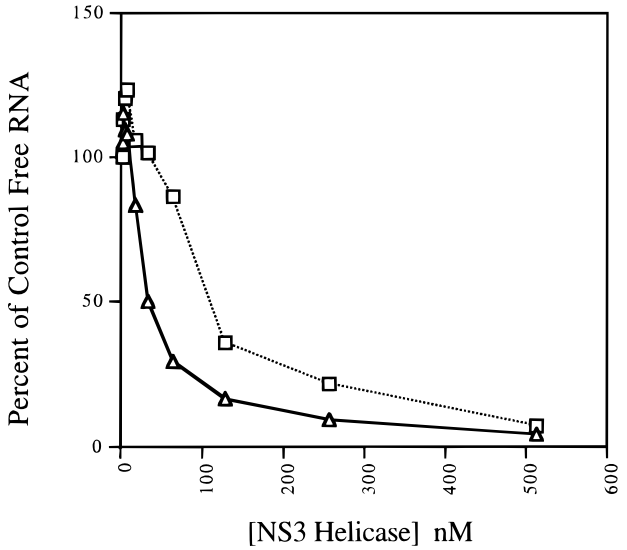

FIGURE 6: Equilibrium binding of ss RNA by NS3 helicase. The indicated concentrations of NS3 helicase were incubated with 1.2 nM of ^{32}P-labeled RNA strand E2 (△) or 1.2 nM of ^{32}P labeled MeRNA strand E2 (□) for 1 h at 22 °C at pH 7.2 as described in Experimental Procedures (strand E2 is the 36 base strand of substrate E). The free and bound RNAs were fractionated by nondenaturing PAGE as described. The percent of the control free RNA (sample incubated with carrier protein minus NS3/4A) is plotted versus the concentration of NS3 Helicase.

Table 1: Stimulation of the ATPase Activity of NS3/4A by RNA and MeRNA

	percent of max ATPase stimulation[a]	
nucleic acid	RNA	MeRNA
125 μM strand E1	13	15
6 μM strand F1	36	48
6 μM strand F2	50	76
1 μM U_{40}	59	77

[a] The maximum stimulation of ATPase activity in each study was determined using the assay as described in Experimental Procedures and a polyU concentration [uridine base] of 0.75 mM. The average calculated k_{cat} for the nucleic acid independent ATPase activities and the nucleic acid stimulated ATPase activities of NS3/4A were 0.98 ± 0.10 and 20.4 ± 0.7 s^{-1}, respectively.

ATPase activity than heterogeneous RNA or MeRNA (Table 1). The sensitivity of NS3 ATPase activity to stimulation by oligos of uridylate has been reported by other investigators (*25*, *29*) and accounts for the effect that a 17 base stretch of uridylate on the 3′ or 5′ side of the central E1 sequence has on stimulation of NS3/4A ATPase activity (compare the effect of strand E1 with strand F1 and F2 on ATPase activity in Table 1). Addition of 125 μM RNA or MeRNA strand E1 induced only a 2.5−3-fold stimulation of NS3/4A ATPase activity (Table 1). The results presented in Figure 4 indicate that 40 and 320 nM concentrations of RNA strand E1 inhibit NS3/4A helicase activity by 50% and 75%, respectively. Even 1 μM RNA strand E1 does not measurably stimulate NS3/4A ATPase activity (data not shown).

DISCUSSION

Oligonucleotides of 2′-*O*-methyl RNA (MeRNA) have been used as antisense probes of RNA processing and studied as possible therapeutic agents (*38*, *39*). MeRNA is resistant to nuclease degradation (38), and though its ribose phosphodiester backbone is sterically similar to DNA, MeRNA:RNA duplexes exhibit greater thermal stability than the corresponding DNA:RNA duplexes (*38*, *40*). RNA helicase substrate E was the substrate routinely used in our laboratory. It consists of a 21 base pair duplex with heterogeneous 17 base and 15 base 3′ overhangs. Since the MeRNA version of substrate E was found to be resistant to unwinding by HCV NS3/4A (Figure 1), duplex and ss MeRNA oligos were used to study the helicase mechanism of NS3/4A and to probe the relationship between its helicase activity and its nucleic acid stimulated ATPase activity.

We found that neither ss nor ds MeRNA (the duplex here was helicase substrate E) inhibited the helicase activity of NS3/4A as well as the same sequence of RNA (Figure 4). The apparent equilibrium binding affinities of RNA and MeRNA for NS3/4A and NS3 helicase differed by only 2−3-fold (Figures 5 and 6), and there was little difference between the apparent steady-state affinities of NS3/4A for RNA and MeRNA, as measured in nucleic acid stimulation of ATPase activity (Table 1). The preference of NS3/4A for oligos of uridylate over RNA or MeRNA of heterogeneous base composition is evident from the table. The relative efficacy of MeRNA and RNA in stimulation of NS3/4A ATPase activity was U40 > strand F2 ≥ strand F1 ≫ Strand E1. Incorporation of the 17 base length of uridylate on the 3′ or 5′ side of the 5′ 21 bases of strand E1 dramatically increased the efficacy of the oligos in stimulating NS3/4A ATPase activity. Whether the different degrees of stimulation of NS3/4A ATPase activity by the oligos listed in Table 1 is due solely to differences in affinity of the enzyme for the oligos, or whether it is due to differences in maximum rate enhancement by these sequences (*25*), the patterns of stimulation by the RNA and MeRNA sequences were similar. In fact the MeRNA oligonucleotides effected a slightly greater ATPase stimulation of NS3/4A than did their RNA counterparts.

This preference for MeRNA for ATPase stimulation contrasts with the effect of MeRNA on NS3/4A helicase activity. Helicase substrates containing MeRNA 3′ strands were not unwound by NS3/4A (Figures 2 and 3). However, the presence of MeRNA in only the 5′ strand of the substrate had no effect on helicase activity (Figure 3). The relative importance of the 3′ and 5′ helicase substrate strands, consistent with HCV NS3 functioning as a 3′−5′ helicase (*29*, *30*), supports a helicase mechanism in which this enzyme, by engaging the 3′ strand during unwinding, passively displaces the 5′ strand.

The two models that have been proposed for helicase function are an inchworm, or passive, mechanism (*41*), as described above, and an active, or rolling, mechanism (*42*). The rolling mechanism requires at least a dimeric quaternary structure, but the passive mechanism is consistent with the helicase functioning as a monomer. It has been reported that HCV NS3 helicase demonstrates no substrate or concentration dependent oligomerization (*43*, *44*). The monomeric state of NS3 and its sensitivity to the composition of only the 3′ strand are both consistent with a passive unwinding mechanism for HCV helicase (*44*). However, the evidence presented here that NS3/4A only engages the 3′ strand of the helicase and that the 5′ strand has a more passive role does not alone rule out the rolling mechanism for HCV

helicase. In the proposed rolling mechanism of a dimeric 3′−5′ helicase, the primary role of the 3′ strand could be accounted for, because, in this model, each monomer binds only to the 3′ strand when in its single strand binding mode (*42*, *45*).

According to the passive model of NS3 helicase activity, after initiation on the single stranded portion of the 3′ strand, NS3 would process along the 3′ strand in a 3′−5′ direction, coupling movement along the strand to NTP hydrolysis and passively unwinding the duplex (*46*, *47*). It is tempting to relate HCV helicase procession along the 3′ strand to the phenomenon of single-stranded nucleic acid stimulation of the enzyme's ATPase activity. Our results suggest a more complex relationship between these two enzymatic activities of HCV helicase. HCV helicase distinguishes between RNA and MeRNA versions of strand E1 and F1 as 3′ strands in helicase substrates (Figures 2 and 3) but not as activators of ATPase activity (Table 1). The presence of 2′-*O*-methyl ribose in the 3′ strand of the RNA duplex might have a specific inhibitory effect upon the procession of a 3′−5′ helicase. However, the relative ineffectiveness of ss MeRNA (compared to ss RNA) as an inhibitor of helicase substrate unwinding by NS3/4A (Figure 4) indicates a specific difference between the affinities of RNA and MeRNA for an RNA binding site on the helicase essential for unwinding activity. There is little difference in the efficacy of the same sequence of MeRNA or RNA in the stimulation of NS3/4A ATPase activity (Table 1, strand E1). There is a dramatic difference between the concentration of heterogeneous base composition RNA oligomer (strand E1) necessary for inhibition of helicase activity (Figure 4) and the concentration necessary to induce measurable stimulation of ATPase activity (Table 1). All these results indicate that the binding of ss RNA by NS3/4A necessary for maximum stimulation of ATPase activity is not directly related to nucleic acid duplex unwinding.

One implication of these conclusions is that the basal (unstimulated) ATPase rate of HCV NS3 is more relevant to its RNA unwinding activity than the fully stimulated ATPase rate induced by oligomers of uridylate. The 20-fold stimulation of the ATPase rate induced by polyU (Table 1) may be an artifactual or vestigial activity of the enzyme or it may be the manifestation of an activity relevant as an accessory function of NS3 in vivo. The 3′ untranslated region of the HCV genome contains a polyU stretch just upstream of the terminal 3′ X tail sequence (*13*, *48*). Perhaps the polyU stimulated ATPase activity of NS3 enhances processing of RNA structure in this region of the viral genome. It is also possible that this phenomenon is related to an in vivo modulation of HCV helicase activity by single stranded 3′ or displaced single-stranded 5′ strands of partially unwound RNA duplex.

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