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The Efficacy of siRNAs against Hepatitis C Virus Is Strongly Influenced by Structure and Target Site Accessibility

Selena M. Sagan,[1,3,*] Neda Nasheri,[1] Christian Luebbert,[1] and John Paul Pezacki[1,2,*]
[1]Steacie Institute for Molecular Sciences, The National Research Council of Canada, Ottawa, ON K1A 0R6, Canada
[2]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1N 6N5, Canada
[3]Present address: Department of Microbiology and Immunology, Stanford University, Sherman Fairchild Building, Stanford, CA 94305-5124, USA
*Correspondence: smsagan@stanford.edu (S.M.S.), john.pezacki@nrc-cnrc.gc.ca (J.P.P.)
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SUMMARY

Hepatitis C virus (HCV) is a global health problem. Designing therapeutic agents that target HCV's RNA genome remains challenging. HCV genomic RNA is large and highly structured with long-range genome-scale ordered RNA structures. Predicting the secondary- and tertiary-structure elements that reveal the accessibility of target sites within HCV RNA is difficult because of the abundance of long-range interactions. Target site accessibility remains a significant barrier to the design of effective therapeutics such as small interfering RNAs (siRNAs) against different strains of HCV. Here we developed two methods that interrogate the folding of HCV RNA, an approach involving viral RNA microarrays (VRMs) and an HCV viral RNA-coated magnetic bead-based (VRB) assay. VRMs and VRBs were used to determine target site accessibility for siRNAs designed against the HCV genome. Both methods predicted potency of siRNAs in cell-culture models for HCV replication that are not easily predicted by informatic means.

INTRODUCTION

Hepatitis C virus (HCV) infection is a rapidly increasing global health concern, with over 200 million people infected worldwide. In most infected individuals, HCV establishes a chronic infection that can lead to cirrhosis, hepatocellular carcinoma, and death. Currently, there is no vaccine available and antiviral therapy, which consists of combination therapy with pegylated-IFNα 2a and ribavirin, achieves sustained response rates in only approximately 50% of infected patients (McHutchison et al., 2006). Although new antiviral agents are in development, the rapid development of resistance suggests that multiple drugs may be needed to limit the emergence of drug-resistant strains (Randall et al., 2003). There is thus an urgent need to develop effective preventative and alternative therapeutic strategies for HCV infection.

HCV is a positive-sense RNA virus that replicates through a double-stranded (ds) RNA intermediate in the cytoplasm of host cells. Its genome encodes an ~3000 amino acid polyprotein which is cleaved, by host and viral proteases, into three structural proteins (core, E1, and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). As for all RNA viruses, the positive-sense HCV RNA genome serves as a template for translation, negative-strand synthesis, and packaging into virions. A number of well-defined *cis*-acting RNA elements have been identified that mediate these processes during the HCV life cycle (Le et al., 1995; Tanaka et al., 1995, 1996; Wang et al., 1995; Wang and Siddiqui, 1995; Kolykhalov et al., 1996; Blight and Rice, 1997; Friebe et al., 2001, 2005; Friebe and Bartenschlager, 2002; Kim et al., 2002; Tuplin et al., 2002, 2004; Lee et al., 2004; You et al., 2004; McMullan et al., 2007; Diviney et al., 2008; You and Rice, 2008). In addition, Simmonds and colleagues have recently used increasingly advanced computational approaches to provide evidence for evolutionarily conserved, genome-scale ordered RNA structures (GORS) of yet unknown function within the genome of HCV and a number of other diverse RNA viruses from plants and animals (Simmonds, 2004; Davis et al., 2008). This suggests that target site accessibility may be an important factor when designing therapeutics that target these highly structured viral genomes, including small RNA species such as small interfering RNAs (siRNAs). HCV is a particularly attractive target for siRNA-based antiviral therapy because it is a cytoplasmically replicating, single-stranded RNA virus whose genome functions as both an mRNA and the template for viral replication.

The RNA silencing pathway uses the RNA-induced silencing complex (RISC) and siRNA duplexes to target complementary mRNAs for endonucleolytic cleavage (Fire et al., 1998; Zamore and Haley, 2005). It has recently been demonstrated that the folded state of the target mRNA can hamper the effectiveness of siRNAs considerably (Holen et al., 2002; Bohula et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Vickers et al., 2003; Xu et al., 2003; Brown et al., 2005; Tafer et al., 2008; Watts et al., 2009). An innovative method called RNA*xs* has been developed in an attempt to address the issue of target site accessibility in mRNAs (Tafer et al., 2008); however, this method cannot account for the large amount of secondary structure present in the highly complex genomes of RNA viruses and has thus far been applied only to cellular mRNAs. Another innovative method

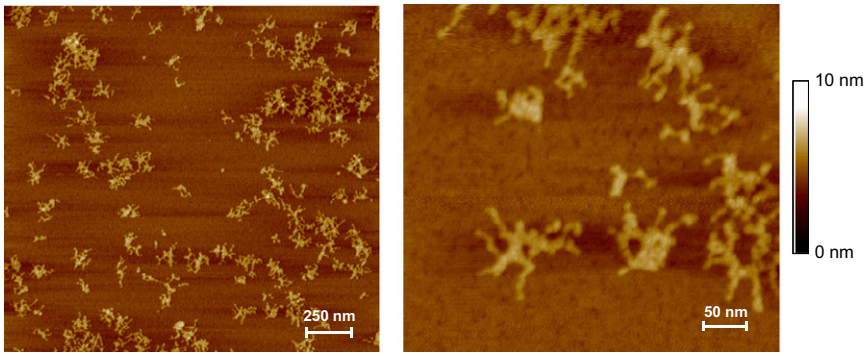

Figure 1. AFM of HCV RNA
HCV RNAs appeared as tightly packed clusters upon deposition, indicating that the tertiary interactions remain intact in the collapsed state. HCV RNAs had diameters of approximately 80–120 nm. The Z scale ranges from 0 to 10 nm.

employing high-throughput selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) was employed to characterize secondary-structure elements within the entire genome of HIV-1 (Watts et al., 2009). Watts et al. were able to correlate HIV-1 mRNA evolutionarily conserved structural elements with folded domains in their respective protein products, implying a role for the folding of genomic RNA in assisting with protein folding during translation (Watts et al., 2009). This approach, although highly innovative, is not designed for the interrogation of target site accessibility of siRNAs. In addition, HCV genomic RNA contains a larger amount of structured RNA or GORS than the HIV-1 genome (Simmonds et al., 2004; Davis et al., 2008).

In this study, we sought to investigate the importance of target RNA structure for the effectiveness of siRNAs directed against the highly structured HCV genome. We demonstrate that in vitro hybridization assays can be used to predict the effectiveness of HCV-specific siRNAs in cell culture. Our results suggest that target site accessibility may be a particularly important parameter for the design of effective siRNAs against the large, highly structured genomes of positive-sense RNA viruses, and have implications for the design and screening of highly effective siRNAs against such targets.

RESULTS

HCV Replicon RNA Retains a Complex Folded Structure upon Deposition

To investigate the physical nature of the highly structured HCV replicon RNA, HCV replicon RNA transcripts were directly visualized by atomic force microscopy (AFM). AFM has been extensively used in the study of DNA (Umemura et al., 2000; Hansma et al., 2004; Liu et al., 2005) and, more recently, has been extended to the investigation of large-scale secondary structure of RNAs (Drygin et al., 1998; Hansma et al., 1999, 2003, 2004; Giro et al., 2004; Alvarez et al., 2005; Kuznetsov et al., 2005; Noestheden et al., 2007; Davis et al., 2008). Upon imaging, the HCV replicon RNA transcripts uniformly adopted a tightly packed condensed state that was largely maintained during the deposition process (Figure 1). HCV replicon RNAs had a regular unit size with a mean radii for the x and y axes of approximately 30 nm and mean heights of 3.9 ± 1.0 nm (n = 10). This indicates that, under appropriate conditions, HCV replicon RNA can be spotted onto surfaces in a complex, highly folded conformation that may be representative of the native conformation.

Design of siRNAs against HCV Replicon RNA

Once it was demonstrated that the HCV replicon RNA could be spotted in a complex, highly folded conformation, we wanted to further investigate whether hybridization to spotted HCV replicon RNAs could be used to predict the potency of HCV replicon-specific siRNAs. Synthetic siRNAs were thus designed targeting different regions of the HCV replicon RNA (Figure 2). siRNAs were designed using the rules outlined by Reynolds and colleagues (Reynolds et al., 2004) and had similar G+C contents to ensure equal binding strengths irrespective of the overall base composition of the siRNAs (Figure 2). The siRNAs were named according to their nucleotide location in the subgenomic replicon RNA (Figure 2). As controls, two previously characterized HCV-specific siRNAs were used: IRES-331 siRNA directed against the HCV internal ribosomal entry site (IRES) region of the HCV replicon RNA (Yokota et al., 2003), and NS5B-7256 siRNA, a highly potent siRNA directed against the NS5B region of the replicon RNA (Wilson et al., 2003). As a negative control, GL3 siRNA was used, which has no sequence complimentarity to HCV replicon RNA. Finally, as a control for target site accessibility, an siRNA (SL3.3-8589) was designed against the 5′ arm of a known stem-loop region (SL3.3) in the NS5B open reading frame (You et al., 2004; Friebe et al., 2005). All siRNAs were fluorophore conjugated at the 5′ end of their antisense (guide) strands so that hybridization could be monitored by fluorescence.

Hybridization of siRNAs to Viral RNA Microarrays Is Restricted by Target Site Accessibility

To investigate the ability of hybridization to spotted HCV replicon RNA to differentiate between accessible and inaccessible target RNA sequences, we prepared viral RNA microarrays (VRMs). VRMs were prepared by spotting the HCV replicon RNA under native conditions on epoxysilane-coated glass slides. The target RNA was diluted in native spotting buffer and was printed in quadruplicate in increasing concentrations on the microarray from 5 to 500 ng/μl (Figure 3A). As negative and positive controls, oligonucleotides complementary to *Campylobacter jejuni* genes were spotted in duplicate (negative control) or quadruplicate (positive controls) in the microarrays at a concentration of 500 ng/μl (Figure 3A). The Cy3 channel (left) shows hybridization of the Cy3-labeled *C. jejuni* DNA controls and hybridization of the guide strand of NS5B-7256 siRNA to the HCV replicon RNA, whereas the Cy5 channel (right) represents hybridization of the Luc988-42 nt DNA control probe to the luciferase region of the HCV replicon RNA, which was used to normalize the data (Figure 3A).

