3' RNA Elements in Hepatitis C Virus Replication: Kissing Partners and Long Poly(U)[†]

Shihyun You and Charles M. Rice*

Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, The Rockefeller University,
1230 York Avenue, New York, New York 10065

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The hepatitis C virus (HCV) genomic RNA possesses conserved structural elements that are essential for its replication. The 3' nontranslated region (NTR) contains several of these elements: a variable region, the poly(U/UC) tract, and a highly conserved 3' X tail, consisting of stem-loop 1 (SL1), SL2, and SL3. Studies of drug-selected, cell culture-adapted subgenomic replicons have indicated that an RNA element within the NS5B coding region, 5BSL3.2, forms a functional kissing-loop tertiary structure with part of the 3' NTR, 3' SL2. Recent advances now allow the efficient propagation of unadapted HCV genomes in the context of a complete infectious life cycle (HCV cell culture [HCVcc]). Using this system, we determine that the kissing-loop interaction between 5BSL3.2 and 3' SL2 is required for replication in the genotype 2a HCVcc context. Remarkably, the overall integrity of the 5BSL3 cruciform is not an absolute requirement for the kissing-loop interaction, suggesting a model in which *trans*-acting factor(s) that stabilize this interaction may interact initially with the 3' X tail rather than 5BSL3. The length and composition of the poly(U/UC) tract were also critical determinants of HCVcc replication, with a length of 33 consecutive U residues required for maximal RNA amplification. Interrupting the U homopolymer with C residues was deleterious, implicating a *trans*-acting factor with a preference for U over mixed pyrimidine nucleotides. Finally, we show that both the poly(U) and kissing-loop RNA elements can function outside of their normal genome contexts. This suggests that the poly(U/UC) tract does not function simply as an unstructured spacer to position the kissing-loop elements.

Hepatitis C virus (HCV) is a major cause of liver disease, with more than 130 million people currently chronically infected worldwide (46, 47). There is no vaccine, and existing antiviral therapies, interferon in combination with ribavirin, induce a sustained response in less than 50% of genotype 1-infected patients. There is thus an urgent need for the development of effective preventative and therapeutic strategies, an effort that will benefit greatly from a detailed understanding of the molecular mechanisms of HCV replication. HCV is a member of the genus *Hepacivirus* in the family *Flaviviridae* (2). The viral genome is a single-stranded, positive-sense RNA molecule approximately 9.6 kb in length. The majority of the genome consists of a single open reading frame that encodes a polyprotein of about 3,000 amino acids. This polyprotein is co- and posttranslationally processed by viral and host proteases to yield the individual gene products, designated C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Core (C) and two envelope glycoproteins (E1 and E2) compose the physical virion, while the remainder of the proteins are involved in RNA replication and virion morphogenesis. NS3 possesses protease activity and is responsible for liberating the majority of the nonstructural proteins from the polyprotein. NS5B is the RNA-dependent RNA polymerase.

The polyprotein-coding region is flanked by 5' and 3' nontranslated regions (NTRs). These NTRs contain *cis*-acting RNA elements (CREs), which are engaged in regulating the

major steps of the viral life cycle. The 5' NTR contains an internal ribosomal entry site, composed of 5' stem-loop II (SLII), 5' SLIII, and 5' SLIV, which initiates translation in a cap-independent manner (6, 13, 18, 36). The 3' NTR consists of a variable region, a poly(U/UC) tract, and a highly conserved terminal region termed the 3' X tail (5, 24, 40a). These 3' RNA structures may play a role in polyprotein expression by enhancing translation from the 5' NTR in a liver cell-specific manner (38a), although this involvement of the 3' NTR remains controversial (12a, 20, 33). The 3' NTR is of primary importance for RNA replication, presumably for the initiation of negative-strand synthesis. The variable region is not absolutely required for this role, although its deletion decreases replication efficiency (14); both the poly(U/UC) tract and the 3' X tail, however, are essential (14, 49). The length of the poly(U/UC) tract is somewhat flexible, ranging from 30 to more than 80 nucleotides among HCV isolates (24). A minimum of 26 U nucleotides has been found to be sufficient for HCV RNA replication in cell culture (14). The highly conserved SL structures of the 3' X tail, 3' SL1, 3' SL2, and 3' SL3 are each absolutely required (14, 49). Although the mechanisms by which the 3' NTR elements act in RNA replication are not clear, it is likely that binding of one or more viral or host proteins to these RNA structures is necessary for the establishment of the replication complex.

In addition to the highly conserved RNA structures within the 5' and 3' NTRs, evolutionarily conserved structures are located within the viral polyprotein-coding region. Through phylogenetic sequence comparisons of HCV isolates and thermodynamic RNA folding analyses, potentially important RNA structures were predicted in the coding regions of core and NS5B (38, 42, 43, 44). The conserved sequence of the core-

* Corresponding author. Mailing address: Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, The Rockefeller University, 1230 York Ave., New York, NY 10065. Phone: (212) 327 7046. Fax: (212) 327-7048. E-mail: ricec@rockefeller.edu.

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coding region, initially attributed to the expression of alternative reading frame proteins (7, 9, 44, 45, 48), has recently been shown to preserve an embedded RNA structure, 5' SLV1, which is required for HCV RNA replication in cell culture and in chimpanzees (32). Within the NS5B-coding sequence, a component of an RNA cruciform structure, 5BSL3.2, has been identified as an essential element required for RNA replication (26, 50). Relocation of 5BSL3.2 was possible but only to the 3' variable region preceding the poly(U/UC) tract, implying a functional link to the 3' end of the genome (15). Subtle changes within the loop region of 5BSL3.2 prevented RNA replication (50), suggesting that sequence specificity in the loop was required for either an RNA-protein or an RNA-RNA interaction. Indeed, a sequence complementary to the 5BSL3.2 loop is found in the loop of a 3' NTR structure, 3' SL2, and participates in a kissing-loop tertiary RNA structure via long-range RNA-RNA interactions (15). The importance of this tertiary RNA structure was confirmed by the rescue of RNA replication by compensating changes in both SLs (15).

The *cis*-acting determinants of HCV replication have been studied primarily in the context of genotype 1b subgenomic replicons, which could be induced to replicate in cell culture by the presence of a selectable marker and emergent adaptive mutations in the nonstructural proteins (2, 3, 28). The recently developed HCV cell culture system (HCVcc) allows viral replication to be studied in the absence of adaptive mutations for the first time (27, 43a, 52a). Here, we examine the importance of the kissing-loop interaction and the intervening poly(U/UC) tract for HCVcc replication. Both the kissing-loop interaction and a minimal length of poly(U) are essential for efficient HCVcc RNA amplification. Our results indicate that the overall secondary structure of the 5BSL3 cruciform that harbors 5BSL3.2 is not required to maintain a functional kissing-loop interaction. Mutants with shorter than optimal poly(U) tracts or tracts interrupted by C residues are unstable, and variants with extended poly(U) quickly arise during passage, implying a strong selective pressure for the length and composition of this element. Remarkably, both the kissing-loop interaction and the poly(U) tract can function outside of their normal genome contexts.

MATERIALS AND METHODS

Cell culture. The human hepatoma cell line Huh-7.5 (4) was propagated in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 0.1 mM nonessential amino acids and 10% fetal bovine serum. Cells were grown at 37°C in 5% CO₂.

Plasmid constructs. Plasmids were created by standard methods. Constructs were verified by restriction enzyme digestion and sequencing of PCR-amplified segments. Descriptions of the cloning strategies are provided below; primer sequences are available upon request. The parental genome, J6/JFH, its replication defective form, J6/JFH(GND), and its luciferase reporter derivative, J6/JFH-5' C19Rluc2Aubi, have been described previously (27, 41).

NS5B CRE mutant (CRE^{MUT}). The NS5B CRE sequence covering 64 amino acids of the carboxy terminus of NS5B was recoded randomly using amino acid recoding analysis (Entelechon, Resensburg, Germany; <http://www.entelechon.com/index.php?id=tools/backtranslation>). The 5B CRE cruciform was disrupted as predicted using Mfold (53). To create the NS5B CRE recoded genome, three rounds of assembly PCR were performed. 5BSL3.3 was recoded by amplification of the J6/JFH plasmid with primers RU-O-6457/RU-O-6185 and RU-O-6186/RU-O-5915, followed by assembly of the primary PCR products with RU-O-6457/RU-O-5915. Using the recoded 5BSL3.3 secondary PCR as a template, 5BSL3.1 was recoded by amplification with RU-O-6457/RU-O-6459 and RU-O-6458/RU-O-5915, followed by assembly PCR with RU-O-6457/RU-O-

5915. Using the recoded 5BSL3.1/5BSL3.3 secondary PCR as a template, 5BSL3.2 was recoded by amplification with RU-O-6457/RU-O-6461 and RU-O-6460/RU-O-5915, followed by assembly PCR with RU-O-6457/RU-O-5915. The final recoded NS5B CRE PCR product was subcloned into pCR2.1-Topo by TA cloning (Invitrogen), followed by sequencing (Biotec Solutions, San Francisco, CA). The final recoded NS5B CRE PCR product was digested with EcoRV and XbaI and cloned into J6/JFH digested with the same enzymes.

Kissing-loop mutant constructs. Point mutations in 5BSL3.2 (C9352U) and 3' SL2 (G9615A) were engineered alone or in combination into J6/JFH-5' C19Rluc2Aubi. For C9352U, J6/JFH was amplified with primers RU-O-5914/RU-O-5920 and RU-O-5919/RU-O-5915, and primary PCR products were assembled with RU-O-5914/RU-O-5915. For G9615A, J6/JFH was amplified with primers RU-O-5914/RU-O-5630 and RU-O-5629/RU-O-5915, and primary PCR products were assembled with the same flanking primer pair. For C9352U/G9615A, the final PCR product containing the G9615A change was amplified with primers RU-O-5914/RU-O-5920 and RU-O-5919/RU-O-5915, and primary PCR products were again amplified with the same flanking primer pair. Final PCR fragments were digested with EcoRV and XbaI and cloned into J6/JFH-5' C19Rluc2Aubi digested with the same enzymes.

Variable length and composition poly(U) constructs. J6/JFH was amplified with the following primer pairs to produce primary PCR products: RU-O-6062/RU-O-6063 for a 27-U poly(U) tract, RU-O-7442/RU-O-7443 for a 16-U poly(U) tract, RU-O-7446/RU-O-7447 for a 7-U poly(U) tract, RU-O-7632/RU-O-7633 for U/Cinter (tract with interspersed C residues), RU-O-7634/RU-O-7635 for U/Ainter (tract with interspersed A residues), RU-O-7636/RU-O-7637 for U/C5'-1 (tract in which one 5' cytidine is replaced with uridine), RU-O-7638/RU-O-7639 for U/C5'-2, RU-O-7640/RU-O-7641 for U/C5'-3, RU-O-7642/RU-O-7643 for U/C5'-4, RU-O-7753/RU-O-7754 for U/C3'-1 (tract in which one 3' cytidine is replaced with uridine), RU-O-7755/RU-O-7756 for U/C3'-2, RU-O-7757/RU-O-7758 for U/C3'-3, RU-O-7759/RU-O-7760 for U/C3'-4, RU-O-7761/RU-O-7762 for U/Cmid-1 (tract in which one centrally located cytidine is replaced with uridine), and RU-O-7763/RU-O-7764 for U/Cmid-3. Primary PCR products were assembled by amplification with RU-O-5914/RU-O-5915. Final PCR fragments were digested with EcoRV and XbaI and cloned into J6/JFH or J6/JFH-5' C19Rluc2Aubi digested with the same enzymes.

Separation of kissing-loop and poly(U) tract. Using NS5B CRE^{MUT} as a backbone, the poly(U/UC) tract was replaced with a 37-U or 37-U/C (every fifth uridine from 3' of poly U is replaced with cytidine) tract. PCR fragments amplified with RU-O-5914/RU-O-5915 using these templates were subcloned into pCR2.1-Topo by TA cloning (Invitrogen). For the constructs with the wild-type kissing-loop structure with a 37-U or 37-U/C poly(U) tract, PCR was performed with RU-O-9469 (NheI site incorporated) and RU-O-5915 using the wild-type 37-U or 37-U/C replicon, followed by pCR2.1-Topo by TA cloning (Invitrogen). Then, the 265 nucleotide (nt) fragment of NheI digestion of the wild-type kissing-loop with 37 U or 37 U/C was subcloned into the TA plasmids containing the 5B CRE recoded with 37 U or 37 U/C, digested with NheI at the 3' SL3. Orientation of the inserted NheI fragment was confirmed and digested with EcoRV and XbaI to be subcloned into the J6/JFH or J6/JFH-5' C19Rluc2Aubi, resulting in duplication of the poly(U) tract and the authentic 3' end of the RNA genome.

In vitro transcription. Plasmid DNA was linearized by digestion with XbaI for 2 h at 37°C, followed by clean up with a QIAquick PCR purification kit (Qiagen, Valencia, CA). Two micrograms of the linearized template was transcribed using the T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's instructions. After 2 h of in vitro transcription, 10 U of DNase I (Ambion, Austin, TX) was added, and after incubation for an additional 20 min at 37°C, transcripts were purified by an RNeasy kit (Qiagen, Valencia, CA). RNA was quantified by absorbance at 260 nm, and the integrity of the transcripts was confirmed by 1% agarose gel electrophoresis.

RNA transfection. RNA was transfected into Huh-7.5 cells by electroporation as described elsewhere (50). Briefly, trypsinized Huh-7.5 cells were washed in ice-cold 1× phosphate-buffered saline (PBS; BioWhittaker, Rockland, ME) and resuspended in 1× PBS at a concentration of 1.25×10^7 cells/ml. One microgram of each RNA was mixed with 5×10^6 cells and pulsed with an ElectroSquare Porator ECM 830 (BTX, Holliston, MA) (820 V, 99 μ s, and 5 pulses). Electroporated cells were diluted in 14 ml of complete medium and plated into 24 wells (nonreporter genomes) or 48 wells in triplicate (reporter genomes) and P100 tissue culture dishes.

For luciferase assays, medium was removed from each well, and cells were washed once with 1× PBS. Cells were lysed for 15 min in 65 μ l of passive lysis buffer (Promega, Madison, WI) per well. Quantification of luciferase activity was performed using 10 μ l of cell lysate and *Renilla* luciferase substrate (Promega) following the manufacturer's instructions. For nonreporter J6/JFH genomes,

replication was monitored by immunohistochemical staining of NS5A as described previously (27).

RT-PCR. For analysis of revertants, total RNA from transfected cells was harvested by an RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed and PCR amplified using a SuperScript III One-Step reverse transcription-PCR (RT-PCR) system with Platinum *Taq* High Fidelity (Invitrogen). Approximately 5 µg of total RNA was denatured at 60°C for 5 min, followed by RT at 55°C for 40 min. Subsequent PCR conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. RT-PCR products were gel purified using a QIAquick gel extraction kit (Qiagen) and sequenced directly or after subcloning into the pCR2.1-Topo TA vector (Invitrogen). For amplification of NS5B CRE^{MUT} RNA, primers RU-O-5935/RU-O-7890 were used; sequencing was performed using primers RU-O-5914 and RU-O-5935. For amplification and sequencing of 7-U or 16-U RNA, primers RU-O-5914 and RU-O-7890 were used. For reengineering of compensatory changes, purified RT-PCR products were digested with *EcoRV* and *XbaI* and ligated to J6/JFH or J6/JFH-5' C19Rluc2AUBi digested with the same enzymes.

RESULTS

The 5BSL3.2 and 3' SL2 kissing-loop interaction is essential for genotype 2a HCVcc replication. The cruciform CRE within the NS5B coding region has been shown to be essential for the replication of a tissue culture-adapted genotype 1b sub-genomic replicon (15, 26, 50). The importance of this structure for the replication of a fully infectious genotype 2a virus (J6/JFH), however, is not known. The amino acid identity between genotypes 1b and 2a over the NS5B CRE region is less than 63%, but the predicted RNA secondary structures are remarkably similar (Fig. 1A) (50), suggesting that NS5B CRE function may be conserved across genotypes. To investigate its significance in the genotype 2a background, amino acids 539 to 585 of J6/JFH NS5B were recoded so as to destroy NS5B CRE RNA secondary structures while retaining the original amino acid sequence. The recoded sequence contained 29 silent mutations throughout the NS5B CRE region and was termed NS5B CRE^{MUT} (Fig. 1A). Analysis of the recoded sequence by Mfold prediction suggested that the NS5B CRE would, indeed, be disrupted (53).

Replication of the NS5B CRE^{MUT} genome was investigated by transfection of in vitro transcribed RNA into Huh-7.5 cells. Immunohistochemical staining (IHC) for NS5A at 2 days post-electroporation indicated that, while wild-type J6/JFH replicated efficiently, there were no detectable NS5A-positive cells with NS5B CRE^{MUT} RNA (Fig. 1B). As NS5B CRE^{MUT}-transfected cells were maintained in culture, however, NS5A-positive cells became detectable, indicating that viable revertants may have arisen (Fig. 1B). To determine whether mutations had emerged, total RNA harvested from NS5B CRE^{MUT}-harboring cells was analyzed by RT-PCR and DNA sequencing. Whereas the rest of the genome sequence was

intact, including the original recoded sequence in the 5B CRE region, a single adenosine residue in the loop of the 3' NTR structure, 3' SL2, was changed to guanosine (Fig. 1C, A9618G). This second-site mutation could compensate for the base pair disturbed by mutation of 5BSL3.2 U9349 to C by restoring the previously identified kissing-loop interaction.

To confirm that the 3' SL2 mutation was responsible for the restoration of NS5B CRE^{MUT} replication, we reengineered the A9618G change into the NS5B CRE^{MUT} genome [CRE^{MUT} (A9618G)]. To facilitate quantitative comparison, these changes were introduced into the J6/JFH-5' C19Rluc2AUBi reporter virus, which encodes *Renilla* luciferase, foot and mouth disease virus 2A, and a ubiquitin monomer upstream of the core protein (41). In vitro transcribed genomes were transfected into Huh-7.5 cells and analyzed for RNA replication by luciferase assay. Measurement of luciferase activities at early time points (2, 5, and 8 h postelectroporation) as an indication of input RNA translation efficiency did not show any significant differences (data not shown). The second-site change in 3' SL2 significantly improved replication of NS5B CRE^{MUT} (A9618G) measured at 6 days postelectroporation, increasing it by over 100-fold (Fig. 1D). Although replication of NS5B CRE^{MUT} was substantially enhanced by the presence of the compensatory mutation, it remained approximately 100-fold reduced from wild type, suggesting that the 5B CRE secondary RNA structure, in addition to the kissing-loop interaction, is necessary to recapitulate the wild-type level of replication. Introduction of the emergent mutation into J6/JFH-5' C19Rluc2AUBi(A9618G) did not affect RNA replication (Fig. 1D, A9618G), presumably because the engineered guanosine in 3' SL2 can form a wobble base pair with the 5BSL3.2 uridine, maintaining the kissing-loop interaction.

The isolation of a pseudorevertant that restored the kissing-loop interaction between 5BSL3.2 and 3' SL2 in the NS5B CRE^{MUT} genome underscores the importance of this interaction in J6/JFH replication. In order to test the significance of the kissing-loop interaction in the context of an intact NS5B CRE, we introduced mutations predicted to disrupt or restore the base-pairing of 5BSL3.2 and 3' SL2 loops in the context of the J6/JFH-5' C19Rluc2AUBi reporter virus (Fig. 1E and F). RNA replication of each genome was measured by luciferase assay at 6 days postelectroporation of Huh-7.5 cells. Mutations predicted to be deleterious to the base-pairing of 5BSL3.2 and 3' SL2, C9352U (U · G) and G9615A (C · A), led to a significant reduction in the RNA replication to levels 10-fold and 1,000-fold lower than the wild type, respectively (Fig. 1F). The mutation of C9352U with G9615A (U · A) was expected to

FIG. 1. The kissing-loop interaction at 3' end of the HCV genome is important in the HCVcc system. (A) Predicted structure of 5B CRE in genotype 2a, JFH-1 strain. The introduced silent mutations are depicted with the changed nucleotides shown in bold (NS5B CRE^{MUT}). The region of 5BSL3.2 involved in the kissing-loop interaction is shaded red. The stop codon is in blue. Watson-Crick base pairs are indicated with filled circles and wobble base pairs are indicated with gray circles. (B) RNA replication as measured by IHC using an anti-NS5A antibody. Nuclei are counterstained blue using hematoxylin 2. The number of days postelectroporation are indicated (prefixed by D) on the images. (C) Predicted structure of the 3' X tail of genotype 2a JFH-1 strain. The region of 3' SL2 involved in the kissing-loop interaction is shaded in red; the identified second-site reversion is indicated. (D) RNA replication of J6/JFH-5' C19Rluc2AUBi containing reengineered reversions at 6 days postelectroporation. Means and standard deviations of triplicate samples are shown. (E) Diagram of the tertiary RNA structure at the 3' end of the HCV genome. The kissing-loop base pairs are shown; the central base pair is in red. Mutations targeting the central base pair are indicated in blue. (F) RNA replication of J6/JFH-5' C19Rluc2AUBi containing kissing-loop mutations at 6 days postelectroporation. Means and standard deviations of triplicate samples are shown. RLU, relative light units; WT, wild type; pol-, polymerase-defective control.

FIG. 2. Selection for longer poly(U) tracts improves replicative fitness. (A) Diagram of the poly(U/UC) tract with flanking regions. Sequences of the wild-type JFH-1 poly(U/UC) tract and the 27-U, 16-U, and 7-U tracts are depicted. (B) RNA replication kinetics of J6/JFH-5' C19Rluc2Aubi with variable poly(U) tracts. Means and standard deviations of triplicate samples are shown. (C) Lengths of the poly(U) tracts over time for 7-U (i) and 16-U (ii) genomes. RNA harvested at the indicated times postelectroporation was RT-PCR amplified, digested with *StuI*/*NheI*, and analyzed by 4% metaphor agarose gel electrophoresis. The corresponding *in vitro* generated transcripts were similarly processed to provide markers of input RNA. (D) RNA replication kinetics of J6/JFH-5' C19Rluc2Aubi with reengineered variable poly(U) tracts. Means and standard deviations of triplicate samples are shown. RLU, relative light units; WT, wild type; pol-, polymerase-defective control.

restore base-pairing and, consistent with this, showed levels of replication comparable to the wild-type (Fig. 1F). The phenotypes of these mutants correspond to the predicted strength of base-pairing in the kissing-loop, $C \cdot G > U \cdot A > U \cdot G > C \cdot A$. These results indicate that the interaction between RNA elements in the NS5B coding region and 3' NTR is a crucial and conserved prerequisite for HCV RNA replication.

Optimal length of the poly(U) tract determined by replicative fitness. The poly(U/UC) tract is positioned between 5BSL3.2 and 3' SL2 and could play a critical role in positioning these RNA elements. In previous replicon studies, a minimal

length of 26 U residues was required for efficient replication (14). We examined the effect of reducing the poly(U/UC) tract from the wild-type length (100 nt) to 27, 16, or 7 U residues in the context of J6/JFH-5' C19Rluc2Aubi (Fig. 2A). Replication was measured over a time course of 2 h to 6 days postelectroporation of Huh-7.5 cells. Equivalent luciferase activities at early times posttransfection indicated that the length and composition of the poly(U/UC) tract did not affect the efficiency of translation (Fig. 2B). Reduction of the poly(U/UC) tract to 27 U residues decreased RNA replication by approximately 10-fold (Fig. 2B). Substitution of the poly(U/UC) tract with 16 U

residues led to kinetics of replication significantly slower than those of the parent. Whereas replication of a 16-U genome was not detectable early after transfection, by day 6 it had increased to 100-fold over the NS5B polymerase-defective mutant. This late onset of replication led us to hypothesize that adaptations might have occurred in the reduced poly(U/UC) tract genomes.

To isolate genomes with increased replication fitness, the 16-U and 7-U sequences were cloned into the nonreporter J6/JFH genome and maintained in culture for up to 14 days. As determined by IHC for NS5A, no detectable replication of the 16-U or 7-U sequence was observed at day 2, although after 3 days and 8 days in culture, respectively, NS5A staining became apparent, possibly indicating that replication-competent variants had been selected in the population (data not shown). In order to assess the length of poly(U) sequences in the adapted populations, total cellular RNA was harvested and amplified by RT-PCR. The RT-PCR products were digested with restriction enzymes with sites flanking the poly(U) sequences and analyzed by agarose gel electrophoresis. For the 7-U sequence, the expected fragments of 206 nt (5' part), 98 nt [poly(U) tract], and 65 nt (3' part) were observed early after transfection, likely as a result of residual input RNA or inefficient replication (Fig. 2C, panel i). At 4 days to 6 days postelectroporation, the amount of the RT-PCR product was markedly decreased. At day 8 and thereafter, however, prominent RT-PCR products were obtained from cells harboring the 7-U sequence. Interestingly, while the 5' and 3' fragments remained the same size, the region spanning the poly(U) tract now migrated more slowly than the 98-nt fragment derived from the input RNA. Similar results were obtained for the 16-U genome, with a gradual increase in the size of the poly(U) fragment observed from 4 days to 14 days posttransfection (Fig. 2C, panel ii). The kinetics of increased length of the poly(U) fragment correlated well with the increased replication observed by NS5A staining.

To determine the precise lengths of poly(U) sequence that had emerged, RT-PCR products generated from day 6 postelectroporation with the 16-U genome were cloned, and individual clones were sequenced. The poly(U) sequences were found to vary, containing stretches of 33, 35, 39, 43, 49, or 60 uridines. To determine whether these increased lengths of poly(U) sequence were the primary compensatory determinants, each of the emergent sequences was cloned into J6/JFH-5'C19Rluc2AUbi and analyzed for replication efficiency by luciferase assay. In contrast to the decreased replication of the 27-U and 16-U genomes, the emergent 33- to 60-U sequences showed replication levels comparable to wild-type J6/JFH-5'C19Rluc2AUbi (Fig. 2D). These results indicate that 33 U residues are sufficient for maximal replication of the J6/JFH genome.

The poly(U) tract composition is important for its function in HCV replication. The length dependence of the poly(U) tract might indicate a function as an unstructured spacer for the kissing-loop partners. In this case, the spacer composition might be less important than its length. The poly(U/UC) tract of natural HCV isolates contains not only uridines but also interspersed cytidines. To test whether the sequence composition of the poly(U/UC) tract was functionally significant, we created subtle mutations predicted not to create secondary

FIG. 3. Interspersed A and C residues in the poly(U) tract inhibit HCV replication. (A) Adenosine or cytosine residues were substituted for uridines in every fifth position from 3' of poly(U) as indicated. (B) RNA replication of J6/JFH (WT), 27U, 16U, and interspersed A and C genomes. Replication was measured by IHC for NS5A at the indicated days postelectroporation.

RNA structures that might influence the kissing-loop interaction. In the context of J6/JFH with a 27-U tract, every fifth uridine from the 3' boundary of the poly(U) was replaced with either adenosine or cytosine (Fig. 3A). These substitutions are predicted to maintain a single-stranded conformation by Mfold. The effects of the A or C substitutions on the replication of J6/JFH with the 27-U tract were initially investigated by analysis of transfected Huh-7.5 cells for NS5A expression. While the 27-U genome replicated at levels slightly decreased from wild-type J6/JFH, 27-U genomes with interspersed A or C bases were significantly impaired, showing no detectable replication at 2 to 4 days postelectroporation (Fig. 3B).

The drastic effect of cytosine substitutions was somewhat surprising since wild-type HCV isolates contain cytidines in the poly(U/UC) tract. We therefore investigated in more detail the effects of interspersed cytidines on J6/JFH RNA replication. In the context of J6/JFH-5'C19Rluc2AUbi with a 27-U tract, we mutated various combinations of U to C (Fig. 4A) and tested their replication competence by luciferase assay of cell lysates at 3 days posttransfection. The level of replication varied depending on the number of interspersed cytidines and was found to be strongly influenced by the number of consecutive uridine bases in the poly(U) tract (Fig. 4B). The location of the consecutive nucleotides was not important, as genomes with uninterrupted uridine sequences located 5' (U/C5'-2), 3' (U/C3'-3), or centrally (U/Cmid-3) replicated similarly (Fig. 4B). Comparison of the replication kinetics of the U/C5'-2 and U/C3'-3 sequences indicated that the interspersed cytosine bases did not affect translation but that replication was decreased to levels comparable to that of the 16-U genome (Fig. 4C). Since the U/C5'-2, U/C3'-3, and 16-U genomes contain consecutive sequences of 16, 15, and 16 U residues, respec-

FIG. 5. Poly(U) lengthening upon passage of genomes with interspersed cytidines. (A) Schematics and RNA replication of the U/C3'-1, U/C3'-2, and U/C5'-1 mutants at day 2 (D2) and day 15 (D15) postelectroporation as measured by IHC for NS5A. (B) Sequences isolated at day 13 (D13) (U/C3'-1 and U/C3'-2 mutants) or day 15 (U/C5'-1 mutant) postelectroporation. The poly(U/UC) tract and flanking regions were amplified by RT-PCR, cloned, and sequenced. Sequences of individual clones are shown.

FIG. 6. Separating the kissing-loop structure and the poly(U) tract. (A) Architectures of tested constructs. The recorded NS5B CRE^{MUT} (X), NS5B CRE^{WT} (WT; heart), polyprotein stop codon (star), poly(U) tract (gray line), and incorporated cytidines (filled circle) are indicated. In the construct designations, U indicates a tract of 37 uridines and U/C indicates a tract of 37 uridines with every fifth uridine from 3' replaced by a cytidine (B) RNA replication of J6/JFH-5' C19Luc2Aubi genomes with duplicated poly(U) tracts. Means and standard deviations of triplicate samples are shown. (C) RNA replication of CRE^{MUT}-U:CRE^{WT}-U/C with mutations in the kissing-loop central base pair (Fig. 1E). RLU, relative light units.

replication. These results also demonstrate that the kissing-loop structure does not require intervening poly(U) to function and, likewise, the poly(U) tract can function outside of its normal context.

DISCUSSION

The advent of the fully infectious system for HCV allows viral RNA replication to be studied in cell culture in the absence of adaptive mutations and selectable markers. The infectious system also allows the rapid selection of compensatory mutations that increase genome fitness in the context of an authentic viral life cycle. Here, we used the HCVcc system to investigate the importance of RNA structural elements encoded in the 3' end of the genome. We along with others have previously reported that an RNA element in the NS5B coding sequence is essential for replication of drug-selected, cell culture-adapted, genotype 1b subgenomic replicons (15, 26, 50). Here, we found that ablation of the NS5B CRE^{MUT} genome in an efficiently replicating, full-length, infectious genotype 2a genome also led to a drastic defect in replication. During maintenance of the impaired NS5B CRE^{MUT} genome in Huh-7.5 cells, we anticipated that multiple reversions might be required to restore individual secondary structures, (5BSL3.1, -3.2, and -3.3) and the overall CRE structure. Instead, a single second-site adenosine-to-guanosine change in the loop of the 3' X structure, 3' SL2, was able to increase replication efficiency by approximately 100-fold. This reversion is expected to restore the kissing-loop interaction between 5BSL3.2 and 3' SL2, suggesting that the integrity of this tertiary structure is a primary requirement for J6/JFH RNA replication. In the context of this altered cruciform structure, we hypothesize that the second-site change of A9618 to G in 3' SL2 may be more advantageous than a simple reversion of C9349 to U in 5BSL3.2, since the second-site change creates a stronger C · G base pair that might compensate for the architectural deformation of 5B CRE. Consistent with this idea, engineering of the wild-type U at position 9349 in the context of the NS5B CRE^{MUT} genome did not allow detectable RNA replication, suggesting that the secondary structure of 5B CRE might participate in optimizing the kissing-loop interaction (our unpublished results).

Given the importance of the kissing-loop interaction for HCV replication, we speculated that the primary function of the intervening poly(U/UC) tract might be as a spacer for the interacting SLs, 5BSL3.2 and 3' SL2. We found that reducing the length of the poly(U/UC) tract to 27 U, 16 U, and 7 U correlated with decreasing replication efficiency. In the adapted subgenomic replicon system, the minimal poly(U) tract length was found to be 26 U (14, 49). In the context of J6/JFH, however, reduction of the poly(U/UC) sequence to 27 U residues hindered replication, and poly(U) tracts with lengths of 33 U residues or longer were strongly selected during passage. Our finding that longer poly(U) sequences recapitulated wild-type J6/JFH replication kinetics suggests that the length of the poly(U/UC) tract is an important and early determinant of RNA accumulation. This difference in minimal sequence length may be attributable to the differing assays employed; the growth kinetics of the reporter virus may have allowed sensitive detection of replication defects, while shorter

FIG. 7. Hypothetical model of the HCV genome 3' end function. Involvement of *trans*-acting factors specific to each RNA structural element may play important role(s) for multiple steps of the HCV life cycle. See Discussion.

poly(U) sequences may have reverted during drug selection of the replicon.

To directly address the significance of the poly(U/UC) sequence composition, we investigated the replication efficiencies of genomes with interspersed A or C nucleotides at various intervals throughout a 27-nt tract. Given that many wild-type HCV sequences of different genotypes include interspersed cytidine residues within the 3' region of the poly(U/UC) tract, it was surprising that a genome with cytidine residues at 5-nt intervals did not show detectable replication. These data agree with previous reports showing that stretches of 26 A, G, or C nucleotides were lethal for subgenomic RNA replication (14). Inclusion of homopolymeric poly(A), poly(G), or poly(C) sequences might create deleterious secondary structures or recruit inappropriate cellular factors [like poly(A) binding protein] that could interfere with the kissing-loop interaction. Our data suggest, however, that even subtle nucleotide substitutions, which would not be predicted to affect the single-stranded nature of the sequence, are detrimental to RNA replication. This finding argues against a role for the poly(U/UC) tract as solely a spacer for the kissing-loop partners and suggests that nucleotides in this region play sequence-specific roles. Interestingly, the number of consecutive uridines in the poly(U) tract correlated well with the ability of the mutant genomes to replicate. This is consistent with previous findings that replication of a transiently expressed subgenomic RNA required at least 20 U residues upstream of a short poly(U/UC) sequence (49). Isolated revertant genomes with high replicative fitness indicated that the location of the U stretch was not critical, as it emerged 5' or 3' to the defective U/C stretch, likely by slipping of the viral RNA polymerase during replication. Although the position of polymerase slipping should be equivalent in a genome where cytidines were rather evenly interspersed, poly(U) sequences were predominantly isolated 5' to the U/C block (Fig. 5B, U/C3'-1). This may indicate slightly higher replication efficiency for a strain with this poly(U/UC) structure, which resembles that of the wild-type configuration.

It remains unclear as to whether the poly(U/UC) tract and the kissing-loop interaction perform independent or linked functions in HCV RNA replication. We have demonstrated that a functional kissing-loop interaction can occur without intervening poly(U) sequence and that the essential role of the poly(U) tract does not require positioning of the homopolymer between the kissing-loop elements; whether a reasonable proximity between these elements is required, however, is still not known. Our data suggest that one role of the poly(U/UC) tract is to bind a functionally important, U-specific *trans*-acting factor. It is possible that the kissing-loop structure functions to

recruit a separate *trans*-acting factor and that these interactions perform independent functions. Alternately, optimal replication may depend on the formation of a higher-order structure requiring interactions between these functional elements. From our data, it seems unlikely that the poly(U) binding factor acts as a primary determinant for positioning 5BSL3.2 and 3' SL2 during the establishment of the tertiary interaction. Rather, we favor a model in which other *trans*-acting factor(s) interact with the highly conserved 3' X tail to aid the formation of the kissing-loop interaction. Our finding that the integrity of 5BSL3 CRE is not essential for replication supports this hypothesis (Fig. 1D, CRE^{MUT}/A9618G). The mechanism(s) by which these RNA elements and their protein binding partners modulate HCV RNA replication remain unclear, but besides initiation of negative-strand synthesis they may also regulate such conflicting demands as translation, replication, and genome packaging (Fig. 7).

Several viral and host proteins have been suggested to interact with the poly(U/UC) tract. Components of the viral replicase complex, NS3, NS5A, and NS5B, have each been shown biochemically to strongly prefer a poly(U) ribohomopolymer (19, 22, 30). It has not been reported, however, if these interactions are length dependent or if they are affected by intervening cytidines. We have found that neither purified NS3 helicase domain nor NS5B shows differential binding to a 35-U ribohomopolymer with or without cytidines interspersed (our unpublished results). It is possible these results might differ if these poly(U) or poly(UC) sequences were tested in the context of an authentic 3' NTR. Several cellular proteins have been implicated in poly(U/UC) tract binding, including polypyrimidine tract binding protein (PTB), heterogeneous nuclear ribonucleoprotein C, glyceraldehyde dehydrogenase, HuR, and La autoantigen (16, 29, 34, 39, 40). PTB, HuR, and La have also been shown to be required for efficient HCV RNA replication (8, 12, 25). None of these cellular factors, however, shows a strict uridine binding specificity or length dependence. In fact, in the case of PTB, the optimal binding site was shown to be 5'-UCUU-3' (10), which is not compatible with our observation that interspersed cytidines are deleterious for HCV replication.

Functionally important U-rich regions are found in other mRNAs. Several viral and cellular mRNAs contain a short U-rich region upstream of the poly(A) signal. This sequence recruits accessory proteins, such as hFip1, PTB, Hu family proteins, cleavage and polyadenylation specificity factor components, and U2AF⁶⁵, which facilitate polyadenylation and complete the formation of the mRNA 3' end (1, 11, 17, 21, 23, 35, 52). In addition, the U-rich region of the myosin phosphatase targeting subunit 1 pre-mRNA has been shown to

mediate an alternative splicing event by interacting with either T-cell intracellular antigen 1 or PTB during tissue-specific developmental regulation (37). The possibility that additional poly(U) binding proteins act in HCV replication remains to be investigated.

In conclusion, we have exploited the infectious HCVcc system to investigate the roles of the 3' *cis*-acting RNA structures. We found that 5BSL3.2 is required for optimal HCV replication and that the kissing-loop interaction in which it participates is essential for this process. The base-pairing interactions of kissing-loop partners as well as the length of the intervening poly(U/UC) sequence are under strong selective pressure. We have found evidence that stretches of uridine bases are an essential attribute of the poly(U/UC) tract, indicating that it likely performs sequence-specific functions during viral replication. Interestingly, the kissing-loop structure and the poly(U) tract can function outside of their normal genome configuration but may work together for optimal fitness.

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