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Hepatitis C Virus Nonstructural Protein 5A (NS5A) Is an RNA-binding Protein*

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) has been shown to antagonize numerous cellular pathways, including the antiviral interferon-α response. However, the capacity of this protein to interact with the viral polymerase suggests a more direct role for NS5A in genome replication. In this study, we employed two bacterially expressed, soluble derivatives of NS5A to probe for novel functions of this protein. We find that NS5A has the capacity to bind to the 3′-ends of HCV plus and minus strand RNAs. The high affinity binding site for NS5A in the 3′-end of plus strand RNA maps to the polypyrimidine tract, an element known to be essential for genome replication and infectivity. NS5A has a preference for single-stranded RNA containing stretches of uridine or guanosine. Values for the equilibrium dissociation constants for high affinity binding sites were in the 10 nM range. Two-dimensional gel electrophoresis followed by Western blotting revealed the presence of unphosphorylated NS5A in Huh-7 cells stably expressing the subgenomic replicon. Moreover, RNA immunoprecipitation and NS5A pull-down experiments showed the capacity of replicon-derived NS5A to bind to synthetic RNA and the HCV genome, respectively. Deletion of all of the casein kinase II phosphorylation sites in NS5A supported stable replication of a subgenomic replicon in Huh-7. However, this derivative could not be labeled with inorganic phosphate, suggesting that extensive phosphorylation of NS5A is not required for the replication functions of NS5A. The discovery that NS5A is an RNA-binding protein defines a new functional target for development of agents to treat HCV infection and a new structural class of RNA-binding proteins.

Hepatitis C virus (HCV)[2] infection is a global health problem. Most cases of acute infection lead to chronic infection, which, in turn, progress to liver cirrhosis and, in the worst cases, liver cancer (1). Current protocols for treating HCV infection fail to produce a sustained virological response in as much as 46% of treated individuals (1). Clearly, more effective strategies to treat HCV infection are needed. For many years, the pursuit of better therapeutics was complicated by the inability to grow HCV in tissue culture and the absence of infectious genomes. The availability of infectious genomes (2–4) rapidly led to the development of subgenomic replicons capable of replicating stably in Huh-7 cell lines and derivatives thereof (5–7).

The subgenomic replicon system has permitted the minimal determinants for genome replication to be determined. Three cis-acting replication elements exist as follows: 5′-nontranslated region (NTR), 3′-NTR, and a region located within the polymerase-coding sequence at the 3′-end of the genome (8–10). In addition, only a subset of the following viral nonstructural proteins is required: NS3, NS4A, NS4B, NS5A, and NS5B (7). None of the structural proteins are required for genome replication (7). Among the essential nonstructural proteins, the most important is NS5B, the viral RNA-dependent RNA polymerase. NS3 is a bifunctional protein. The amino-terminal domain is a serine protease responsible for releasing the individual proteins from the NS3-NS5B polyprotein. The carboxyl-terminal domain is an RNA helicase. Although many genome replication functions can be envisaged for an RNA helicase, it is not clear how this activity functions in HCV genome replication. NS4A is a cofactor for NS3 that binds to the protease domain and modulates both the protease and helicase activities (11, 12). NS4B functions to establish membranous webs in cells that are thought to be the site of RNA synthesis (13). This function for NS4B may require the NS4AB precursor (14), although interaction between the processed forms of NS4A and NS4B has been observed (15).

NS5A is essential for genome replication; however, a clear genome replication function for this protein is not known (16). NS5A interacts with membranes in the cell as a result of an amphipathic helix present at the amino terminus of the protein (Fig. 1). The encoded protein has a molecular mass of 49 kDa; however, 56- and 58-kDa forms of this protein have been observed in mammalian cells (17). The 56-kDa form is thought to arise from phosphorylation of the protein in conserved clusters of serine and threonine residues (clusters II and III in Fig. 1). The 58-kDa form is thought to arise from additional phosphorylation in cluster I; this form is often referred to as the hyperphosphorylated form. Formation of the 58-kDa form requires the presence of NS4A and perhaps a direct interaction between these two proteins (17–19) (Fig. 1). Recently, NS5A was expressed in bacteria, and this unphosphorylated form also migrates in SDS-polyacrylamide gels as a 56-kDa species, suggesting that the proline-rich nature of the protein rather than phosphorylation leads to the "aberrant" mobility in gels (20).

To date, most studies of NS5A have illuminated functions for this protein in resisting the response of cells initiated by interferon-α, perhaps by inhibiting the double-stranded RNA-activated protein kinase (21). NS5A has the capacity to induce endoplasmic reticulum stress, resulting in the activation of nuclear factor κB (22). NS5A has also been shown to interact with components of numerous cellular signaling pathways (16). However, two important observations point to a more direct role for NS5A in genome replication. First, mutations that enhance the capacity of subgenomic replicons to replicate RNA in

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[2] The abbreviations used are: HCV, hepatitis C virus; NS, nonstructural protein; NTR, nontranslated region; TR, terminal region; DTT, dithiothreitol; BME, β-mercaptoethanol; IRES, internal ribosome entry site; eIF3, eukaryotic initiation factor 3; Sxl, Sex-lethal protein; IEF, isoelectric focusing; oligo, oligonucleotide.

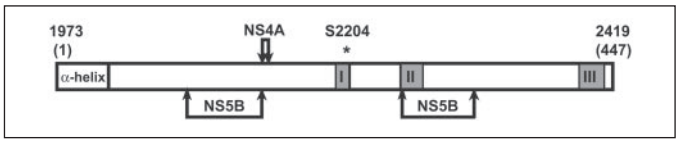

FIGURE 1. **Functional domains of HCV NS5A.** The amino-terminal 32 amino acids form an amphipathic helix that is responsible for interaction of NS5A with membranes (27). NS5A has three clusters of conserved serines (*I*, 2194–2210; *II*, 2246–2269; and *III*, 2380–2409) that can be phosphorylated (16). The *asterisk* indicates the site of the S2204I adaptive mutation. Phosphorylation within cluster I requires expression of NS4A and potentially an interaction with residues 2135–2139 of NS5A (17). NS5A (residues 2077–2134 and 2249–2306) has been to shown to interact functionally and physically with the viral RNA-dependent RNA polymerase, NS5B (23).

Huh-7 cells map to the NS5A-coding sequence, in many cases changing a conserved serine in cluster I and reducing or eliminating production of the 58-kDa form of NS5A (6). In addition, NS5A has been shown to interact with NS5B, and this interaction is essential for maintenance of subgenomic replicons in Huh-7 cells (23, 24) (Fig. 1).

We recently succeeded in producing NS5A in bacteria and purifying soluble forms of this protein suitable for biochemical characterization (20). In this report, we show that unphosphorylated NS5A has the capacity to bind to elements located in the 3′-ends of HCV plus and minus strand RNA. This protein binds uridylate- and guanylate-rich, single-stranded RNA with highest affinity. In addition, we provide evidence that replicon-derived NS5A retains this activity. The observations reported here have significant implications on roles for NS5A in regulating the switch from genome translation to genome replication, recruitment of polymerase and helicase to viral RNA, and enhancement of polymerase and helicase processivity.

EXPERIMENTAL PROCEDURES

Materials—All RNA oligonucleotides were from Dharmacon Research, Inc. (Boulder, CO). DNA oligonucleotides for binding assays, dT_{15} and $dU_{15}C$, were from Qiagen. All restriction enzymes, T4 polynucleotide kinase, and Deep Vent DNA polymerase were from New England Biolabs, Inc. T4 DNA ligase was from Invitrogen. DNA primers for PCRs were from Integrated DNA Technologies. [α-^{32}P]UTP (>6,000 Ci/mmol) was from PerkinElmer Life Sciences; [γ-^{32}P]ATP (>7,000 Ci/mmol) was from ICN. All other reagents were of the highest grade available from Sigma, Fisher, or VWR Scientific.

Construction of S2204I and Δ2380–2409 HCV Subgenomic Replicon Plasmids—Oligo 1 (HCV-5A-4912-PstI-for, 5′-GCG CTG CAG ACG CGT ACA CCA CGG GC-3′) and oligo 2 (HCV-5A-5575-BglII-rev, 5′-GCG AGA TCT CTC GAG GGA ATT TCC-3′) were used to subclone the NS5A-coding sequence from the plasmid pHCVbart.rep1b/Ava-II into pUC-18 plasmid by using PstI and BglII sites to give pUC18-NS5A-WT. Oligo 3 (HCV-5A-S2204I-StyI-for, 5′-GCG TCT AGA CCT TGG CCA GCT CAT CAG CTA TCC AGC TGT CTG CGC CTT-3′) and oligo 2 were used to introduce the mutation S2204I into pUC18-NS5A-WT. The S2204I subclone was digested with BlpI and XhoI and ligated into BlpI/XhoI-digested pHCVbart.rep1b/Ava-II.

Oligo 4 (HCV-5A-ΔCluster3-for, 5′-GAC GGC GAC GCG GGA GAG GAG GCT AGT GAG G-3′) and oligo 5 (HCV-5A-ΔCluster3-rev, 5′-CCT CAC TAG CCT CCT CTC CCG CGT CGC CGT C-3′) were used to construct Δ2380–2409. The overlapping PCR products were amplified using oligo 6 (HCV-5A-XhoI-for, 5′-GCG AAA TTC CCT CGA GCG ATG CCC ATA-3′) and oligo 7 (HCV-MfeI-rev, 5′-GCG GGT GGT GTC AAT TGG TGT CTC-3′), digested, and ligated into XhoI/MfeI-digested pHCVbart.rep1b/Ava-II.

Bacterial Expression of Recombinant NS5A—Escherichia coli extract containing recombinant NS5A was prepared as described previously (20). Briefly, BL21(DE3)pCG1 cells containing NS5A were grown in 10 ml of NZCYM supplemented with 25 μg/ml kanamycin, 20 μg/ml chloramphenicol, and 0.1% dextrose at 37 °C to an A_{600} of 0.8 before isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The cells were grown for an additional 4 h at 20 °C. The induced cells were analyzed by SDS-PAGE, and the NS5A concentration was quantitated by densitometric analysis of the Coomassie-stained gel.

Expression and Purification of NS5A Derivatives—Three NS5A derivatives, NS5A-His, His-Δ-NS5A, and His-Δ-NS5A-S2204I, were expressed in *E. coli* and purified from the soluble fraction as described previously (20).

Constructs for in Vitro Transcription—The 5′-NTR (terminal 386 nucleotides), 3′-NTR (terminal 247 nucleotides), and 3′-NTR containing a deletion of the polypyrimidine tract (3′-NTRΔpoly(U)) were amplified by PCR from pHCVbart.rep1b/AvaII provided to us by Professor Charles Rice (Rockefeller University) (6). The 3′-end of the HCV antigenome (3′-TR, terminal 374 nucleotides) was amplified by PCR from pBRTHCV provided to us by Professor John McCarthy (University of Manchester Institute of Science and Technology, UK) (25). The amplified products were cloned into a pUC18 vector that contains a promoter for T7 RNA polymerase, a cloning region, and sequences encoding a hepatitis δ virus ribozyme capable of co-transcriptional cleavage (26).

In Vitro Transcription Reactions—Plasmid DNA containing subgenomic HCV sequences (pHCVbart.rep1b/AvaII) or 3′-NTRΔpoly(U) was linearized with ScaI and purified by using QIAEX kit (Qiagen). The poly(rU/C) tract was deleted from subgenomic RNA by linearizing the transcription template MfeI. The (+)5′-NTR plasmid was linearized by using SacII. Transcription reactions (20 μl) consisted of 350 mM HEPES, pH 7.5, 32 mM magnesium acetate, 40 mM DTT, 2 mM spermidine, 28 mM NTPs (where NTPs are a mixture of ATP, GTP, UTP, and CTP), 0.5 μg of template, and 0.5 μg of T7 RNA polymerase. For the filter binding assays, the subgenomic RNA was labeled internally by adding [α-^{32}P]UTP to the *in vitro* transcription reaction. Reactions were incubated for 3 h at 37 °C. Magnesium pyrophosphate was removed by centrifugation. RQ1 DNase (Promega) was added to remove the DNA template. RNA was precipitated by using lithium chloride. The RNA pellet was suspended in TE and desalted by using a 1-ml Sephadex G-25 (Sigma) spin column. RNA concentration was determined by using absorbance at 260 nm and an extinction coefficient of 85.9 μM^{-1} cm^{-1} for subgenomic RNA, 68.6 μM^{-1} cm^{-1} for the subgenomic RNA with a poly(rU/C) tract deletion, 4.10 μM^{-1} cm^{-1} for (+)5′-NTR RNA, and 1.55 μM^{-1} cm^{-1} for the (+)3′-NTR Δpoly(U) RNA.

The (+)3′-NTR and (−)3′-TR were prepared by *in vitro* transcription of the EcoRI-digested plasmids described above. RNA with a free 5′-OH was produced by supplementing the transcription reaction with guanosine. The final transcription reaction contained the following: 25 ng/μl digested DNA, 60 mM HEPES, pH 7.5, 40 mM DTT, 2 mM spermidine, 25 mM magnesium acetate, 8 mM guanosine, 2.6 mM each NTP, and 25 μg of T7 RNA polymerase. Uridine comprises 45% of the (+)3′-NTR sequence. For this RNA, transcription proceeded more efficiently by increasing the UTP concentration to 5.2 mM. Reactions were incubated for 2 h at 37 °C, followed by digestion with RQ1 DNase (1 unit/μg DNA template) for 30 min at 37 °C. After phenol/chloroform extraction and ethanol precipitation, the transcripts were gel-purified on a 5% denaturing polyacrylamide gel containing 3.5 M urea and 50% formamide for (+)3′-NTR and 5% denaturing polyacrylamide gel with 7 M urea for (−)3′-TR. The RNA was extracted from the gel by using an Elutrap (Schleicher & Schuell) and concentrated by ethanol precipitation. RNA concentration was determined by using absorbance at 260 nm and

extinction coefficients of 2.63 and 3.98 μM^{-1} cm^{-1} for the (+)3′-NTR and (−)3′-TR, respectively. The 5′-OH-containing RNAs were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Purification and Trace Labeling of RNA Oligonucleotides—The synthetic RNA oligonucleotides were purified on a 23% denaturing polyacrylamide gel. Labeling reactions, typically 20 μl, contained 0.1 μM [γ-^{32}P]ATP, 10 μM RNA, 1× kinase buffer, and 0.4 units/μl T4 polynucleotide kinase. Reactions were incubated 1–2 h at 37 °C and stopped by incubating for 15 min at 65 °C. Unincorporated nucleotide was removed by passing through a 1-ml Sephadex G-25 spin column. The absence of labeled nucleotide contamination was verified by thin layer chromatography followed by PhosphorImaging. The integrity of the labeled RNA was verified by denaturing PAGE followed by PhosphorImaging.

RNA Filter Binding Assays—Binding reactions, typically 50 μl, contained 2 nM radiolabeled RNA (2 nM) and NS5A (0–500 nM) in binding buffer (50 mM HEPES, pH 7.5, 5 mM magnesium chloride, 10 mM BME). The binding reaction was initiated by addition of the diluted NS5A to the remaining components. Reactions were incubated on ice for 30 min. Membranes were pre-soaked in the binding reaction buffer and assembled from top to bottom as follows: polysulfone (Pall Scientific), nitrocellulose (Schleicher & Schuell), and Hybond-N$^+$ nylon (Amersham Biosciences), in a slot-blot apparatus (Amersham Biosciences). After assembly, 20 μl of each binding reaction was applied to each slot and filtered through the membranes. Membranes were air-dried and visualized by PhosphorImaging (Amersham Biosciences) and quantified by using ImageQuant software (Amersham Biosciences). Binding data were fit to the following hyperbolic Equation 1,

$$\theta = \theta_{max}[P]/([P] + K_{0.5}) \quad \text{(Eq. 1)}$$

where θ is the percentage of bound RNA; θ_{max} is the maximal percentage of RNA competent for binding; [P] is the concentration of NS5A; and $K_{0.5}$ is the apparent dissociation constant. Fitting was performed by using KaleidaGraph 3.5 software (Synergy Software). In one case, the data were fit to a cooperative binding model using the following Equation 2,

$$\theta = \theta_{max}[P]^n/([P]^n + K_{0.5}{}^n) \quad \text{(Eq. 2)}$$

where n is the Hill coefficient, and the other variables are as described above.

UV Cross-linking Assay—In a typical UV cross-linking experiment, radiolabeled rU_{15} (100 nM) was incubated with NS5A (0–500 nM) in binding buffer (50 mM HEPES, pH 7.5, 10 mM BME, 5 mM magnesium chloride) for 30 min on ice and then irradiated with 254 nm light for 15 min at 4 °C. Proteinase K digestion reaction employed 14 μg of proteinase K incubated with the cross-linked sample for 2 h at 50 °C. Each cross-linked sample was mixed with an equal volume of 2× SDS loading buffer, heated for 3 min at 65 °C prior to loading on an 8% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed, and cross-linked species were visualized by PhosphorImaging.

Fluorescence Polarization Assay—Experiments were performed by using a Beacon fluorescence polarization system (Amersham Biosciences). NS5A (0–100 nM) and 3′-fluorescein-labeled rU_{20} (FL-rU_{20}) were gently mixed in binding reaction buffer (50 mM HEPES, pH 7.5, 50 μM EDTA, 0.1 mg/ml bovine serum albumin, and 10 mM NaCl) and incubated for 3 min at 37 °C. Binding of NS5A was measured by the change in polarization. All steps were performed in reduced light. Data were fit to a hyperbola by using KaleidaGraph.

Cell Culture and G418 selection of HCV Subgenomic Replicons—Huh-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin/streptomycin (Invitrogen), and 0.1 mM nonessential amino acids (Invitrogen).

To select for subgenomic replicons that can stably replicate in Huh-7 cells, 1.6×10^6 Huh-7 cells were transfected with 2 μg of *in vitro* transcribed replicon RNA using TransMessenger transfection system (Qiagen), and 1×10^5 cells were seeded in 100-mm diameter dishes. 12–14 h post-transfection, the cells were placed under G418 selection (500 μg/ml) for 2–3 weeks. Colonies were stained with crystal violet. S2204I and pol$^-$ subgenomic replicons served as positive control and negative control, respectively.

Two-dimensional Gel Electrophoresis and Western Blot Analysis—Isoelectric focusing (IEF) of the NS5A samples was performed using Zoom IPGRunner system (Invitrogen) and Zoom strips, pH 4–7 (Invitrogen). Cell lysates for two-dimensional analysis were prepared as described in the manufacturer's protocol with some modification. *E. coli* cells expressing 5 μg of NS5A, 1.3×10^7 Huh-7 parental cells, or cells stably expressing the S2204I HCV replicon were lysed by sonication in 950 μl of lysis buffer (1× Zoom two-dimensional protein solubilizer (Invitrogen), 3 mM Tris base, 21 mM DTT, supplemented with protease inhibitor mixture (Roche Applied Science)). Five μl of 99% *N,N*-dimethylacrylamide was added to the lysates for alkylation and incubated for 30 min on ice, followed by 15 min of incubation at room temperature. Ten μl of 2 M DTT was added to quench any excess *N,N*-dimethylacrylamide, and the lysates were centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatants were collected, and the protein concentrations were measured by the Bradford assay. For isoelectric focusing electrophoresis, 10 μl of the prepared lysates were diluted in 1.1× Zoom two-dimensional protein solubilizer, 10 mM DTT, 1% pH 4–7 Zoom carrier ampholytes (Invitrogen), and 0.02% bromphenol blue to a final volume of 140 μl. A Zoom strip, pH 4–7, was hydrated with each sample for 1 h at room temperature. IEF was performed at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 105 min. After IEF, the strips were equilibrated in 1× NuPAGE LDS Sample buffer/1× Reducing Agent (Invitrogen) at room temperature for 15 min on a rotary shaker. The equilibrated strips were applied to 8% SDS-polyacrylamide gel and electrophoresed with the purified recombinant His-Δ-NS5A as the marker. After electrophoresis, proteins were transferred to nitrocellulose membrane and probed by rabbit polyclonal anti-NS5A antibody as described previously (20).

To reprobe the membrane to mouse monoclonal anti-phosphoserine antibody (Sigma), the membrane was stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50 °C for 30 min. Membrane was subsequently washed and probed with 1:1000 dilution of the anti-phosphoserine antibody and 1:1000 dilution of goat anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology). The proteins containing phosphoserine were detected by ECL Western blot detection reagents (Amersham Biosciences) and exposed to a BioMax MR x-ray film (Eastman Kodak).

UV Cross-linking and Immunoprecipitation of Huh-7 Cell Lysates with RNA—1.0×10^7 parental Huh-7 cells or S2204I replicon cells from T-75 flasks were trypsinized and washed with phosphate-buffered saline (PBS). Cells were suspended in 200 μl of hypotonic buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, supplemented with protease inhibitor mixture) on ice and lysed by the addition of 25 μl of hypotonic buffer containing 2.5% Nonidet P-40. The samples were freeze-thawed five times in liquid nitrogen and centrifuged at 16,000 × *g* for 30 min. Protein was divided into 250-μg aliquots and stored at −80 °C.

Synthetic rU_7 with 4-thio modification on the fourth uracil (4-S-rU_7) was end-labeled with [γ-^{32}P]ATP and used in the cell lysate UV cross-linking experiments. 1 μM of the radiolabeled 4-S-rU_7 was incubated

with 250 μg (50 μl) of the Huh-7 cell lysates on ice for 30 min and then irradiated at 302 nm for 5 min at 4 °C. The samples were diluted with 200 μl of the HNAET buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.67% bovine serum albumin, 1 mM EDTA, 0.33% Triton X-100) and pre-cleared by incubating with 50 μl of the pre-washed Pansorbin cells (Calbiochem) for 1 h at 4 °C on a rotary shaker. The supernatant was then incubated with 4 μl of the rabbit polyclonal anti-NS5A antibody overnight at 4 °C. 50 μl of the pre-washed Pansorbin cells were added and incubated for 2 h at 4 °C. The cell pellets were washed three times with 0.5 ml of HNAETS buffer (HNAET containing 0.5% SDS), followed by 0.5 ml of HNE buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 1 mM EDTA). Each cell pellet was suspended in 40 μl of 1× SDS sample buffer (112.5 mM Tris, pH 6.8, 2.5% SDS, 25% glycerol, 2.5% BME, and 0.025% bromphenol blue) and incubated at 90 °C for 10 min. The proteins were resolved on 8% SDS-polyacrylamide gels, and the gels were fixed and dried for PhosphorImaging analysis.

NS5A Pull-down Assay—Biotinylated HCV replicon RNA was prepared by *in vitro* transcription using T7 RNA polymerase. Typically, a 20-μl transcription reaction contains 0.5 μg of ScaI-linearized pHCV-Luc-WT plasmid DNA, 350 mM HEPES, pH 7.5, 32 mM $MgCl_2$, 40 mM DTT, 2 mM spermidine, 28 mM NTPs, 50 μM Biotin-11-CTP (PerkinElmer), and 0.5 μg of T7 RNA polymerase.

To prepare cell lysates for the pull-down assay, 3×10^7 Huh-7 cells or S2204I replicon cells were suspended in 1 ml of cold hypotonic buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl) and lysed by a Dounce homogenizer. The lysate was centrifuged at 900 × *g* for 5 min, and the S9 fraction was kept at −80 °C. In the pull-down experiments, 2 μg of biotinylated HCV replicon RNA was mixed with 50 μl of streptavidin magnetic beads (New England Biolabs) and incubated at room temperature for 30 min. 250 μg (~70 μl) of cell lysate and 100 μg (10 μl) of tRNA were added to the beads and incubated on ice for 30 min. The beads were pulled down by a magnet, and the supernatant was removed. After three washes with 100 μl of hypotonic buffer, the beads were suspended in 1× SDS sample buffer and heated at 95 °C for 10 min prior to 8% SDS-PAGE separation and Western blot analysis.

Formaldehyde Cross-linking of NS5A-HCV RNA Complexes—2 μg of the *in vitro* transcribed biotinylated HCV replicon RNA was added to 125 μg of cell lysate supplemented with 100 μg of tRNA, and the mixture was incubated on ice for 30 min. Cross-linking was initiated by adding 16% formaldehyde (Polysciences, Warrington, PA) to 1% final concentration. After incubation at room temperature for 15 min, the reaction was quenched by the addition of 0.25 M glycine (final concentration) followed by a 5-min incubation at room temperature. 50 μl of the streptavidin magnetic beads were added to the quenched sample and incubated at room temperature for 30 min. The beads were pulled down by a magnet, and the supernatant was removed. After three stringent washes with 100 μl of HNAETS buffer and two washes with 100 μl of hypotonic buffer, the beads were suspended in 50 μl of hypotonic buffer, and 1 μl (32.5 mg/ml, Sigma) of RNase A was added to the suspension. The ribonuclease treatment was allowed to go for 30 min at room temperature. After adding 50 μl of 2× SDS sample buffer, the sample was heated at 95 °C for 10 min, and the solubilized proteins were analyzed by 8% SDS-PAGE and Western blotting.

Metabolic Labeling of Proteins—S2204I or Δ2380–2409 stable cells were incubated in methionine- and cysteine-deficient Dulbecco's modified Eagle's medium (Invitrogen) or phosphate-deficient Dulbecco's modified Eagle's medium (Invitrogen) for 6 h before Express ^{35}S-protein labeling mix (200 μCi/ml; PerkinElmer Life Sciences) or [^{32}P]orthophosphate (250 μCi/ml; PerkinElmer Life Sciences) was added. After an 18-h incubation, cells were washed twice with cold PBS and lysed with SDS lysis buffer (50 mM Tris-Cl, pH 7.6, 0.5% SDS, 1 mM EDTA, 20 μg/ml of phenylmethylsulfonyl fluoride). The cell lysates were passed through a 27-gauge needle several times to shear cellular DNA. After a 10-min incubation at 75 °C, the lysates were clarified by centrifugation. Immunoprecipitation was performed as described above.

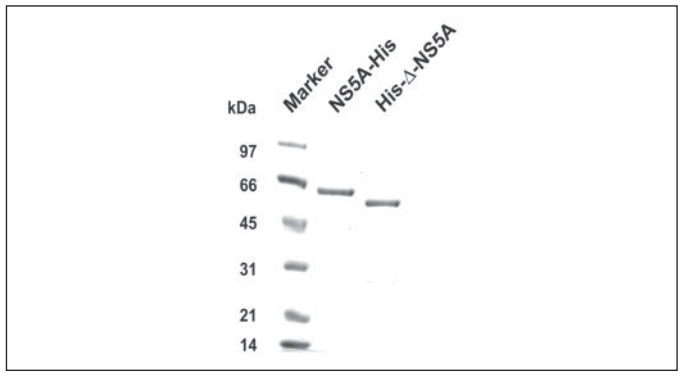

FIGURE 2. **Purified proteins employed in this study.** NS5A-His (600 ng) and His-Δ-NS5A (600 ng) were resolved by SDS-PAGE by using a 4–15% gradient gel.

RESULTS

HCV NS5A Binds to HCV Subgenomic Replicon RNA

We have described the expression and purification of two soluble derivatives of HCV NS5A that were suitable for biochemical characterization (20). The first derivative, NS5A-His, consists of the full-length NS5A protein containing a carboxyl-terminal hexahistidine tag. The second derivative, His-Δ-NS5A, contains an amino-terminal hexahistidine tag in place of the first 32 amino acids of wild-type NS5A that have been implicated in membrane binding (27). Although results presented in this report employed the more soluble His-Δ-NS5A derivative, essentially identical results were obtained with the full-length protein. The purity of NS5A-His and His-Δ-NS5A is shown in Fig. 2.

During development of the purification procedures for the NS5A derivatives, we noted that removal of nucleic acid from the extract was essential in order to observe reproducible binding of NS5A to various ion-exchange and affinity resins (20). The capacity of nucleic acid to interfere with NS5A binding to chromatography resins suggested the possibility that NS5A was a nucleic acid-binding protein. This possibility was tested directly by using a filter binding assay (28, 29). His-Δ-NS5A (0–500 nM) was mixed with labeled HCV subgenomic replicon RNA (1 nM). The binding reaction was incubated on ice for 30 min prior to separating free RNA from NS5A-bound RNA. Binding reactions were loaded into a slot-blot apparatus containing three membranes: polysulfone, nitrocellulose, and nylon. The polysulfone membrane retains any large protein-RNA aggregates. The nitrocellulose membrane retains the soluble protein-RNA complexes. The nylon membrane retains any free RNA, permitting more accurate quantitation. As the concentration of His-Δ-NS5A increased, more His-Δ-NS5A-RNA complex was retained on the nitrocellulose membrane (Fig. 3*A*). Very few, if any, large aggregates formed given the minimal retention of label on the polysulfone membrane (Fig. 3*A*). The fraction of RNA bound was plotted as a function of [His-Δ-NS5A], and the data were fit to an equation describing a hyperbola (Eq. 1), yielding a $K_{0.5}$ value of 320 ± 25 nM (Fig. 3*B*). An NS5A derivative containing the S2204I change functioned as well as wild-type NS5A (Fig. 3*B*). This change enhances stable replication of HCV subgenomic replicons (6). When the 3′-NTR was deleted from the subgenomic RNA, there was an apparent loss of high affinity binding sites (Fig. 3*C*).

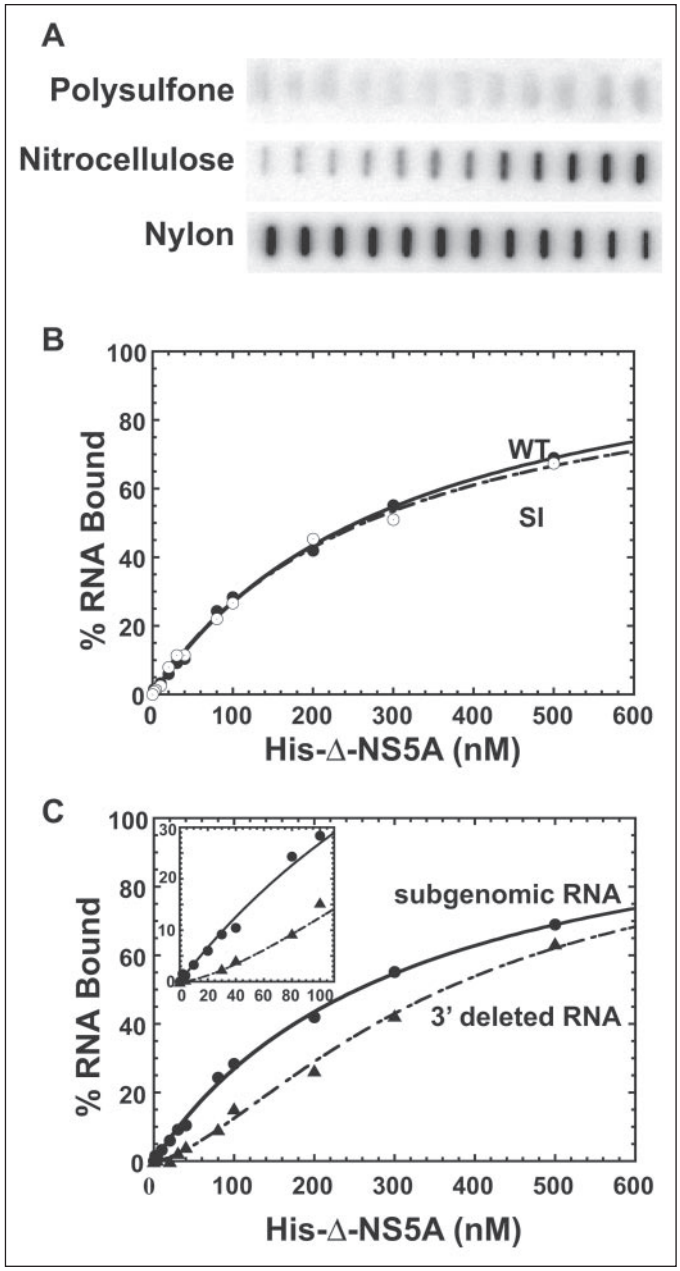

FIGURE 3. **NS5A binds to HCV subgenomic replicon RNA.** Interaction between NS5A and subgenomic RNA was monitored by using a filter binding assay. Radiolabeled RNA (1 nM) was incubated with His-Δ-NS5A (0–500 nM), loaded onto a slot-blot apparatus, and filtered through the following three membranes: polysulfone, nitrocellulose, and nylon (*top* to *bottom*). *A,* RNA bound to each membrane as a function of NS5A concentration visualized by PhosphorImaging. *B,* quantitation of the data shown in *A* yielded a percentage of RNA bound that was plotted as a function of NS5A concentration and fit to Equation 1. Both wild-type (*WT*) His-Δ-NS5A (●) and the S2204I (*SI*) derivatives (○) were evaluated. The $K_{0.5}$ value of 320 ± 25 nM is for both. *C,* an RNA derivative with a deletion of the poly(rU/C) tract in the subgenomic RNA (▲) did not contain the high affinity sites observed in the wild-type RNA (●). The *inset* expands the initial portion of the graph.

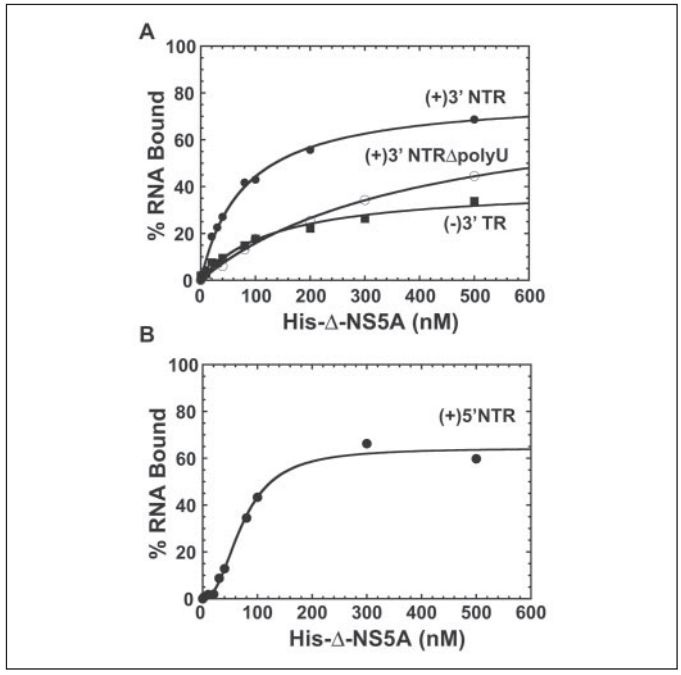

FIGURE 4. **NS5A binds to the 3′-ends of HCV plus and minus strand RNAs.** *A,* interaction between NS5A and the three 3′ RNAs was monitored, quantitated, and fit as described in the legend to Fig. 3. The $K_{0.5}$ values are 80 ± 10, 130 ± 20, and 400 ± 50 nM for the (+)3′-NTR (●), (−)3′-TR (■), and (+)3′-NTRΔpoly(U) (○), respectively. The end point for the (−)3′-TR was 40 ± 3%, suggesting that all of the sites in this RNA are not as accessible as those in the (+)3′-NTR. *B,* interaction between NS5A and the 5′-NTR was fit best by a cooperative binding model (Equation 2 under "Experimental Procedures") with the following parameters: $n = 2.3 \pm 0.2$, $K_{0.5} = 73 \pm 4$ nM, and an end point of 64 ± 2 nM.

HCV NS5A Binds to the 3′-Ends of HCV Plus and Minus Strand RNA

In order to begin to delimit the sites on HCV RNA bound by NS5A, *in vitro* transcripts representing the 3′-ends of HCV plus (3′-NTR) and minus strand (3′-TR) RNAs were evaluated. Because the 3′-ends of HCV plus and minus strand RNAs are thought to be the sites for initiation of RNA synthesis, binding of NS5A to these regions of HCV RNAs would support a role for the RNA binding activity of NS5A in genome replication. The analysis was performed as described above, and the quantitation is shown in Fig. 4. His-Δ-NS5A bound to both the 3′-NTR and 3′-TR with similar affinity, $K_{0.5}$ values of 80 ± 10 and 130 ± 20 nM, respectively (Fig. 4*A*). Although the extrapolated end point of the binding reaction for 3′-NTR was 80 ± 4%, the extrapolated end point of the binding reaction for 3′-TR was only 40 ± 3%. These data suggest that the sites to which NS5A binds on the 3′-TR are not accessible in every molecule of RNA present in the population.

Molecular genetic studies of the HCV 3′-NTR have shown that two elements of the RNA are absolutely essential for genome replication: the polypyrimidine tract and the terminal 98 nucleotides, commonly referred to as the 3′-X tail (9, 10, 30). Deletion of the polypyrimidine tract reduced substantially the capacity of NS5A to bind to this RNA (Fig. 4*A*).

Finally, an experiment was performed to determine whether NS5A would bind to the 5′-NTR. As shown in Fig. 4*B*, NS5A binds to this element. However, the affinity of the interaction is reduced substantially relative to NS5A binding to the 3′-NTR. In addition, a good fit of the data required a cooperative binding model (Eq. 2), suggesting that multiple molecules of NS5A may be required to form a stable NS5A-5′-NTR complex.

NS5A Binds to Uridylate- and Guanylate-rich RNA

In order to begin to define the sequence specificity, if any, for NS5A, we evaluated the capacity of His-Δ-NS5A to bind to a series of oligonucleotides: rU_{15}, rG_{15}, rA_{15}, rC_{15}, $(UAG)_5$ (representative of a single-stranded, heteropolymeric RNA), and the terminal 20 nucleotides from the 3′-NTR (referred to as t3′-NTR) that folds into a hairpin (representative of double-stranded, heteropolymeric RNA). His-Δ-NS5A was capable of binding both rU_{15} and rG_{15} with high affinity ($K_{0.5}$ values of 50 ± 5 and 15 ± 5 nM, respectively) (TABLE ONE). His-Δ-NS5A did not bind to any of the other RNAs tested (TABLE ONE), suggesting that NS5A binds single-stranded stretches of uridylate- and/or guanylate-

TABLE ONE

NS5A is a G/U-rich RNA-binding protein

RNA binding to the indicated RNAs was evaluated by using a filter-binding assay as described under "Experimental Procedures."

RNA employed	Binding parameters	
	$K_{0.5}$	End point
	nM	%
rU15	50 ± 5	90 ± 5
rG15	15 ± 5	95 ± 5
rA15	>500	<5
rC15	>500	<5
$(UAG)_5$	>500	<5
t3′-NTR	>500	<5
rU30	70 ± 5	100 ± 5

containing RNA. Increasing the length of oligo(rU) from 15 to 30 nucleotides did not increase the affinity of His-Δ-NS5A for this RNA (TABLE ONE), suggesting that the His-Δ-NS5A RNA-binding-site size is no greater than 15 nucleotides.

In order to confirm that the inability to observe binding of His-Δ-NS5A to rC_{15} was not due to the limited sensitivity of the direct filter binding assay, we performed a competition experiment. His-Δ-NS5A (80 nM) and labeled rU_{15} (2 nM) were mixed with increasing concentrations of rC_{15} (0–200 nM), and the fraction of His-Δ-NS5A-rU_{15} complex remaining was determined and plotted as function of rC_{15} concentration (Fig. 5). Again, neither rC_{15} (Fig. 5) nor rA_{15} (data not shown) was capable of competing effectively with rU_{15}. Competition by rC_{15} was not observed even when the concentration was increased to 800 nM. Control experiments showed that unlabeled rU_{15} competed quite well for the labeled rU_{15} (Fig. 5). We also used this approach to evaluate whether His-Δ-NS5A could bind dT_{15} or dU_{15}. Binding to dU_{15} was ~120-fold weaker than rU_{15} binding (Fig. 5); binding to dT_{15} was not observed (Fig. 5). Together, these data suggest NS5A interacts with both the sugar and base of the bound nucleic acid.

Cross-linking of His-Δ-NS5A to rU15

Multiple attempts were made to interrogate the NS5A-RNA complex by using a native gel mobility shift assay. Unfortunately, these attempts were not successful. Therefore, in order to provide some physical evidence for the association of NS5A with RNA and to obtain information on the stoichiometry of binding, we performed a UV cross-linking experiment. His-Δ-NS5A (0–500 nM) was incubated with labeled rU_{15} (100 nM) for 30 min prior to exposing the mixture to 254 nm light for 15 min. Cross-linked products were resolved by SDS-PAGE and visualized by PhosphorImaging (Fig. 6*A*). His-Δ-NS5A cross-linked to rU_{15}, and the cross-linking was concentration-dependent (Fig. 6*A*). The fastest migrating species was consistent with a 1:1 stoichiometry of His-Δ-NS5A:rU_{15}. A slower migrating species was also observed that was consistent with two molecules of His-Δ-NS5A bound to rU_{15}, suggesting that less than 10 nucleotides are required for NS5A binding to RNA. The specificity of the cross-linking was confirmed by showing that the cross-linked complexes could be competed away by using unlabeled rU_{15} but not by using unlabeled rC_{15} (Fig. 6*B*).

Determination of the Equilibrium Dissociation Constant for His-Δ-NS5A Binding to rU20

Because the filter binding approach is a nonequilibrium method, apparent dissociation constants ($K_{0.5}$) obtained by using this approach may not accurately reflect the true equilibrium dissociation constant (K_d). In order to obtain a true K_d value for the His-Δ-NS5A-rU complex, we turned to a fluorescence polarization assay. In this assay, protein is mixed with a fluorescein-conjugated nucleic acid, and complex formation is detected as an increase in anisotropy of the emitted light. His-Δ-NS5A (0–168 nM) was incubated with fluorescein-labeled rU_{20} (0.1 nM). The change in anisotropy was plotted as a function of the concentration of His-Δ-NS5A. The data were fit to an equation describing a hyperbola, yielding a K_d value of 10 ± 2 nM (Fig. 7). This K_d value is actually in good agreement with the $K_{0.5}$ value obtained by using the filter binding approach.

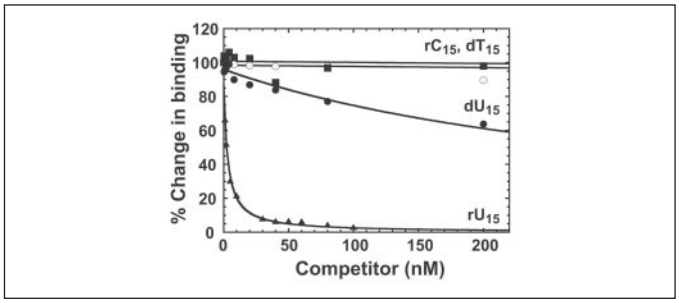

FIGURE 5. **NS5A interacts with both the base and the sugar of bound nucleic acid.** Using the filter binding assay in a direct mode is not always sensitive enough to detect weak binding. Therefore, investigation of the binding specificity of NS5A employed a competitive assay. Competitor RNA or DNA oligonucleotides (0–200 nM) (rU_{15} (▲), rC_{15} (○), $dU_{15}C$ (●), or dT_{15} (■)) were mixed with 2 nM radiolabeled rU_{15} prior to the addition of 80 nM His-Δ-NS5A. The samples were evaluated as described in the legend to Fig. 3. Shown is the percentage of radiolabeled rU_{15}-NS5A complex formed as a function of competitor oligonucleotide.

Unphosphorylated NS5A in Huh-7 Cells Replicating a Subgenomic Replicon

The protein evaluated in this study was not phosphorylated. In mammalian cells, NS5A has been shown to be phosphorylated (6, 31). In order to determine whether unphosphorylated NS5A is present in Huh-7 cells replicating a subgenomic replicon, we prepared extracts, resolved proteins by two-dimensional gel electrophoresis, and detected the various forms of NS5A by Western blotting (Fig. 8*A*). The replicon employed contained the S2204I mutation and is referred to as the SI replicon. Forms of NS5A with pI values of ~4.5, 5.0 and 6.5 were observed (Fig. 8*A*). The form with a pI value of 5.0 is clearly phosphorylated as this region of the blot stained with an anti-phosphoserine antibody (Fig. 8*B*). That the proteins detected by using antibodies against NS5A and phosphoserine are indeed NS5A was confirmed by evaluating extracts prepared from Huh-7 cells (Fig. 8, *C* and *D*). Forms of NS5A with pI values of 4.5 and 6.5 were not detected by the anti-phosphoserine antibody (compare Fig. 8, *A* to *B*). The more acidic form may arise from threonine phosphorylation. However, the more neutral form was the most likely candidate for unphosphorylated NS5A. In order to determine the pI for unphosphorylated NS5A, we evaluated extracts prepared from *E. coli* expressing full-length NS5A. As shown in Fig. 8*E*, the pI value for the unphosphorylated protein is in the 6.5 range. That this protein was unphosphorylated was confirmed by the absence of reactivity with the anti-phosphoserine antibody (Fig. 8*F*). Taken together, these data demonstrate the existence of unphosphorylated, processed NS5A at steady state in Huh-7 cells stably replicating a HCV subgenomic replicon.

Replicon-derived NS5A Binds to RNA

Binding to rU_7—The presence of unphosphorylated protein in Huh-7 cells replicating HCV RNA does not prove that this protein is competent for RNA binding. Indeed, the presence of the other HCV nonstructural proteins could preclude interaction of NS5A with RNA. In order to

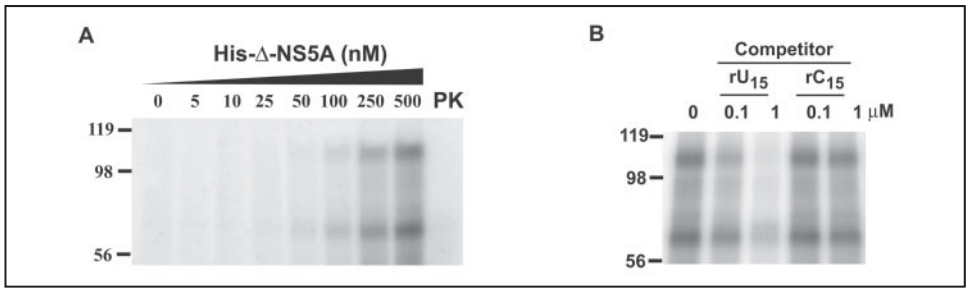

FIGURE 6. **NS5A cross-links to RNA.** *A,* cross-linking in the absence of competitor. Radiolabeled rU_{15} (100 nM) was incubated with an increasing amount of His-Δ-NS5A (0–500 nM) prior to exposure to 254 nm light. Shown is the PhosphorImager of the cross-linked products resolved by SDS-PAGE on an 8% gel. The *PK lane* is a cross-linking reaction containing 500 nM NS5A that was treated with proteinase K prior to SDS-PAGE (see "Experimental Procedures"). *B,* cross-linking in the presence of competitor. In order to show the specificity of cross-linking, experiments were performed in the presence of the indicated concentrations of rU_{15} or rC_{15}.

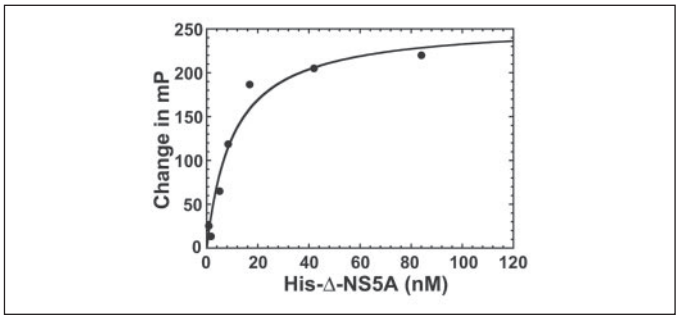

FIGURE 7. **Binding of NS5A to RNA measured by using a fluorescence polarization assay.** His-Δ-NS5A (0–100 nM) was incubated with a fluorescein-labeled rU_{20} (0.1 nM) and the fluorescence polarization (*mP*) was measured by using a Beacon fluorescence polarization instrument. The change in fluorescence polarization was plotted as a function of His-Δ-NS5A concentration and fit to a hyperbola by using KaleidaGraph, yielding a K_d value of 10 ± 2 nM.

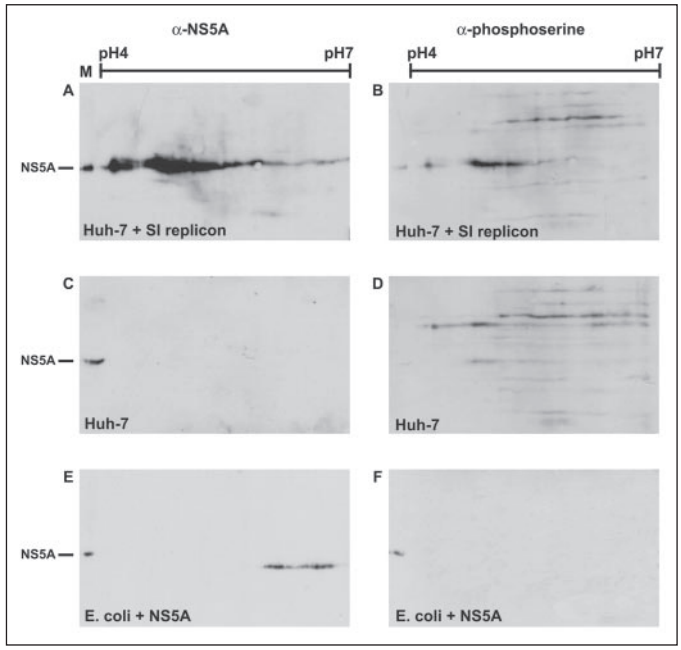

FIGURE 8. **Unphosphorylated NS5A protein exists in Huh-7 cells expressing the S2204I subgenomic replicon.** Huh-7 cells stably expressing the S2204I (SI) HCV subgenomic replicon were lysed as described under "Experimental Procedures," and the sample was subjected to two-dimensional gel electrophoresis and Western blot analysis. Parental Huh-7 cells and *E. coli* cells expressing recombinant NS5A were used as controls. Lysate prepared from the SI replicon cells was probed by using a rabbit polyclonal anti-NS5A antibody (*A*) and by a mouse monoclonal anti-phosphoserine antibody (*B*). NS5A spots were detected across the entire ranges; the phosphorylated NS5A species is centered around pH 5.0. Reactivity with anti-NS5A antibody was absent in the parental Huh-7 cell sample (*C*). The background phosphoserine containing proteins in the Huh-7 cells can be seen in *D*. The spots for the bacterially expressed unphosphorylated NS5A centered around pH 6.5 (*E*), and no phosphorylated NS5A was detected in this sample (*F*). *M* indicates the marker lane, where the purified recombinant His-Δ-NS5A was loaded.

demonstrate RNA binding activity for NS5A derived from Huh-7 cells stably replicating HCV subgenomic RNA, we performed an RNA immunoprecipitation experiment. Briefly, extracts were prepared, mixed with end-labeled rU_7 containing 4-thiouridine at position 4, and exposed to 302 nm light. NS5A was immunoprecipitated; proteins were resolved by SDS-PAGE, and the dried gel was exposed to a PhosphorImaging screen. NS5A was cross-linked to the labeled oligonucleotide (Fig. 9*A*, *lane + SI replicon*). The size of the cross-linked species was in the 60-kDa range, consistent with a single molecule of NS5A (56 kDa) bound to a single oligonucleotide (2.3 kDa). Cross-linked cellular proteins were not present in this region of the gel (Fig. 9*A*, *lane Huh-7*), confirming the specificity of the protocol. We conclude that NS5A produced in Huh-7 cells is a single-stranded RNA-binding protein capable of binding to U-rich RNA.

Binding to HCV Genome—Given the existence of a poly(U) tract in the 3′-end of HCV genomic RNA, it is reasonable to conclude that the Huh-7 cell-derived NS5A will bind to this RNA. However, other NS5A-binding sites may exist in genomic RNA. In order to ask this question, we developed an NS5A pull-down experiment. HCV RNA was synthesized *in vitro* in the presence of biotinylated CTP. Under the transcription conditions employed, one to four biotinylated cytidine residues should have been incorporated into HCV genomic RNA. The translational efficiency and specific infectivity of this RNA was within 2-fold of unmodified RNA (data not shown). Biotinylated RNA was mixed with cell lysate, and complexes were pulled down by using streptavidin beads. Bound protein was eluted from the beads by using SDS, and the presence of NS5A was determined by Western blotting after SDS-PAGE. NS5A was not observed in Huh-7 cells (Fig. 9*B*, *lane 1*). However, NS5A was observed when the intact genome was employed (Fig. 9*B*, *lane 2*). Deletion of the 3′-NTR did not prevent NS5A binding (Fig. 9*B*, *lane 3*), suggesting that the polypyrimidine tract is not the only site on HCV RNA to which NS5A binds. NS5A recovery from the replicon lysate was dependent upon RNA as NS5A was not recovered when biotinylated DNA was employed (data not shown).

Although the NS5A pull-down experiment confirms that NS5A binds to HCV RNA, this experiment does not provide any information on whether or not the binding is direct. In order to obtain evidence for direct binding, we performed the following experiment. Lysates containing NS5A (Fig. 9*C*, *lane 1*) were used to form complexes as described above (Fig. 9*C*, *lane 3*). Washing the beads under very stringent conditions released NS5A from the beads (Fig. 9*C*, *lane 4*). If the complexes were cross-linked by using formaldehyde, then NS5A recovery was resistant to stringent washing and required digestion of the RNA by RNase A (Fig. 9*C*, *lane 2* and data not shown). If the interaction of NS5A was mediated by a protein factor, then retention by cross-linking would require an NS5A-protein cross-link that would preclude the observance

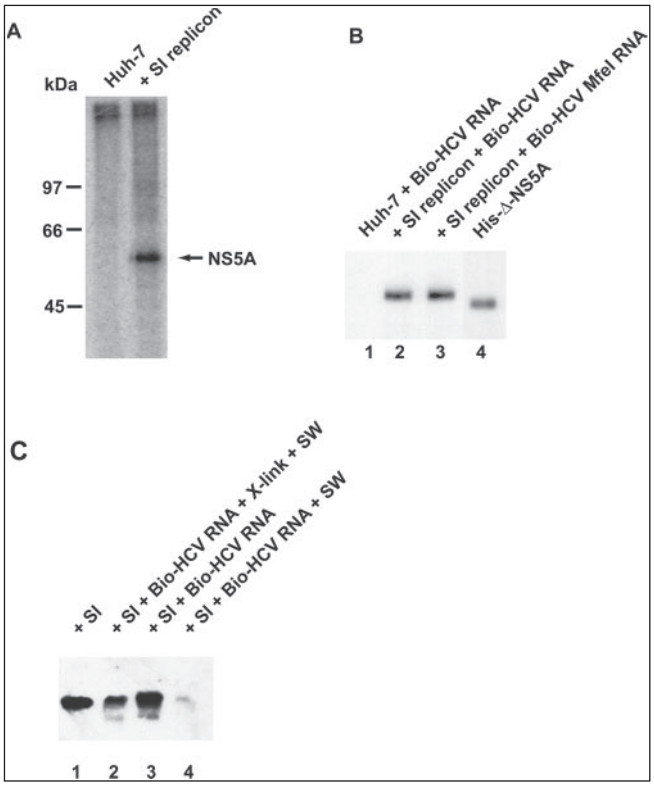

FIGURE 9. **Replicon-derived NS5A binds RNA.** *A,* cross-linking to rU7. The parental Huh-7 cells and SI replicon cells were lysed as described under the "Experimental Procedures." 250 μg (50 μl) of the total protein was incubated with 1 μM radiolabeled 4-S-rU_7 on ice for 30 min. After UV cross-linking at 302 nm for 5 min, the NS5A protein was immunoprecipitated using a rabbit polyclonal anti-NS5A antibody and Pansorbin cells. The immunoprecipitated complexes were analyzed by 8% SDS-PAGE and PhosphorImaging. A predominant NS5A cross-linked species was detected in the SI replicon sample. No band in that molecular range was present in the Huh-7 parental cells. *B,* binding to HCV genome. Biotinylated HCV replicon RNA (2 μg) was incubated with 250 μg of cell lysate. Streptavidin beads (50 μl) were added to pull down the RNA-protein complex. The samples were heated in 1× SDS sample buffer and analyzed by 8% SDS-PAGE and Western blotting. NS5A was detected when S2204I replicon cell lysate was incubated with the biotinylated HCV RNA (*lane 2*). Deletion of the 3′-untranslated region from the HCV replicon RNA did not reduce binding significantly (*lane 3*). Pull-down experiment with the parental Huh-7 cell lysates was used as a negative control (*lane 1*), and the purified recombinant His-Δ-NS5A (*lane 4*) was loaded as a positive control. *C,* cross-linking to the HCV genome. Biotinylated HCV replicon RNA (2 μg) was incubated with 125 μg of cell lysate. Cross-linking was performed by adding 1% formaldehyde for 15 min and then quenched by adding glycine (0.25 M final concentrations). Streptavidin beads (50 μl) were added to pull down the RNA-protein complex. The samples were heated in 1× SDS sample buffer and analyzed by 8% SDS-PAGE and Western blotting. NS5A was detected when S2204I replicon cell lysate was incubated with the biotinylated HCV RNA, cross-linked, pulled down, stringently washed (*SW*), and treated with RNase A (*lane 2*). NS5A was also detected when S2204I replicon cell lysates were incubated with the biotinylated HCV RNA and pulled down (*lane 3*). Very little, if any, NS5A was detected when S2204I replicon cell lysate was incubated with the biotinylated HCV RNA, pulled down, and stringently washed (*lane 4*).

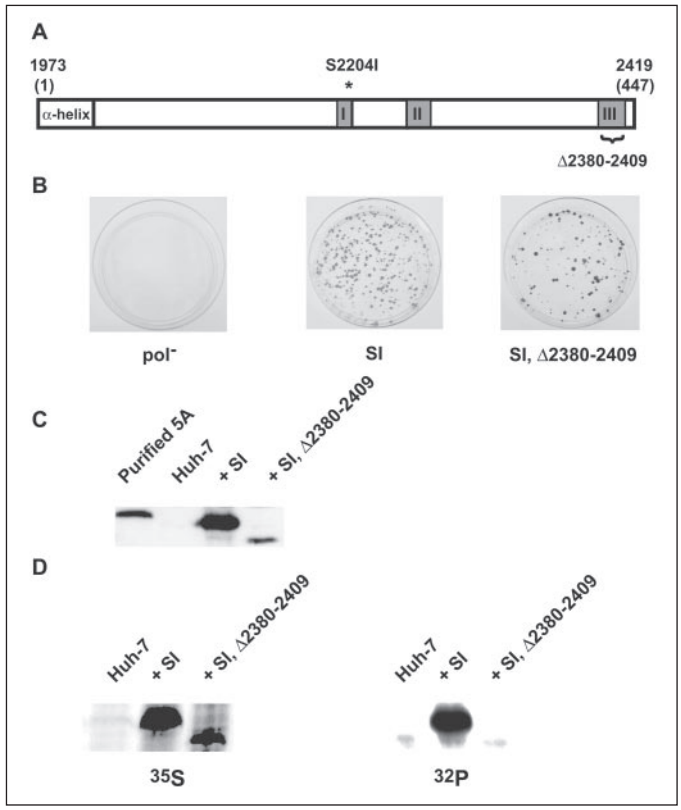

FIGURE 10. **Phosphorylation sites in the carboxyl terminus of NS5A are not essential for replication.** *A,* construct of Δ2380–2409. In the context of S2204I adaptive mutation, the third region in NS5A that contains a cluster of conserved serine residues was deleted. *B,* Δ2380–2409 replicates in G418 selection assay. 2 μg of *in vitro* transcribed subgenomic replicon RNA was transfected to 1.6×10^6 Huh-7 cells. 1×10^5 cells were seeded in 100-mm diameter dishes and were subjected to G418 selection. The replication-deficient pol^- replicon that contains a GDD to GAA mutation in the viral polymerase did not produce any viable colonies, whereas Δ2380–2409 replicon replicated with efficiency similar to the S2204I replicon. *C,* Western blot analysis of NS5A expressed in the Δ2380–2409 replicon cells. Proteins from Huh-7 cells that stably supported S2204I or Δ2380–2409 replicon were separated on an 8% SDS-polyacrylamide gel and were probed with a rabbit anti-NS5A polyclonal antibody. Purified recombinant NS5A and the parental Huh-7 cells were used as positive and negative controls. *D,* metabolic labeling of S2204I and Δ2380–2409 replicon cells. Parental Huh-7 cells, SI replicon cells, and Δ2380–2409 replicon cells were incubated with ^{35}S-protein labeling mix (*left*) or [^{32}P]orthophosphate (*right*) in Dulbecco's modified Eagle's medium for 18 h. After lysis, NS5A was immunoprecipitated with a rabbit anti-NS5A antibody. Immunoprecipitates were separated on 8% SDS-polyacrylamide gels and visualized by PhosphorImaging.

of appropriately sized NS5A. Therefore, we conclude that binding of replicon-derived NS5A to HCV RNA is mediated by a direct binding of the protein to RNA.

Primary Sites for NS5A Phosphorylation Are Dispensable for RNA Replication in Huh-7 Cells and RNA Binding in Vitro

A second approach that we took to evaluate the potential role of NS5A phosphorylation in RNA binding was to engineer a replicon that expressed an NS5A mutant incapable of being phosphorylated by casein kinase II. We and others (20, 31) have shown that casein kinase II is responsible for most of the observed phosphorylation of NS5A and that these sites are restricted to a carboxyl-terminal domain of NS5A referred to as cluster III (Fig. 10*A*). We deleted residues 2380–2409 in cluster III in the background of the S2204I adaptive mutation. This mutant was capable of stable replication in Huh-7 cells (Fig. 10*B*, *panel SI,Δ2380–2409*). Western blotting confirmed the expression of the truncated NS5A derivative (Fig. 10*C*, *lane SI,Δ2380–2409*). Although both wild-type NS5A (SI) and the deletion mutant (SI,Δ2380–2409) were efficiently labeled with [^{35}S]methionine and -cysteine (Fig. 10*D*, *panel ^{35}S*), only the wild-type protein was labeled with [^{32}P]orthophosphate (Fig. 10*D*, *panel ^{32}P*). The S2204I mutation is known to prevent formation of the p58 form of NS5A, *i.e.* phosphorylation in cluster I (Fig. 10*A*) (31). Together, these studies suggest that extensive phosphorylation of NS5A is not required for the replication functions of NS5A.

DISCUSSION

NS5A Is a Single-stranded-RNA-binding Protein with Specificity for Uridylate- and Guanylate-rich RNA—HCV NS5A has been implicated in interactions with myriad cellular proteins (16, 21, 32). However, a more direct role in genome replication has been suggested by the finding that NS5A interacts in solution with NS5B, the viral RNA-dependent RNA polymerase (23). In order to begin to employ a biochemical approach to sort out the various functions of NS5A, we developed a

procedure to express and purify NS5A from the soluble fraction of *E. coli* (20). During development of the purification protocol, we noted that nucleic acid in the cell extract prevented NS5A from binding to several chromatographic resins. This observation suggested the possibility that NS5A was a nucleic acid-binding protein.

Our initial experiments showed that purified NS5A (Fig. 2) had the capacity to bind to HCV subgenomic replicon RNA with an affinity in the micromolar range (Fig. 3). At least one region of this RNA that was bound by NS5A was the 3′-NTR (Fig. 4), and high affinity binding to this RNA required the polypyrimidine tract. NS5A also had the capacity to bind the 3′-end of HCV minus strand RNA (referred to as 3′-TR in Fig. 4). In order to define the specificity of binding, we evaluated the capacity of NS5A to bind to a variety of single- and double-stranded RNA and DNA oligonucleotides (TABLE ONE). NS5A had a clear preference for U-rich and G-rich RNA (TABLE ONE). This preference was scrutinized further by using a variety of approaches, including a competition filter binding assay (Fig. 5), UV cross-linking (Fig. 6), and fluorescence polarization (Fig. 7). Most interestingly, NS5A interacts with both the base and the sugar moieties of nucleic acid as the affinity of NS5A for rU_{15} was much greater than for dU_{15}, and no detectable binding to dT_{15} was observed (Fig. 5). More importantly, HCV NS5A present in cell-free extracts prepared from Huh-7 cells stably replicating HCV subgenomic RNA was also capable of binding to a synthetic RNA oligonucleotide and full-length HCV genomic RNA (Fig. 9), suggesting that RNA binding activity of NS5A is retained in a cellular milieu in the presence of the other nonstructural proteins.

Putative NS5A-binding Sites in the 5′- and 3′-Ends of HCV Plus and Minus Strand RNA—Given the observed preference of NS5A for U- and G-rich RNA, we asked whether elements of the appropriate composition existed in either the 3′-NTR or 3′-TR. The polypyrimidine tract is the primary location for high affinity NS5A-binding sites in the 3′-NTR (Fig. 11*A*). U/G stretches exist in every stable stem of the 3′-TR (Fig. 11*B*). In addition, U/G stretches also exist in the 5′-NTR, and many of these tracts are in elements known to contribute to the internal ribosome entry site (IRES) (Fig. 11*C*).

The polypyrimidine tract in the 3′-NTR is absolutely essential for genome replication and virus viability (9, 10, 30). Efficient genome replication is observed when only 26 uridylate residues are present in this region of the genome (9). Most interestingly, a genome containing only six uridylate residues is quasi-replication-competent as replication-competent revertants can be obtained (9). In contrast, a genome containing only a single uridylate residue is lethal for genome replication (9). These data, combined with the observation that the polypyrimidine tract is the site for NS5A binding to the 3′-NTR, suggest that binding of NS5A to the 3′-NTR may be necessary for efficient RNA synthesis. This possibility does not exclude a role for cellular proteins binding to the polypyrimidine tract (33). Indeed, the 3′-NTR has been suggested to be important for translation (34), although a role for the polypyrimidine tract in translation is unlikely (10).

Sites other than the 3′-NTR are utilized by NS5A. The quantity of NS5A recovered from an RNA pull-down experiment was unchanged by deleting the 3′-NTR from HCV RNA (Fig. 9*B*). This observation confirms the existence of low affinity binding sites in the HCV genome and the capacity for NS5A to act as a nonspecific RNA-binding protein. Of course, the number of nonspecific sites greatly exceeds the number of specific sites. As discussed in greater detail below, the function of this nonspecific RNA binding activity could be to reduce the level of secondary structure present in the genome.

The 3′-TR is also assumed to be essential for genome replication. Clearly, production of full-length plus strand RNA requires initiation at the 3′-end of the minus strand, so the complex secondary structure associated with this cis-acting replication element has been suggested to function in replicase assembly (35). In the 3′-TR, single-stranded U/G stretches are not predicted to exist in the most thermodynamically stable form of the RNA (Fig. 11*B*). This prediction provides an explanation for the reduced end point observed for binding of NS5A to this RNA (Fig. 4). It is possible that a fraction of the RNA was trapped in a misfolded conformation, exposing U/G-rich elements that could be bound by NS5A. Alternatively, given the intrinsic dynamics (breathing) of nucleic acid duplexes (36, 37), it is possible that NS5A was capable of trapping open regions of the appropriate sequence composition when present in the single-stranded conformation.

The HCV 5′-NTR is absolutely essential for HCV multiplication (38). The 5′-NTR can be divided into three domains, I–III. Domain I is required for genome replication (38). Domains II and III form the IRES that is required for production of the HCV polyprotein (38). Domains II and III form a tertiary structure (39, 40) that facilitates interaction with the 40 S ribosomal subunit (41), and this binary complex then recruits eIF3 to form a 43 S preinitiation complex (39). Interactions between the IRES and 40 S ribosomal subunit are mediated by the interaction of ribosomal proteins with most of the accessible surface of domains II and III. The interaction between eIF3 and domain III is restricted to the bulge in stem IIIb (Fig. 11*C*). At least one potential site for NS5A binding to the 5′-NTR is present in each of the three domains, with the highest frequency of sites located in domain III (Fig. 11*C*). Particularly intriguing is the presence of a binding site in the domain IIId loop. This loop is absolutely essential for 40 S ribosome binding, and antisense oligonucleotides directed to this loop inhibit 40 S ribosomal subunit binding and translation (42).

Functional Implications for the Interaction between NS5A and 5′- and 3′-Ends of HCV Plus and Minus Strand RNA—One of the most obvious functions for binding sites in the 3′-end of plus and minus strand RNAs is recruitment of replicase components to the appropriate RNA. NS5A is known to interact with NS5B (15, 24), and NS5B interacts with NS3-4A, the viral helicase (15, 25). However, once assembled, the presence of a single-stranded RNA-binding protein with even low affinity for RNA could help to stabilize single-stranded RNA intermediates produced by the helicase, for example in the 3′-TR, and to increase the efficiency of replication by the polymerase by diminishing the level of secondary structure. In fact, given the substantial accumulation of NS5A in cells persistently replicating HCV RNA (43), too high of an affinity for RNA could inhibit translation of cellular and viral mRNA and genome replication.

A function for NS5A binding to the IRES and the 3′-NTR of HCV RNA could be to cause the switch from translation of the genome to replication of the genome. It is well documented that RNA templates for translation in eukaryotes are functionally circular because of interactions between proteins bound to the 5′- and 3′-ends of the RNA. Viral RNAs are likely no different (44). Whereas the 40 S subunit and eIF3 bind to the HCV IRES, many cellular proteins have been implicated in binding to the 3′-NTR. Whether or not proteins bound to the 3′-NTR participate in translation or genome circularization is not known. However, it is safe to assume that these proteins need to be displaced in order for genome replication to occur. NS5A binding to subdomain IIId of the IRES could prevent ribosome binding, and preinitiation complex formation and binding to the polyuridine tract could provide a steric block to formation of protein-bridged interactions between the 5′- and 3′-NTRs. Although it is not absolutely clear whether circularization of the HCV genome is required for genome replication, the possibility has been suggested (45). This level of organization of the genome could be

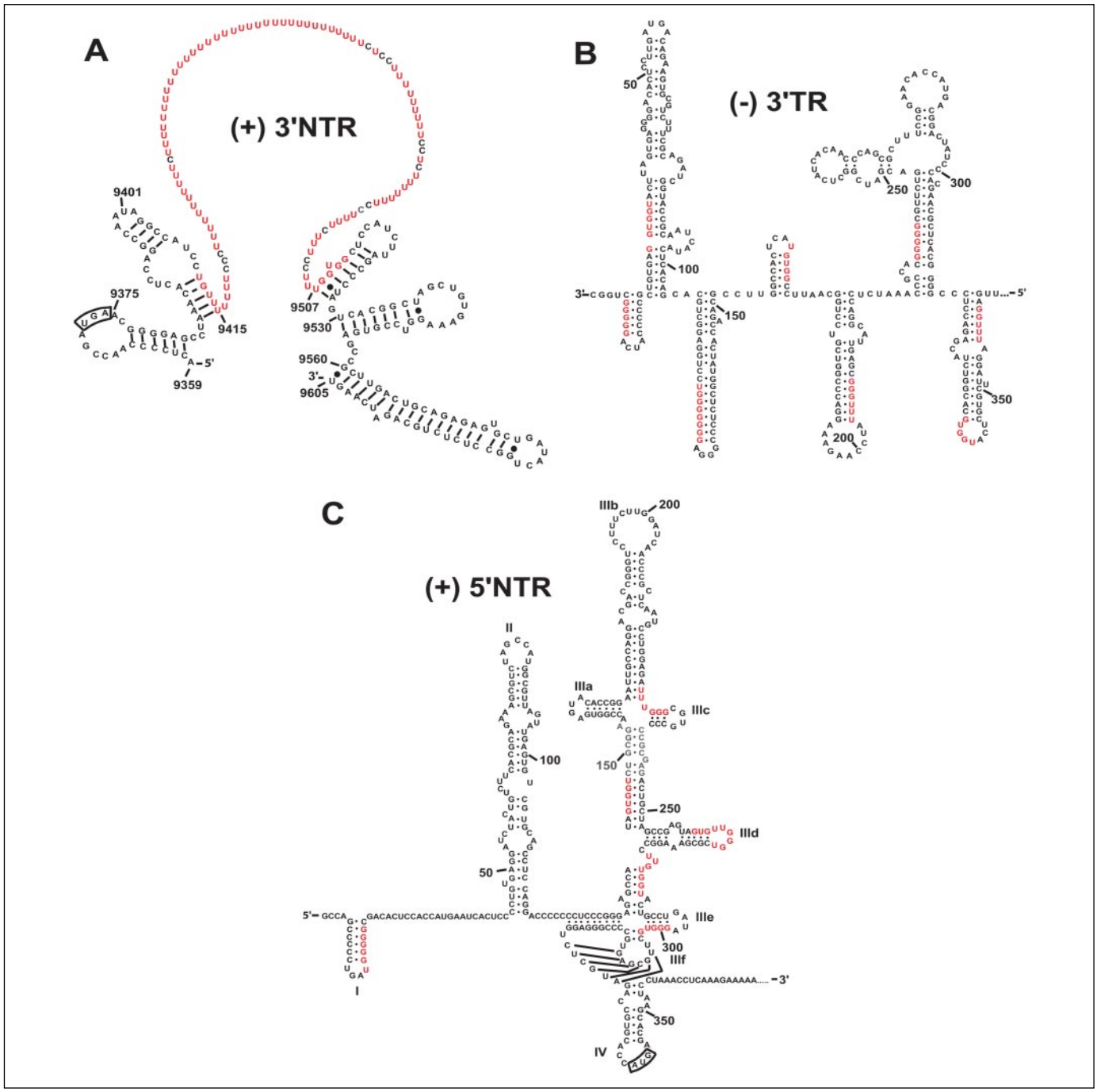

FIGURE 11. **Potential NS5A-binding sites in HCV RNA.** Highlighted in *red* are G/U stretches observed in (+)3′-NTR (*A*), (−)3′-TR (*B*), and (+)5′-NTR of HCV RNA (*C*).

mediated by interaction between NS5A proteins bound to the 5′- and 3′-NTRs. Alternatively, NS5A bound to the 5′-NTR could interact with NS5B bound to the 3′-NTR.

Role of Phosphorylation—NS5A can be phosphorylated in mammalian cells (17). It is generally assumed that all of the NS5A in cells is phosphorylated because the protein migrates in SDS-polyacrylamide gels as 56–58-kDa species instead of the expected 49-kDa species. However, we have shown that unphosphorylated, untagged NS5A co-migrates with the 56-kDa form produced in Huh-7 cells (20). Therefore, it was possible that unphosphorylated protein is present in the cell. The existence of unphosphorylated NS5A in Huh-7 cells was confirmed by using two-dimensional gel electrophoresis and Western blotting to evaluate extracts prepared for cells stably replicating an HCV subgenomic replicon (Fig. 8). RNA immunoprecipitation experiments were used to show that NS5A protein produced in the replicon cells is capable of binding to RNA (Fig. 9). However, more studies will be required to determine whether this binding was due to unphosphorylated protein, phosphorylated protein, or both.

Three potential clusters of phosphorylation exist (Fig. 1). Cluster II contains, at best, a single conserved site of phosphorylation. In contrast, cluster III has multiple phosphorylation sites and is the primary reason that NS5A can be metabolically labeled with orthophosphate (Fig. 10*D*). Cluster III can be deleted without any impact on the RNA binding

activity of NS5A,[3] and these same deletions support genome replication in Huh-7 cells (Fig. 10*B*). The role of cluster I is not as straightforward. This region is absolutely required for genome replication and is the site of mutations that permit HCV genomes to replicate in Huh-7 cells (6). Again, preliminary studies suggest the NS5A derivatives with changes in cluster I retain RNA binding activity. Because NS5A phosphorylation is not essential for RNA binding, it is possible that NS5A phosphorylation provides a mechanism to activate cryptic functions of NS5A, for example by regulating the interferon-induced, double-stranded RNA-activated protein kinase or interacting with proteins involved in cellular signaling pathways. Notwithstanding, phosphorylation may alter the specificity or affinity of NS5A for RNA. Additional studies will be required to address these possibilities directly.

Cellular Proteins with Comparable Specificity—The sequence of NS5A lacks any motifs consistent with the RNA binding activity of the protein. Actually, this circumstance is not completely unexpected for two reasons. First, many RNA-binding motifs, for example the RNA recognition motif, are based on the tertiary fold and often lack sequence similarity (46). Second, many of the sequence-specific interactions of proteins with RNA employ the carbonyl and amide moieties of the backbone rather than the side chains of amino acids, thus providing an explanation for minimal sequence similarity, if any (46).

Because we could not use the NS5A sequence to gain insight into NS5A structure or function, we asked whether the NS5A binding specificity was unique. If proteins of known structure and function with similar binding specificity exist, then these proteins may represent useful starting points for development of a structural model for NS5A and provide additional clues into possible virus-host interaction functions for NS5A. Several cellular proteins with specificity essentially identical to that of NS5A, *i.e.* capable of binding to poly(rU) and/or poly(rG) but incapable of binding to poly(rC) or poly(rA), were identified: lupus antigen (47, 48), cleavage and stimulation factor 64 (49), TIA-1 (50), sex-lethal protein (Sxl) (51), *Trypanosoma cruzi* RNA-binding proteins (52), and fragile X mental retardation protein (53). With the exception of fragile X mental retardation protein, which has a KH (hnRNA homology) domain, all of these proteins contain one or more RNA recognition motifs. Most of these proteins exist in the cytoplasm, at least occasionally, and in this compartment, these proteins function as post-transcriptional regulators of gene expression (48, 50, 52, 53). Some of these proteins also exist occasionally in the nucleus, for example Sxl and TIA-1, and in this compartment, these proteins function as splicing regulators. Most interestingly, lupus antigen binds to both the 5′- and 3′-NTRs of HCV RNA (54, 55).

Recently, it has become clear that the amino-terminal domain of NS5A binds zinc (56) by using a new zinc coordination motif in the context of a novel fold (57). This domain crystallized as a dimer. A groove exists at the interface of the two molecules that has a positive electrostatic potential with dimensions appropriate for binding to single-stranded RNA (57). Therefore, NS5A may define a novel structural class of RNA-binding proteins.

In conclusion, the finding that HCV NS5A is an RNA-binding protein provides the second activity of this protein with implications for genome replication and represents another functional target for the development of anti-HCV therapeutics. The capacity of cellular pre-mRNA splicing, mRNA stability, and translation to be regulated by proteins with binding specificities identical to that of NS5A begs the question: does NS5A employ these activities as well?

[3] L. Huang and C. E. Cameron, unpublished observations.

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Hepatitis C Virus Nonstructural Protein 5A (NS5A) Is an RNA-binding Protein

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