Intragenic Complementation of Hepatitis C Virus NS5A RNA Replication-Defective Alleles

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Hepatitis C virus NS5A has three structural domains, is required for RNA replication and virion assembly, and exists in hypo- and hyperphosphorylated forms. Accumulated data suggest that phosphorylation is involved in modulating NS5A functions. We performed a mutational analysis of highly conserved serine residues in the linker region between domains I and II of genotype 2a JFH1 NS5A. As with genotype 1b Con1 NS5A, we found that specific serine residues were important for efficient hyperphosphorylation of JFH1 NS5A. However, in contrast with Con1 replicons, we observed a positive correlation between hyperphosphorylation and JFH1 replicon replication. We previously demonstrated *trans*-complementation of a hyperphosphorylation-deficient, replication-defective JFH1 replicon. Our results suggested that the defective NS5A encoded by this replicon, while lacking one NS5A function, was capable of performing a separate replication function. In this report, we examined an additional set of replication-defective NS5A mutations in *trans*-complementation assays. While some behaved similarly to the S232I replicon, others displayed a unique *trans*-complementation phenotype, suggesting that NS5A *trans*-complementation can occur by two distinct modes. Moreover, we were able, for the first time, to demonstrate intragenic complementation of replication-defective NS5A alleles. Our results identified three complementation groups: group A, comprising mutations within NS5A domain I; group B, comprising mutations affecting serine residues important for hyperphosphorylation and a subset of the domain I mutations; and group C, comprising a single mutation within the C-terminal region of domain II. We postulate that these complementation groups define three distinct and genetically separable functions of NS5A in RNA replication.

Hepatitis C virus (HCV) is an enveloped positive-sense single-stranded RNA virus of the genus *Hepacivirus* in the family *Flaviviridae*. The ~9.6-kb HCV genome encodes an ~3,000-amino-acid (aa) polyprotein that is cleaved by viral and cellular proteases into 10 mature proteins: core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (reviewed in references 1 and 2). Core is the viral capsid protein, while E1 and E2 are highly glycosylated envelope proteins important for viral entry. P7, a small transmembrane ion channel protein, and NS2, a membrane-associated protease, are required for assembly and release of infectious virions. The remaining nonstructural (NS) proteins are essential components of the HCV RNA replication complex. NS5B is an RNA-dependent RNA polymerase that catalyzes synthesis of positive- and negative-strand RNA. NS3 and its cofactor NS4A function as a serine protease responsible for releasing mature NS proteins from the polyprotein precursor. NS3 also possesses RNA helicase and NTPase activities essential for RNA replication. NS4B is an integral membrane protein likely involved in formation of intracellular membranous compartments where viral replication occurs. The final viral component of the replication complex, NS5A, is a multifunctional protein whose precise roles in RNA replication and other aspects of the HCV life cycle are still being elucidated.

Interactions between NS5A and a variety of host cell proteins, as well as other HCV NS proteins, have been described (reviewed in reference 3). While the biological relevance of many of these interactions remains unclear, others play important roles in HCV replication (3). NS5A consists of an N-terminal amphipathic alpha helix (amino acids 5 to 25) that targets NS5A to intracellular membranes and three structural domains (I, II, and III) separated by regions of low-complexity sequence (LCS I and LCS II) (4–7). Domain I (aa 28 to 213) contains a zinc-binding motif, is relatively well conserved among HCV isolates, and is essential for RNA rep-

lication. NS5A domain I has been crystallized in two distinct, non-overlapping homodimer configurations (8, 9). A groove formed between the monomers in the Tellinghuisen et al. (9) dimer is a potential RNA binding pocket, and several studies have confirmed the capacity of NS5A to bind RNA (10–13). The RNA binding groove is not present in the NS5A dimer reported by Love et al. (8), suggesting the possibility that different dimer conformations of NS5A could possess distinct biological properties. Domains II (aa 250 to 342) and III (aa 356 to 447) are less well conserved than domain I and are natively unfolded (14, 15). Domain II is important for RNA replication and may also contribute to the RNA binding properties of the protein (10, 11). Domain III is largely dispensable for RNA replication, but it is essential for virion assembly (16, 17).

When NS5A is expressed in tissue culture cells in the context of a contiguous NS3-NS5A polyprotein, at least two alternatively phosphorylated forms of the protein, which can be distinguished by electrophoretic mobility, are produced (18, 19). Hypophosphorylated NS5A (p56) is phosphorylated at one or more residues in the central and C-terminal part of the protein, while hyperphosphorylated NS5A (p58) is believed to also be phosphorylated at one or more highly conserved serine residues within the surface-exposed LCS I (aa 214 to 249) connecting domains I and II

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(20, 21). Differential phosphorylation potentially regulates several functions of NS5A in RNA replication and virion assembly (reviewed in reference 22).

Like many positive-sense RNA viruses, HCV RNA replication occurs within virus-induced intracellular membrane alterations that provide a structural site for assembly and function of the replication machinery and may also function to shield the virus from host cell antiviral responses (23, 24). As a consequence of the membrane compartmentalization and sequestration of viral replication complexes, diffusion of virus-encoded replication proteins between replication complexes encoded by different viral genomes may be limited, leading to a preference for *cis*-dominant replication functions. For HCV, *cis*-acting RNA replication functions have been reported for NS3, NS4A, and NS5B, while *trans*-acting functions have been reported for NS5A and NS4B (25–28).

We previously used *trans*-complementation assays with HCV replicons encoding NS5A proteins with different sensitivities to the HCV replication complex inhibitor dechlorasvir (DCV) (BMS-790052) to demonstrate two distinct functions of NS5A in RNA replication, one that was efficiently rescued by *trans*-complementation and another that displayed a strong *cis*-dominance. In this study, we examined the correlation between NS5A hyperphosphorylation and replication in the JFH1 subgenomic replicon system, while also expanding our previous results by examining *trans*-complementation and intragenic complementation of a range of NS5A replication-defective mutants. Our results identify three distinct functions of NS5A required for HCV RNA replication.

MATERIALS AND METHODS

Cells, inhibitors, and recombinant DNA clones. Human hepatoma (Huh-7.5) and baby hamster kidney (BHK1) cells were maintained as previously described (29). A plasmid encoding a bicistronic replicon with the Con1 HCV 5' untranslated region (5'UTR) and internal ribosomal entry site (IRES) sequences, a *Renilla* luciferase (Rluc) reporter gene, and an encephalomyocarditis virus (EMCV) IRES followed by the NS3 through 3'UTR region from the JFH1 strain has been described (26). NS5A amino acid substitutions were introduced into the replicon plasmid by replacing a BspE1-BspE1 fragment with PCR-generated fragments and were confirmed by sequence analysis. A Con1 replicon with a Rluc reporter has been described (30). Dechlorasvir (BMS-790052) has been described (31).

Replicon assays. Transient replication assays were performed as previously described (26, 29). Briefly, replicon transcripts, generated with a Ribomax T7 express system (Promega Corp., Madison, WI) from XbaI-linearized plasmids, were transfected into Huh7.5 cells by using DMRIE-C (liposome formulation of the cationic lipid DMRIE [1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide] and cholesterol) reagent (Invitrogen, Corp., Carlsbad, CA). Ten micrograms of RNA was used for single-replicon transfections, while 20 μ g (10 μ g of each replicon) was used for cotransfections. After 24 h, transfected cells were transferred to 96-well tissue culture plates (~10,000 cells/well in 200 μ l) and treated with serial dilutions of inhibitors (1 μ l/well) in dimethyl sulfoxide (DMSO). After an additional 72 h, plates were harvested for *Renilla* luciferase assays as described previously (29). Replication capacities were measured as replication windows (uninhibited signal/background signal) as described previously (26, 29). Background signals were derived from wells treated with ≥ 1 μ M DCV, and uninhibited signals were from DMSO-treated wells. Dose-response curves and inhibitor half-maximal effective concentrations (EC_{50} s) were generated with IDBS XLfit (dose response one-site model 205) as described previously (32).

Transient protein expression and immunoblotting assays. HCV nonstructural proteins were expressed from replicon plasmids in BHK-21

cells by using a modified vaccinia virus-T7 (MVA-T7) expression system or following transfection of replicon RNA into Huh7.5 cells as previously described (26, 33). Western blot analysis of NS5A and NS3 proteins was performed as previously described (26). NS5A (7B5) and NS3 (2E1) monoclonal antibodies (BioFront Technologies, Tallahassee, FL) were used at 1:1,000 dilutions. A rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA) was used at a 1:10,000 dilution.

RESULTS

Several serine residues in the LCS I linker region connecting NS5A domains I and II are highly conserved among NS5A isolates, including between the genotype 1b Con1 isolate and the genotype 2a JFH1 isolate (Fig. 1A). In Con1 subgenomic replicons, serine-to-alanine replacements of a subset of serine residues in this region have been shown to decrease NS5A hyperphosphorylation and enhance replication (34). To assess the importance of the conserved serine residues in the JFH1 strain, individual serine-to-alanine substitutions were introduced into a subgenomic JFH1 replicon containing a *Renilla* luciferase (Rluc) reporter gene. The mutant replicons were assessed for replication in transient replication assays, and NS5A expression was examined by Western blot analysis following vaccinia virus-T7 polymerase-mediated (VV-T7) expression of the replicon-encoded proteins. Serine-to-alanine substitutions at positions 225, 229, 232, and 235 markedly reduced replication levels while also decreasing NS5A hyperphosphorylation (Fig. 1B). Very similar NS5A expression patterns were observed following transfection of replicon RNA directly into Huh7.5 cells (Fig. 1B, bottom blot), confirming that the effect of the mutations on hyperphosphorylation was not an artifact of the VV-T7 expression system. The most severe replication defects were seen with replicons bearing S229A and S235A amino acid substitutions, which were indistinguishable from a negative-control GND replicon with a glutamic acid-to-asparagine substitution at the catalytic site of NS5B. In agreement with previous results (26), a JFH1 replicon with an S232I amino acid substitution was also severely impaired for both hyperphosphorylation and replication. In contrast, alanine replacement of serine residues at positions 222, 228, 230, and 238 did not appreciably impair replication capacity or NS5A hyperphosphorylation (Fig. 1B). These results suggest that phosphorylation of serine residues at positions 225, 229, 232, and 235 might be important for replication of the JFH1 replicon. To further assess this possibility, glutamic acid residues were introduced as phosphoserine mimics at these positions. Replicons with S225E, S232E or S235E amino acid substitutions replicated at >80% of the wild-type (WT) level (Fig. 1C), consistent with replication being facilitated by phosphorylation of these residues. In contrast, a replicon with an S229E amino acid substitution displayed the same replication-null phenotype as a replicon with an S229A substitution, indicating that introduction of negative charge at this position was not sufficient to promote efficient replication, and distinguishing this serine residue from serine residues at positions 225, 232, and 235. Similar levels of NS5A were expressed from each of these replicons, suggesting that the associated replication phenotypes were not related to differences in protein stability (Fig. 1C). Introducing glutamic acids at positions 229, 232, and 235 yielded NS5A polypeptides that migrated slightly slower on SDS-polyacrylamide gels than polypeptides with alanine residues at these positions (Fig. 1C). Similar shifts in gel migration upon replacing serine with glutamic acid have been observed with other proteins (35, 36). For a control,

FIG 1 Highly conserved serine residues in the LCS I region of NS5A are important for JFH1 replication and NS5A hyperphosphorylation. (A) Alignment of Con1 and JFH1 NS5A amino acid residues 222 to 238 is shown, and the conserved serine residues are boxed. (B) JFH1 replicons with *Renilla* luciferase (Rluc) reporter genes and with the indicated amino acid substitutions were transiently expressed in Huh7.5 cells in the presence (1 μ M) and absence (DMSO) of DCV as described in Materials and Methods. Replication capacities were determined from luciferase activities approximately 96 h after transfection and are plotted as replication windows (relative light units [RLU] from DMSO-treated cells/RLU from DCV-treated cells) relative (rel.) to the value for the parental control (WT), which was set at 100. Values are means plus standard deviations (error bars) from at least two independent experiments performed in duplicate. A replicon with a mutation affecting the catalytic site of NS5B (GND) was used as a negative replication control. Western blots showing NS5A proteins transiently expressed from the indicated replicons by using a vaccinia virus expression system (VV-T7) or following transfection of replicon RNA into Huh-7 cells (RNA trx) are shown below the graph (see Materials and Methods for details). The positions of hyperphosphorylated (hyper-P) and hypophosphorylated (hypo-P) NS5A phosphoforms are indicated to the right of the top blot. Not all of the mutants were included on the bottom blot. (C) Replicons with the indicated amino acid substitutions were transiently expressed in Huh7.5 cells, and replication windows were measured

hyperphosphorylation and replication phenotypes of Con1 replicons with isoleucine or alanine replacements for S232 were examined. As expected (34, 37), in the context of the Con1 replicon, these amino acid substitutions resulted in increased replication and decreased hyperphosphorylation relative to the wild-type replicon (Fig. 1D).

A replication-defective JFH1 replicon with an S232I substitution can be rescued (*trans*-complemented) by coexpression of a helper replicon encoding a functional NS5A (26, 27). To determine whether replication defects associated with S229A, S229E, and S235A amino acid substitutions could also be *trans*-complemented, Rluc replicons harboring these mutations were transfected into Huh7.5 cells together with a helper replicon that carried a neomycin-resistant marker (neo) but did not possess a luciferase reporter (JFH1-neo), thus permitting replication of the defective replicons to be monitored by luciferase enzyme activity. The control S232I replicon was efficiently *trans*-complemented, with a replication capacity of $\sim 14\%$ (replication window of 271) (Fig. 2A) of the WT JFH1 control replicon. Replicons harboring S229A, S229E, and S235A amino acid substitutions were similarly *trans*-complemented, with mean replication windows of 157, 182, and 185, respectively. In contrast, and in agreement with previous results (27, 34), a replicon with an active site mutation in NS5B (GND) was not *trans*-complemented (Fig. 2A).

We previously showed that replication of a *trans*-complemented JFH1-S232I replicon was sensitive to the NS5A inhibitor DCV even when the helper replicon was DCV resistant (26). To determine whether the S229A, S229E, and S235A replicons behaved similarly, DCV EC_{50} s were calculated from luciferase signals derived from *trans*-complementation assays performed with DCV-sensitive (WT-neo) and DCV-resistant (F28S-neo) helper replicons (Fig. 2B). A replicon variant with a F28S amino acid substitution in NS5A was chosen for these experiments because it is highly resistant to DCV (mean EC_{50} of 566 compared to 0.056 nM for the WT replicon [Fig. 2B]) and because it replicates as well as the WT JFH1 replicon in transient replication assays (26). Very similar levels of *trans*-complementation were obtained with the DCV-sensitive and DCV-resistant helper replicons in the absence of DCV (data not shown). As shown in Fig. 2B, the defective replicons (replicons with S232I, S229A, S229E, and S235A) were sensitive to DCV ($EC_{50} \leq 0.05$ nM) regardless of whether the helper replicon was DCV sensitive or DCV resistant, indicating that the rescued replicon did not adopt the inhibitor sensitivity phenotype of the helper replicon. In agreement with previous results (26), we also observed that the DCV sensitivity of sensitive (WT) and resistant (F28S) Rluc replicons did not substantially change when they were coexpressed with a neo replicon of the opposite sensitivity, indicating that neither the DCV-sensitive phenotype nor the DCV-resistant phenotype was *trans*-dominant (Fig. 2B). Taken together, these results suggest that the hyperphosphoryla-

as described above for panel B. The Western blot below the graph shows NS5A proteins expressed from the replicons by using the VV-T7 expression system. (D) The replication capacities of the indicated Con1 replicons were plotted as replication windows following transient expression in Huh7.5 cells as described above for panel B. A Western blot showing NS5A proteins expressed from the replicons by using the VV-T7 expression system is shown below the blot. WT refers to a replicon without adaptive mutations, and GDD* refers to a replicon in which the GDD motif at the NS5B catalytic site was changed to AAG.

FIG 2 Replication of defective replicons bearing mutations affecting conserved serine residues in the central region of NS5A are *trans*-complemented by replication-competent replicons. (A) Rluc replicons with the indicated amino acid substitutions were transiently expressed in Huh7.5 cells in the presence and absence of a helper replicon that did not have a luciferase reporter (JFH1-neo). Replication windows were determined from DCV-treated and untreated cells as described in the legend to [Fig. 1B](#). *trans*-complementation was measured as an increase in the replication window upon coexpression of the helper replicon. The replication window of the parental Rluc replicon (WT) is shown for comparison (mean replication window of 1,891). A replicon with an inactivating NS5B mutation (GND) was used as a negative control (mean replication window of 1.5). (B) DCV EC_{50} , calculated from luciferase assays performed 96 h after coexpression of the indicated replication-defective Rluc replicons with DCV-sensitive (JFH-neo WT) or DCV-resistant (JFH-neo F28S) helper replicons, are plotted on a log10 scale. DCV EC_{50} s from transient expression assays with the JFH1 parental (WT) and DCV-resistant (F28S) replicons in the absence (black bars) and presence of helper replicons are shown for comparison. Values are means $\pm$ standard deviations (error bars) from two or more independent experiments each performed in duplicate.

tion-deficient NS5A proteins encoded by the replication-defective replicons participated in replication of the defective Rluc replicons and were inhibited by DCV.

The *trans*-complementation experiments thus far described used defective replicons with mutations altering serine residues implicated in NS5A hyperphosphorylation ([Fig. 1](#)) (34). To determine whether replicons with NS5A mutations outside this region could also be *trans*-complemented, a set of mutations associated with severe replication defects was identified by alanine replacement mutagenesis ([Fig. 3A](#)). Alanine scanning of the region immediately downstream of the NS5A N-terminal amphipathic α helix identified three amino acid substitutions (P29A, L31A, and P32A) that decreased replication to levels similar to those of the GND negative-control replicon. Cysteine-to-alanine substitutions at NS5A residues involved in zinc binding (C39A and C80A) also yielded replication-defective replicons, as did valine-to-alanine and glycine-to-alanine substitutions at NS5A residues 121 (V121A) and 337 (G337A). Analogous amino acid substitutions in the Con1 replicon have also been shown to be associated with replication defects (7, 10, 38). Finally, a replicon with linked P32A and S232I amino acid substitutions was also severely impaired for

FIG 3 Identification of point mutations associated with severe RNA replication phenotypes. (A) Replication capacities of JFH1 replicons with the indicated amino acid substitutions were assessed in transient replication assays as described in the legend to [Fig. 1B](#) and are plotted relative to the parental control (WT). (B) Replicons with the indicated amino acid substitutions were transiently expressed in Huh7.5 cells, and luciferase assays were performed at 4, 30, 96, and 144 h after transfection. Relative light units (RLU) are plotted for each replicon at the indicated time points. Values are the means plus standard deviations (error bars) from two experiments each performed in duplicate. A replicon with a mutation affecting the GDD catalytic motif in NS5B (GND) served as a negative control. (C) HCV polyproteins were transiently expressed from the indicated replicons by using a VV-T7 expression system, and NS3 and NS5A proteins were detected by Western analysis of parallel blots. Two independent blots are shown to illustrate the potential effect of C39A, C80A, and V121A amino acid substitutions on NS5A hyperphosphorylation. α -NS3, anti-NS3 antibody.

replication ([Fig. 3A](#)). The replication defects of a subset of these mutant replicons (P32A, C39A, V121A, and G337A) were confirmed by examining luciferase activities at various time points after transfection of Rluc replicon RNAs into Huh7.5 cells. While the luciferase values generated from the JFH1-WT parental replicon increased over 144 h, the signals from the mutant replicons steadily decreased in a pattern that was very similar to that of the negative-control GND replicon ([Fig. 3B](#)). Mature NS3 and NS5A proteins were detected by Western blot analysis when the replica-

tion-defective replicons were expressed in a vaccinia virus-T7 polymerase transient expression system (Fig. 3C), indicating that the mutations did not severely affect polyprotein processing. As expected, less hyperphosphorylated NS5A was observed from the replicon with the linked P32A plus S232I mutations. In addition, replicons with C39A, C80A, and V121A amino acid substitutions also appeared to express less hyperphosphorylated NS5A than the WT control (Fig. 3C).

Having identified a set of replication-defective replicons, we next asked whether any of these mutant replicons could be *trans*-complemented. In fact, several of the mutant replicons were rescued by coexpression with a helper replicon; however, the levels of replication were variable and, in most cases, were much lower than that observed with the S232I replicon (Fig. 4A). A replicon with a G337A substitution was *trans*-complemented most efficiently (mean replication window of 120, or ~6.4% if the WT JFH1 replicon). In contrast, the P29A, L31A, P32A, V121A, and P32A plus S232I replicons were *trans*-complemented less efficiently, with mean replication windows ranging from 7 to 42 (0.4 to 2.2% of the WT JFH1 replicon). The C39A and C80A replicons were *trans*-complemented very poorly, if at all, with mean replication windows of 4 and 2, respectively (Fig. 4A). Except for the C39A and C80A variants, replication windows generated from luciferase signals obtained when the NS5A mutant replicons were coexpressed with helper replicons were sufficiently large to calculate DCV EC_{50} s. DCV EC_{50} data from *trans*-complementation assays with DCV-sensitive (WT-neo) and DCV-resistant (F28S-neo) helper replicons are shown in Fig. 4B. Representative dose-response curves from experiments with two of the mutant replicons (L31A and V121A) are also included in Fig. 4C. The results from these experiments were distinct from those observed with the S232I replicon. The P29A, P31A, P32A, V121A, and P32A plus S232I replicons were DCV sensitive when rescued with a DCV-sensitive helper replicon (WT-neo), but they fully adopted the resistant phenotype when the helper replicons were DCV resistant (F28S-neo) (Fig. 4B and C), suggesting that the helper-encoded NS5A was predominantly involved in replication of the defective Rluc replicon. The G337A replicon was also sensitive to DCV when it was *trans*-complemented with a DCV-sensitive replicon, but an intermediate DCV sensitivity was observed when the G337A replicon was rescued with the DCV-resistant replicon (Fig. 4B). Dose-response curves generated from *trans*-complementation assays with the G337A Rluc replicon are shown in Fig. 5A and B. While a normal sigmoidal curve was obtained when the helper was DCV sensitive (WT-neo [Fig. 5A]), a biphasic curve with two apparent inflection points was observed when the helper replicon was DCV resistant (F28S-neo [Fig. 5B]). These results suggested the possibility that distinct DCV-sensitive and DCV-resistant replicon populations were involved in the replication of the G337A replicon when the helper replicon was DCV resistant. To explore this possibility, we compared the G337A replicon dose-response curves with those obtained when JFH1-WT and JFH1-F28S Rluc replicons were transiently expressed separately and together in Huh7.5 cells. As expected, normal sigmoidal curves with single inflection points were obtained from the individually expressed replicons (Fig. 5C and D). However, when equal amounts of the WT and F28S replicon RNAs were cotransfected into Huh7.5 cells, a biphasic curve with two inflection points was obtained (Fig. 5E). This is the expected result if two distinct, and similarly replicating, Rluc replicon populations were

FIG 4 Replicons with different replication-inactivating mutations in NS5A display distinct *trans*-complementation phenotypes. Rluc replicons with the indicated amino acid substitutions were transiently expressed in the presence and absence of a helper replicon that did not have a luciferase reporter (JFH1-neo). Replication windows were determined from luciferase activities as described in the legend to Fig. 1B. The replication window observed from a parental Rluc replicon (WT) is shown for comparison. A replicon with an inactivating NS5B mutation (GND) was used as a negative control. (B) DCV EC_{50} s, calculated from luciferase assays performed 96 h after coexpression of the indicated Rluc replicon with a DCV-sensitive (JFH-neo WT) or DCV-resistant (JFH-neo F28S) helper replicon, are plotted on a log10 scale. For comparison, DCV EC_{50} s obtained from transient expression assays with the JFH1 parental (WT) and DCV-resistant (F28S) replicons in the absence of a helper replicon are also plotted (black bars). EC_{50} s could not be calculated for the C39A and C80A replicons because of small replication windows. Values are means $\pm$ standard deviations (error bars) from two or more independent experiments with each experiment performed twice. ND, not determined. (C) Representative DCV dose-response curves derived from *trans*-complementation assays with defective V121A and L31A Rluc replicons and DCV-sensitive (WT-neo) and DCV-resistant (F28S-neo) helper replicons are shown. The dose-response curves were plotted relative to luciferase activity in the absence of inhibitor (100% control). Data points are means $\pm$ standard deviations. Solid lines show the DCV concentration at 50% of control (EC_{50}).

FIG 5 Dose-response curves from *trans*-complementation assays reveal DCV-sensitive and DCV-resistant replicon populations. Replicons were transiently expressed in Huh7.5 cells, and DCV dose-response graphs were generated from luciferase assays performed ~96 h posttransfection. The dose-response curves were plotted relative to luciferase activity in the absence of inhibitor (100% control). (A and B) Dose-response graphs derived from *trans*-complementation assays with the G337A Rluc replicon and DCV-sensitive (WT-neo) and DCV-resistant (F28S-neo) helper replicons, respectively. The data in panel B appear to be biphasic with two inflection points (arrows) and could not be fitted to a curve unless points were excluded (not shown). (C and D) Results generated from individual transfections of WT and DCV-resistant (F28S) Rluc replicons. (E) Data obtained following cotransfection of equal amounts of WT and F28S Rluc replicon RNA. (F) Overlay of the data shown in panels C to E. (G and H) Dose-response graphs derived from *trans*-complementation assays with the S232I replicon and the indicated helper replicons. Double inflection points in the dose-response curves plotted in panels B, E, and H are indicated by arrows. Dashed lines indicate the projected DCV concentrations at the inflection point. Solid lines mark the DCV concentration at 50% of control (EC_{50}).

present, one that was DCV sensitive (first inflection point) and another that was DCV resistant (second inflection point). Superimposing the dose-response curves from the individual WT (Fig. 5C) and F28S replicons (Fig. 5D) onto the curve generated from the cotransfection (WT + F28S [Fig. 5E]) confirmed that the two inflection points corresponded to the DCV EC_{50} s expected for sensitive and resistant replicon populations (Fig. 5F). The similarity between the WT + F28S curve (Fig. 5E) and the curve generated when the G337A Rluc replicon was *trans*-complemented with

the DCV-resistant replicon (F28S-neo) (Fig. 5B) suggests that distinct DCV-sensitive and DCV-resistant replicon populations were also present in the latter case, implying that NS5A proteins encoded by both the helper replicon (DCV resistant, F28S-neo) and the defective replicon (DCV sensitive, G337A-Rluc) were involved in replication of the defective Rluc replicon.

These results prompted us to reexamine the dose-response curves from *trans*-complementation assays with the replicons bearing mutations to serine residues in NS5A LCS I (Fig. 2). Rep-

representative examples of dose-response curves derived from *trans*-complementation of the defective S232I replicon are shown in Fig. 5G and H. Similar curves were also obtained from *trans*-complementation of the S229A, S229E, and S235A replicons (data not shown). A plateau observed in the lower portion of the curve (higher DCV concentrations) generated from the S232I replicon that was rescued with the DCV-resistant replicon (F28S-neo) (Fig. 5H, arrow) suggests that a mixture of DCV-sensitive and DCV-resistant replicon populations was also present in this case, although the percentage of resistant replicons was lower than in the case of the G337A replicon, and the observed plateau did not noticeably alter the DCV EC₅₀ calculated from the luciferase signal (Fig. 2B and 5). Overall, these results indicated that NS5A *trans*-complementation can occur by two distinct, but not mutually exclusive, modes, a relatively efficient mode with the rescued replicon retaining its original DCV sensitivity and a relatively inefficient mode with the rescued replicon adopting the DCV sensitivity of the helper replicon.

The results from the *trans*-complementation assays hinted at the possibility that different NS5A mutations might be affecting different NS5A functions, thus suggesting that intragenic *trans*-complementation of NS5A alleles might also be possible. To test this possibility, pairs of replication-defective Rluc replicons were cotransfected into Huh7.5 cells, and replication was assessed from the resulting luciferase activity. The P32A, S232I, and G337A alleles were chosen as representatives for testing in combination with the panel of NS5A mutations. As shown in Fig. 6, intragenic complementation of NS5A alleles was observed, with each of the test replicons displaying a distinct complementation pattern. The increased luciferase signals observed upon complementation were sensitive to DCV (Fig. 6) and to an HCV protease inhibitor (data not shown), confirming that they resulted from replicon replication. However, since both defective replicons carried Rluc reporters, it was not possible to determine the relative contribution to replication of the individual replicons. The P32A allele was successfully complemented by replicons with mutations affecting the central serine residues of NS5A (S229A/E, S232I, and S235A) and with the G337A mutant replicon, but it was not complemented by replicons with mutations affecting other amino acid residues within the N-terminal region of NS5A (P29A, L31A, C39A, C80A, V121A, or P32A plus S232I) (Fig. 6A). In addition to being complemented by the P32A allele, the S232I allele was complemented by the P29A, L31A, and G337A alleles, but not by the C39A, C80A, and V121A alleles (Fig. 6B). Finally, the G337A allele was complemented by all of the mutant replicons, including the replicon with two amino acid substitutions (P32A plus S232I) (Fig. 6C). On the basis of their abilities to complement the P32A, S232I, and G337A replicons, we classified the replication-defective NS5A alleles into three complementation groups (groups A, B, and C [Table 1]), consistent with NS5A performing at least three distinct RNA replication functions (see the model in Fig. 7).

DISCUSSION

The function of NS5A hyperphosphorylation in HCV replication remains ambiguous. In one model, NS5A phosphorylation modulates HCV replication, with hypophosphorylated NS5A playing a role in RNA replication and hyperphosphorylation inducing a transition to virion assembly (reviewed in reference 22). Much of the evidence supporting this model comes from studies performed with the genotype 1b Con1 replicon. In the present study,

FIG 6 Intragenic complementation of replication-null NS5A alleles. Combinations of replication-null Rluc replicons with the indicated amino acid substitutions in NS5A were transiently expressed in Huh7.5 cells. Replication windows were derived from luciferase readings taken from DCV-treated and untreated cells 96 h after transfection as described in the legend to Fig. 1B.

we examined the relationship between NS5A hyperphosphorylation and replication of a genotype 2a JFH1 replicon by performing a mutational analysis of conserved serine residues in the LCS I between NS5A domains I and II. On the whole, our results suggest a positive correlation between NS5A hyperphosphorylation and JFH1 replicon replication: serine-to-alanine amino acid substitutions that reduced NS5A hyperphosphorylation impaired replication (S225A, S229A, S232A, and S235A), while substitutions that did not appreciably reduce hyperphosphorylation did not impair replication (S222A, S228A, S230A, and S238A). Moreover, JFH1 replicons with glutamic acid residues as phosphoserine mimics at positions 225, 232, and 235 replicated at close to WT levels, suggesting that phosphorylation of one or more of these residues could be important for efficient replication. In contrast, S229 was unique in that it could not be functionally replaced by either alanine or glutamic acid. For each of the serine-to-alanine substitutions that we examined, the effects on NS5A hyperphosphorylation were very similar to those observed by analogous substitutions in the Con1 replicon (34), suggesting that hyperphosphorylation in these strains occurs via similar pathways. However, while we observed a positive correlation between hyperphosphorylation and replication in the JFH1 replicon (Fig. 1B),

TABLE 1 Intragenic complementation of replication-defective NS5A alleles^a

JFH1-Rluc replicon	Complementation of the following replication-defective NS5A allele ^b :			Complementation group(s) ^c
	P32A	S232I	G337A	
P29A	—	+	+	A
L31A	—	+	+	A
P32A	—	++	+	A
C39A	—	—	+	A, B
C80A	—	—	+	A, B
V121A	—	—	++	A, B
S229A	+	—	++	B
S229E	++	—	++	B
S232I	++	—	++	B
S235A	++	—	++	B
G337A	+	++	—	C
P32A + S232I	—	—	+	A, B

^a Pairs of replication-defective replicons were assessed for complementation (Fig. 6).^b —, replication window of <2; +, replication window of 10 to 100; ++, replication window of >100.^c Group A, unable to complement P32A; group B, unable to complement S232I; group C, unable to complement G337A.

the opposite effect has been observed with the Con1 replicon (34) (Fig. 1D). One possible explanation for this difference was provided by Neddermann et al. (39) who proposed that a critical ratio of hyper- and hypophosphorylated NS5A might be required for efficient replication. According to this model, the difference between the Con1 and JFH1 strains may reflect different optimal ratios of the NS5A phosphoforms. In any event, available data from both strains point toward a critical role of the conserved serine residues in LCS I in modulating both NS5A hyperphosphorylation and replicon replication.

trans-Complementation assays with HCV replicons have been used to identify *cis*- and *trans*-acting functions of HCV NS proteins required for RNA replication. To date, NS3, NS4A, and NS5B have displayed *cis*-acting requirements, while at least some RNA replication defects of NS5A and NS4B can be provided in *trans* (25–27, 40). In this study, we identified two distinct modes by which replicons with NS5A defects can be *trans*-complemented by coexpression with a functional helper replicon. The first mode was characterized by the defective replicon completely adopting the NS5A inhibitor sensitivity phenotype of the helper replicon (Fig. 4). This was the dominant mode of *trans*-complementation observed with replicons harboring mutations in the N-terminal region of NS5A (P29A, L31A, P32A, and V121A). We propose that this type of *trans*-complementation involves the exchange of the NS5A encoded by the helper replicon into the replication complex of the defective replicon. This “direct” *trans*-complementation occurred relatively inefficiently, with maximum replication capacities ~2% that of the WT JFH1 replicon, consistent with findings from previous studies indicating that very little exchange of NS5A occurs between replication complexes encoded by different replicons (26, 40). Jones et al. (27) reported similarly low *trans*-complementation efficiencies of certain NS4B mutants. They suggested that NS4B *trans*-complementation might occur only when helper and defective RNA genomes are incorporated into the same replication foci, a potentially rare event. Our results suggest that *trans*-complementation of some NS5A mutations might be similarly limited.

In the second mode of *trans*-complementation, the defective replicon did not completely adopt the inhibitor sensitivity phenotype of the helper replicon but instead maintained at least a partially sensitive phenotype, even when the helper replicon was highly resistant to the inhibitor, thus implying that the sensitive NS5A encoded by the defective replicon participated in an RNA replication function that was blocked by the NS5A inhibitor. This type of *trans*-complementation was observed with defective replicons that had mutations affecting key serine residues required for efficient hyperphosphorylation (S229A/E, S232I, and S235A) and also with a replicon with a G337A amino acid substitution in the C-terminal region of NS5A domain II (Fig. 2 and 4). We propose that this mode of *trans*-complementation occurs when the defective NS5A is capable of performing a function as a component of the replication complex but lacks another function that is provided by the helper replicon and that may occur outside the replication complex. This type of *trans*-complementation might be viewed as “indirect” *trans*-complementation, since it does not require the helper-encoded NS5A to be exchanged into the replication complex of the defective replicon. The indirect mode of *trans*-complementation can be relatively efficient, as exemplified by the replication capacities achieved with replicons with mutations affecting hyperphosphorylation-associated serine residues (~8 to 14% that of the WT control replicon [Fig. 2A]). In cases where indirect *trans*-complementation occurred, distinct sensitive and resistant replicon populations were observed (Fig. 5), implying that both the helper-encoded and defective-replicon-encoded NS5A proteins were involved in replication of the defective replicon. These results could be explained if “direct” and “indirect” modes of *trans*-complementation were operating si-

FIG 7 Model of NS5A complementation groups and associated functions. A diagram of the domain organization of the 466-amino-acid JFH1 NS5A protein is shown, based on analogy to the proposed organizational domain structure of the Con1 NS5A protein (7). The positions of three structural domains (domains I, II, and III) and interconnecting low-complexity linker sequences (LCS I and LCS II) are indicated. A membrane-associated N-terminal amphipathic α helix (helix) is connected to domain I by a proposed hinge (aa 28 to 32). Zinc-coordinating residues in domain I (C39, C57, C59, and C80) are shown as vertical lines. The approximate positions of residues shown by mutational analysis to be critical for RNA replication are indicated. The horizontal lines beneath the diagram indicate the complementation groups (A, B, and C) to which the mutations affecting these residues were assigned (Table 1). We propose that each complementation group is associated with a distinct NS5A function (also labeled A, B, and C to match the complementation groups) required for RNA replication. The schematic below the NS5A diagram illustrates the proposed functions retained by the indicated NS5A alleles. Note that C39A, C80A, and V121A belong to complementation groups A and B and therefore are deficient for functions A and B while retaining function C.

multaneously, such that the inhibitor-sensitive replication resulted from indirect *trans*-complementation, and the inhibitor-resistant replication resulted from direct *trans*-complementation.

Replicons with C39A and C80A NS5A amino acid substitutions were *trans*-complemented very poorly, if at all, even compared to the other replicons that were inefficiently *trans*-complemented (Fig. 4A). C39 and C80 are well-characterized residues that, together with C57 and C59, coordinate a zinc atom that is probably required to maintain the structural integrity of NS5A domain I (7, 9). The inability to successfully *trans*-complement replicons with these mutations could be explained if the misfolded proteins interact nonproductively with other replication complex components and occlude the integration of helper replicon-encoded NS5A into the defective replication complex. Alternatively, since the C39A and C80A amino acid substitutions are predicted to block NS5A dimerization (41), it is also possible that direct *trans*-complementation requires the formation of heterodimers between NS5A molecules expressed from the helper and defective replicons.

In addition to *trans*-complementation, we were able to demonstrate intragenic complementation of replication-defective NS5A alleles. Our results identified three NS5A complementation groups (groups A, B, and C [Table 1]), each of which is potentially associated with a distinct RNA replication function (Fig. 7). Complementation group A, defined by the inability to complement a replicon with a P32A allele, includes mutations affecting residues in the N-terminal region of NS5A domain I (P29A, L31A, P32A, C39A, C80A, and V121A). Residues 29, 31, and 32 are highly conserved among sequences in the European HCV database (42) and are located in a proposed hinge region linking the membrane-anchoring N-terminal amphipathic α -helix to the zinc-binding motif of domain I. Residues C39 and C80 are important for zinc binding, RNA binding, and NS5A dimerization (7, 41), and V121, located in β -sheet 5 downstream of the zinc-binding motif, is required for an interaction with the cellular protein FKBP8 (38). Among the domain I alleles, P29A, L31A, and P32A were capable of complementing the S232I allele, while C39A, C80A, and V121A were not. These results indicate that the C39A, C80A, and V121A alleles also belong to complementation group B, which was defined by the inability to complement the S232I allele and which also includes the S229A, S229E, and S235A alleles (Table 1). Replicons with C39A, C80A, and V121A amino acid substitutions appeared to express reduced levels of hyperphosphorylated NS5A compared to the WT replicon control (Fig. 3C), potentially establishing a phenotypic link between these alleles and the LCS I serine alleles. In fact, accumulating evidence suggests a functional association between LCS I and NS5A domain I. For example, Hwang et al. (13) defined a minimal NS5A RNA binding domain (amino acids 33 to 249) that requires Zn^{2+} and includes LCS I, and Lim et al. (41) recently showed that the LCS I linker region, in addition to domain I, is important for efficient NS5A dimerization. These RNA binding and dimerization studies were carried out with nonphosphorylated NS5A (13, 41), implying that NS5A hyperphosphorylation is not required for these activities. These findings are consistent with a model in which a hypophosphorylated NS5A dimer or multimer binds RNA and is directly involved in RNA replication, possibly as an integral component of the replication complex. We postulate that it is this function that is retained by the S232I allele but is lacking in complementation group A domain I alleles. A separate pool of NS5A, possibly made available via hyperphosphorylation, could perform a non-replication-complex

function, for example by interacting with cellular components to establish a favorable environment for RNA replication. This second function would be retained by the P29A, L31A, and P32A alleles but would be lacking in complementation group B alleles. Recent studies demonstrating an NS5A domain I-mediated interaction with phosphatidylinositol 4-kinase III α (PI4KIII α), required for maintaining the integrity of the membranous replication compartment (43–45), identify a function that may be affected by mutations belonging to complementation group B.

Complementation group C was defined by the inability to complement the G337A NS5A allele. All of the other NS5A mutants that we examined, including a double mutant with P32A and S232I amino acid substitutions, were capable of complementing the G337A allele, suggesting that this amino acid substitution is associated with a third NS5A function required for replication (Fig. 7). G337 is located near the C terminus of NS5A domain II within the second of two cyclophilin A (CypA) binding sites identified within JFH1 NS5A (46). It will be interesting to determine whether the G337A amino acid substitution affects CypA binding and whether complementation group C is functionally defined by the interaction between NS5A and CypA. Lim et al. (41) recently showed that NS5A proteins with C39A and C80A amino acid substitutions retained the ability to bind CypA, suggesting that these mutations do not affect the overall structural integrity of NS5A domain II. These results are consistent with our finding that these alleles can complement the G337A-associated replication defect.

Our results suggest that NS5A performs at least three functions required for RNA replication. These functions are associated with mutations in domains I and II which comprise complementation groups A, B, and C. NS5A also plays a critical role in virion assembly, and mutations affecting this process map to the C-terminal region of NS5A domain III (16, 17, 47, 48). At least some of the NS5A mutations that affect virion assembly do not affect RNA replication, indicating that the assembly function can be genetically separated from RNA replication functions (16, 17, 47). These findings suggest the likelihood that the assembly mutations define a fourth NS5A complementation group. On the whole, these results support the hypothesis that NS5A is a multifunctional, modular protein with several key roles in the HCV life cycle.

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