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Mutational Analysis of Hepatitis C Virus Nonstructural Protein 5A: Potential Role of Differential Phosphorylation in RNA Replication and Identification of a Genetically Flexible Domain

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Nonstructural protein 5A of the hepatitis C virus (HCV) is a highly phosphorylated molecule implicated in multiple interactions with the host cell and most likely involved in RNA replication. Two phosphorylated variants of NS5A have been described, designated according to their apparent molecular masses (in kilodaltons) as p56 and p58, which correspond to the basal and hyperphosphorylated forms, respectively. With the aim of identifying a possible role of NS5A phosphorylation for RNA replication, we performed an extensive mutation analysis of three serine clusters that are involved in phosphorylation and hyperphosphorylation of NS5A. In most cases, alanine substitutions for serine residues in the central cluster 1 that enhanced RNA replication to the highest levels led to a reduction of NS5A hyperphosphorylation. Likewise, several highly adaptive mutations in NS4B, which is also part of the replication complex, resulted in a reduction of NS5A hyperphosphorylation too, arguing that alterations of the NS5A phosphorylation pattern play an important role for RNA replication. On the other hand, a deletion encompassing all highly conserved serine residues in the C-terminal region of NS5A that are involved in basal phosphorylation did not significantly affect RNA replication but reduced formation of p56. This region was found to tolerate even large insertions with only a moderate effect on replication. Based on these results, we propose a model of the role of NS5A phosphorylation in the viral life cycle.

Hepatitis C virus (HCV) presents the only member of the genus *Hepacivirus* that belongs to a group of genetically diverse positive-strand RNA viruses of the family *Flaviviridae* (47). These viruses are characterized by an enveloped nucleocapsid that harbors a single-stranded RNA genome. In the case of HCV, the genome has a length of about 9,600 nucleotides and it carries at both termini highly structured nontranslated regions (NTRs) that provide indispensable signals for RNA translation and replication (13, 14, 26). The 5′ NTR functions as an internal ribosome entry site (IRES), directing translation of the HCV polyprotein that is cleaved by viral and cellular proteases into at least 10 different products (reviewed in references 3 and 40). These are core, envelope protein 1 (E1), E2, p7, nonstructural proteins 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (from the N terminus to the C terminus of the polyprotein).

Studies with subgenomic HCV replicons in the human hepatoma cell line Huh-7 have shown that proteins NS3 to NS5B are sufficient for RNA replication (32). Defined functions have been ascribed to most of them. NS3 is a two-domain molecule that carries in its N terminus a serine-type protease (18) and in the C-terminal remainder nucleoside triphosphatase and helicase activities (23). NS4A is the cofactor of the NS3 protease (4, 43), and NS5B is the RNA-dependent RNA polymerase (31). NS4B is a highly hydrophobic protein that can induce the formation of intracellular membrane vesicles to which the cytoplasmic replication complex (RC) appears to be anchored (10, 17, 19).

A still enigmatic protein is NS5A, which is also a component of the RC. NS5A is a phosphoprotein, mainly phosphorylated on serine and to a much lesser extent, on threonine residues (reviewed in reference 36). This reaction is carried out by one or more cellular kinases that appear to belong to the CMCG group of serine-threonine kinases to which casein kinase II (CKII), cyclin-dependent kinases (CDKs), and mitogen-activated protein kinases (MAPKs) belong (20, 24, 38, 41). Moreover, the p70S6K kinase or enzymes with the same specificity seem to be involved in NS5A phosphorylation (9). In one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two NS5A phospho variants can be detected, designated p56 and p58 according to their apparent molecular weights. Basal phosphorylation, which leads to the generation of p56, requires domains in the center and in the C terminus of NS5A. The p58 protein presents the hyperphosphorylated form of p56 (21, 44). Centrally located serine residues 2197, 2201, and 2204 are important for this modification (Fig. 1) (44). However, it is not clear whether these residues are phospho acceptor sites or affect phosphorylation in a more indirect way. The only phospho acceptor sites that have been mapped by biochemical approaches are serine residues 2321 (39) and 2194 (22), but only the latter is highly conserved among HCV strains.

Both the degree and the requirements for hyperphosphorylation vary between different HCV isolates. In the case of the HCV-H isolate (genotype 1a), at least two phosphorylated forms of NS5A were observed in the absence of other HCV proteins (41). In contrast, for the genotype 1b HCV-BK, HCV-J, and HCV-Con1 isolates, more stringent requirements were found (1, 21, 25, 28, 34). For instance, phosphorylation of Con1- and BK-NS5A is only detected when NS5A is expressed in the context of a fully processed NS3-to-NS5A polyprotein fragment. Small deletions in NS4B and even single-amino-acid substitutions in NS3 or NS4A that do not affect polyprotein cleavage

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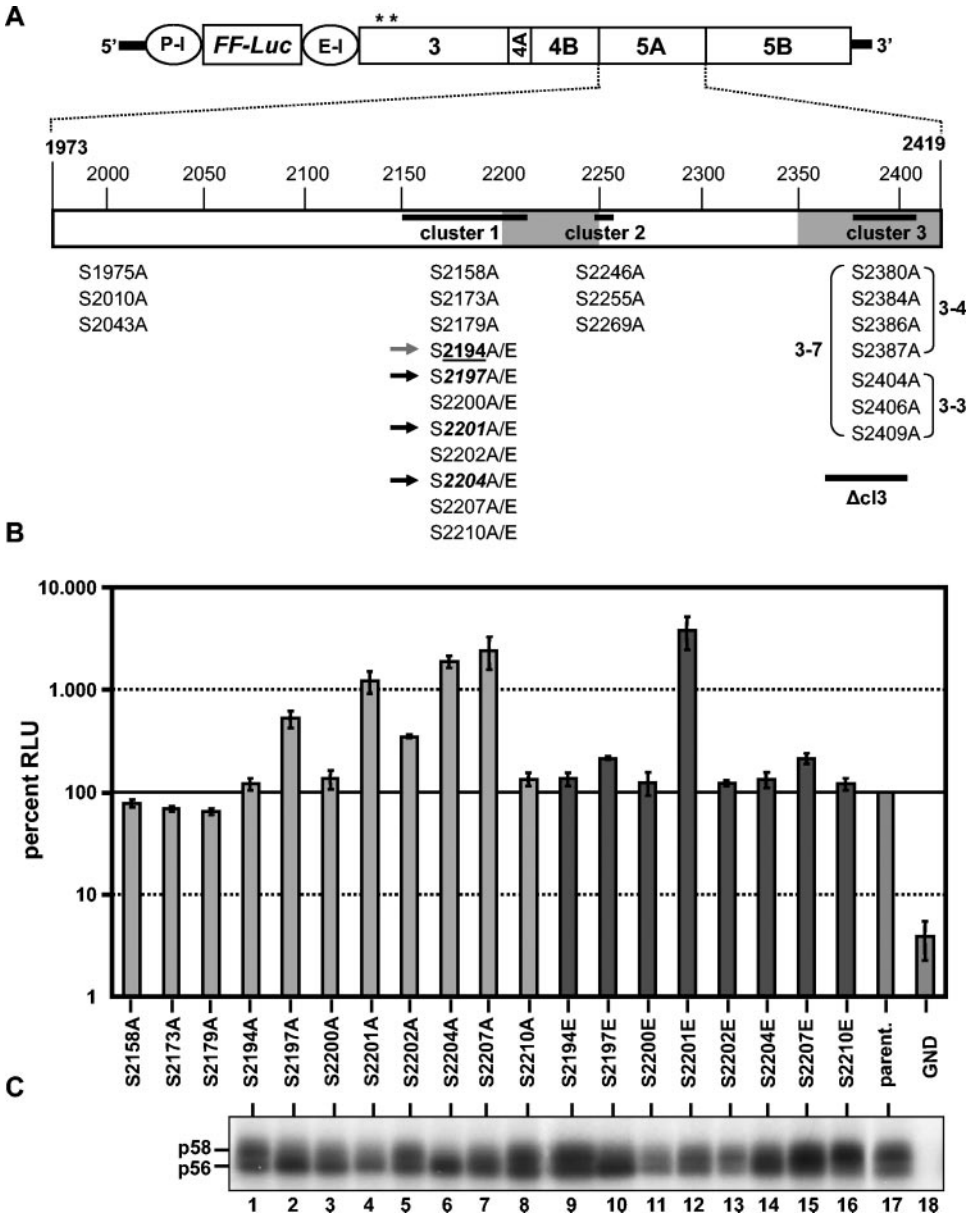

FIG. 1. Amino acid substitutions affecting highly conserved serine residues in NS5A and their impact on RNA replication and hyperphosphorylation. (A) The structure of the subgenomic HCV replicon used for transient RNA replication analyses is shown in the upper panel. The HCV 5′ and 3′ NTRs are indicated by thick lines, the PV IRES (P-I) directing the translation of the reporter gene coding for firefly luciferase (*FF-luc*) is depicted as an oval. The EMCV IRES (E-I) directs the translation of the HCV NS3 to -5B coding sequence. The positions of cell culture adaptive mutations in NS3 are indicated by asterisks. The lower panel shows a schematic representation of NS5A indicating the major phospho acceptor site (S2194, grey arrow) and sites required for hyperphosphorylation (S2197, S2201, and S2204, black arrows). Regions important for basal phosphorylation are shaded in grey. Serine residues highly conserved between multiple HCV isolates and genotypes were grouped into three clusters, with cluster 1 including the sites involved in hyperphosphorylation as well as the major phospho acceptor site. Amino acid substitutions generated in this study are indicated below the scheme. In the case of cluster 1, in addition to alanine substitutions, selected replacements by glutamic acid were analyzed. In the case of cluster 3, substitutions affecting 3, 4, or 7 aa residues were combined (mutants 3-3, 3–4, and 3–7, respectively). The deletion removing cluster 3 completely is indicated below (Δcl3). Numbers refer to the positions within the polyprotein of the HCV Con1 isolate (EMBL database accession no. AJ238799). (B) Result of transient replication assays. Mutants specified below the graph were transfected into Huh-7 cells. Replication was measured by determining the relative light units (RLU) at 48 h postelectroporation and normalization for transfection efficiency by using the luciferase activity measured 4 h after transfection. The value determined with the parental replicon (parent.) was set as 100% and used as a reference to normalize the replication of all other replicons. The replicon carrying an inactivating mutation in the NS5B RNA polymerase (GND) served as a negative control. Values are means and standard deviations from at least three independent experiments, each measured at least in duplicate. (C) Phosphorylation analysis of NS5A by using the vaccinia virus T7 hybrid system. NS3-to-NS5B polyprotein fragments carrying a given mutation in NS5A were transfected into Huh-7 cells, and proteins were radiolabeled by using [^{32}P]orthophosphate. NS5A proteins were isolated by immunoprecipitation and separated by SDS-PAGE (10% polyacrylamide). Mock-transfected cells served as a negative control (lane 18). The two phosphoprotein variants p56 and p58 are marked.

abolish formation of p58. These results imply that rather subtle changes have a tremendous effect in *cis* on the accessibility of sites in NS5A for phosphorylation: e.g., by induction of conformational changes (25, 34). Since phosphorylation of NS5A is a conserved feature among different HCV isolates and genotypes and also among other members of the *Flaviviridae*, it is supposed to play an important role in the viral life cycle (38).

Multiple cellular proteins interacting with NS5A and resulting in very different effects on cell functions have been described (reviewed in reference 46). For instance, NS5A appears to play a role in resistance against alpha interferon (IFN-α) by interfering with the activity of the protein kinase PKR that is responsible for the translational arrest and the induction of apoptosis in IFN-α-treated cells (15). In addition, several lines of evidence show that NS5A is critically involved in RNA replication. First, this protein is part of the membrane-associated RC (17). Second, NS5A interacts with the cellular protein human vesicle-associated membrane protein-associated protein A (h-VAP-A). This interaction appears to be very important for RNA replication, as demonstrated by RNA interference experiments and expression of dominant-negative protein fragments (16). In addition, while the manuscript for this article was under review, Evans and coworkers reported that the interaction between h-VAP-A and NS5A is phosphorylation dependent in a way that hyperphosphorylation of NS5A disrupts the interaction and therefore negatively regulates HCV RNA replication (12, 49). Third, a hot spot for cell culture adaptive mutations that enhance RNA replication resides in the center of NS5A (5, 27, 30). Fourth, several reports demonstrated that mutations in NS5A inhibit RNA replication (11, 37, 42). Fifth, a recent study by Tellinghuisen and colleagues demonstrated that NS5A is a zinc metalloprotein and that alteration of the zinc binding sites not only resulted in a loss of zinc ion coordination but also blocked RNA replication (45). These results clearly support the crucial role of NS5A for RNA replication.

Alanine substitutions for highly conserved serine residues in cluster 1 of NS5A and their effects on RNA replication and hyperphosphorylation. In order to study the role of NS5A phosphorylation for RNA replication, we first searched for highly conserved serine residues that could serve as phospho acceptor sites. This approach is based on the assumption that important phosphorylation sites should be conserved among different virus isolates and genotypes. However, we cannot exclude the possibility that nonconserved residues may contribute to NS5A phosphorylation of our Con1 isolate. For instance, the major phosphorylation site in NS5A of the H77 isolate was mapped to amino acid S2321, a position that is not conserved among different isolates (39).

Based on amino acid sequence alignments of about 80 different HCV isolates, we identified 24 highly conserved serine residues in NS5A that were grouped in three clusters (Fig. 1A). Cluster 1 includes the major phospho acceptor S2194 and the potential sites of hyperphosphorylation (S2197, S2201, and S2204), cluster 2 comprises three conserved serine residues, and cluster 3 resides at the C terminus of NS5A and overlaps with the region required for basal phosphorylation. Given their high degree of conservation, three distantly spaced serine residues in the N terminus of NS5A flanking the amphipathic α-helix that anchors NS5A to intracellular membranes (7, 11) were included in the analysis (Fig. 1B).

The importance of these residues for RNA replication was studied by introducing alanine substitutions into the subgenomic replicon pFK PI-luc/E1202G+T1280I, which carries two weakly adaptive mutations in NS3 (E1202G and T1280I; Fig. 1A). These mutations were shown to increase replication efficiency synergistically when combined with adaptive mutations in NS5A (27). This replicon is composed of the complete HCV 5′ NTR, the poliovirus (PV) IRES that directs the translation of the firefly luciferase reporter gene, the IRES of the encephalomyocarditis virus (EMCV) mediating translation of the NS3-to-NS5B coding region, and the authentic 3′ NTR. Standard recombinant DNA technologies were used for all plasmid constructions. Site-directed mutagenesis was done by PCR-based technologies, and amplified fragments were subcloned into the parental replicon vector by using the appropriate restriction sites. The integrity of the final construct was verified by sequence analysis using the automatic sequencer ABI 370 (Applied Biosystems, Darmstadt, Germany). Replication efficiencies of the RNAs carrying various single-amino-acid substitutions were determined in a transient replication assay as described recently by using 5 μg of in vitro-transcribed replicon RNA that was transfected by electroporation into 400 μl of a cell suspension containing 10^7 Huh-7 cells/ml (27). Aliquots of transfected cells were seeded in 10-cm^2 dishes and incubated for 4, 24, 48, and 72 h. Luciferase activities were determined in cell lysates in duplicate measurements as described elsewhere (27). The results were normalized for transfection efficiency by using the luciferase activity measured 4 h after transfection and expressed as a percentage relative to that of the parental replicon at 48 h after transfection.

Alanine substitutions for the highly conserved serine residues at the N terminus of NS5A (S1975, S2010, and S2043) did not affect RNA replication significantly (data not shown). The same was true for the analogous replacements of S2158, S2173, and S2179 at the N terminus of cluster 1 (Fig. 1B). Exchange of S2194 for Ala that removed the major phospho acceptor site also had no effect on RNA replication. This result is in agreement with an earlier report that demonstrated no absolute requirement of S2194 for HCV RNA replication (6). A different phenotype was found for most of the remaining alanine substitutions in cluster 1. In the case of the known adaptive sites S2197 and S2204, alanine substitutions led to an enhanced RNA replication (Fig. 1B). Interestingly, these mutations have so far not been found in replicons isolated from Huh-7 cells. Instead, proline and cysteine residues at position 2197 or isoleucine and arginine residues at position 2204 have been described showing a high flexibility for mutations at these sites that confer cell culture adaptation. A peculiar phenotype was observed with the alanine substitutions affecting the three consecutive serine residues at positions 2200 to 2202. While the mutations affecting S2201 and S2202 increased RNA replication, albeit to different extents, the replacement affecting S2200 had no significant effect (Fig. 1B), arguing that these three consecutive serine residues are functionally not interchangable. Surprisingly, the alanine substitution for S2207 that so far has not been identified as a site for cell culture adaptation enhanced RNA replication as efficiently as the substitution affecting S2204, which is the major hot spot for cell culture adaptive mutations (5, 29). The fact that the S2207A substitution has not yet been described cannot be ascribed to the co-

don usage because a single nucleotide substitution is sufficient to change the UCU serine codon into the GCU alanine codon.

It should be pointed out that the adaptive phenotype caused by substitutions S2197A, S2201A, S2204A, and S2207A was also observed in the context of a nonadapted wild-type luciferase replicon that carried only the HCV IRES for translation of the luciferase gene, although replication efficiencies were significantly lower than those of the replicons carrying the PV IRES and adaptive mutations in NS3 (not shown). This observation excluded the possibility that the presence of these mutations and the PV IRES affected NS5A hyperphosphorylation and RNA replication.

To study the effect of these mutations on hyperphosphorylation of NS5A, NS3 to -5B polyprotein fragments carrying defined NS5A mutation(s) were expressed with the vaccinia virus/T7 hybrid system in Huh-7 cells. Two hours after infection with the modified vaccinia virus Ankara carrying a copy of the bacteriophage T7 RNA polymerase gene, cells were transfected with pTM-derived constructs (4) by using the Effectene transfection reagent according to the instructions of the manufacturer (QIAGEN, Hildesheim, Germany). The pTM vector contains the promoter for T7 RNA polymerase and the EMCV IRES that allows efficient translation of the downstream HCV coding region. To analyze the phosphorylation status of NS5A, cells were incubated for 6 h in phosphate-free medium supplemented with 100 μCi of [^{32}P]orthophosphate (Amersham Pharmacia Biotech, Freiburg, Germany). NS5A was isolated by immunoprecipitation with a monospecific antiserum and analyzed after SDS-PAGE (10% polyacrylamide) and autoradiography. As shown in Fig. 1C, the alanine substitutions affecting residues S2201, S2204, and S2207, which enhance RNA replication most efficiently, led to a significant decrease of NS5A hyperphosphorylation, which was not the case for the moderately adaptive mutation S2202A. This result indicates that alanine substitutions for those serine residues in cluster 1 that strongly increase RNA replication (more than fivefold as compared to the parental replicon) correlate with a significant reduction of NS5A hyperphosphorylation. Moreover, the data suggest that serine residue 2207 is also involved in hyperphosphorylation as well as cell culture adaptation.

Most glutamic acid substitutions for serine residues in cluster 1 of NS5A do not confer cell culture adaptation and have a minor impact on hyperphosphorylation. To evaluate the effect of constitutive phosphorylation at specific sites on RNA replication and NS5A hyper-phosphorylation, we replaced highly conserved serine residues in cluster 1 by glutamic acid residues that mimic the presence of a phosphoserine. As shown in the right panel of Fig. 1C, save for one, the glutamic acid substitutions did not lead to increases of RNA replication. The exception was the S2201E mutant that replicated as efficiently as the replicons carrying the most adaptive alanine substitutions (S2204A and S2207A). This result suggests that phosphorylation at this position does not interfere with an enhanced RNA replication. We therefore speculate that serine residue 2201 of wild-type NS5A is not phosphorylated, because an otherwise wild-type replicon should replicate to much higher levels. Moreover, the presence of a negative charge rather than the presence of a phospho group at position 2201 may be sufficient for the enhancement of RNA replication.

In the case of the other serine residues involved in cell culture adaptation (S2197, S2202, S2204, and S2207) mimicking of a phosphoserine and therefore generation of a constitutive phosphorylation at these positions by the glutamic acid substitutions led to replication levels comparable to wild type, arguing that phosphorylation at these sides may limit replication efficiency.

Analysis of the phosphorylation pattern of the substitutions after expression in the context of an NS3-to-NS5B polyprotein fragment revealed different NS5A patterns with various ratios of p56 and p58. In some cases, the pattern was similar to that of the parental NS5A (e.g., S2194E, compare lanes 9 and 17), whereas for some mutants p56 or p58 was more pronounced (e.g., S2197E and S2210E, respectively). Interestingly, the inverse correlation between the reduction of p58 levels and high-level enhancement of RNA replication we observed with the alanine substitutions was less clear in the case of the S2201E mutant. The glutamic acid substitution at this site enhanced RNA replication very strongly but had no clearly visible effect on p56 and p58 production (compare lane 12 with lane 17). One explanation could be that this particular substitution led to phosphorylation of an alternative serine residue in very close proximity resulting in p58 production. Alternatively, the protein migrating with an apparent molecular mass of 58 kDa may represent a fraction of basal phosphorylated NS5A that due to the presence of the negatively charged glutamic acid residue has an electrophoretic mobility indistinguishable from that of hyperphosphorylated NS5A. To address the second possibility, a metabolic ^{35}S labeling of NS3 to NS5B was performed by using Huh-7 cells that constitutively produce T7 RNA polymerase from a retrovirally transduced expression cassette (T.P. and R.B., unpublished observations). These cells were used in order to avoid pleiotropic effects that might be exerted by the vaccinia virus MVA strain. Upon liposomal transfection of pTM NS3-to-NS5B expression vectors, proteins were radiolabeled for 4 h with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine. NS5A was isolated by immunoprecipitation, and one-half was treated with phosphatase, whereas the other half was mock incubated. Proteins were separated by SDS-PAGE (10% polyacrylamide) and visualized by autoradiography. As shown in Fig. 2A, p58 was clearly produced with the S2201E mutant (lane 3). Upon treatment with phosphatase, all proteins collapsed into a single protein species, arguing that the glutamic acid substitution per se did not alter the electrophoretic mobility of NS5A. In summary, this result suggests that with the exception of S2201E, mutations enhancing RNA replication to the highest levels result in a reduction of NS5A hyperphosphorylation.

Influence of adaptive mutations in NS4B and NS5B on hyperphosphorylation of NS5A. As described above, cell culture adaptation can also be achieved by mutations in NS3, NS4B, and NS5B. Moreover, for most HCV isolates analyzed thus far, at least one additional viral protein is required for NS5A hyperphosphorylation. These observations prompted us to test whether adaptive mutations in proteins other than NS5A would affect p58 production. To this end, we focused our analysis on four mutations in NS4B and one in NS5B that enhance RNA replication to various extents (29) (Fig. 2B). The substitutions in NS4B increase RNA replication most efficiently, whereas the NS5B mutation is only weakly adaptive (29).

To study the impact of the adaptive mutations in NS4B and

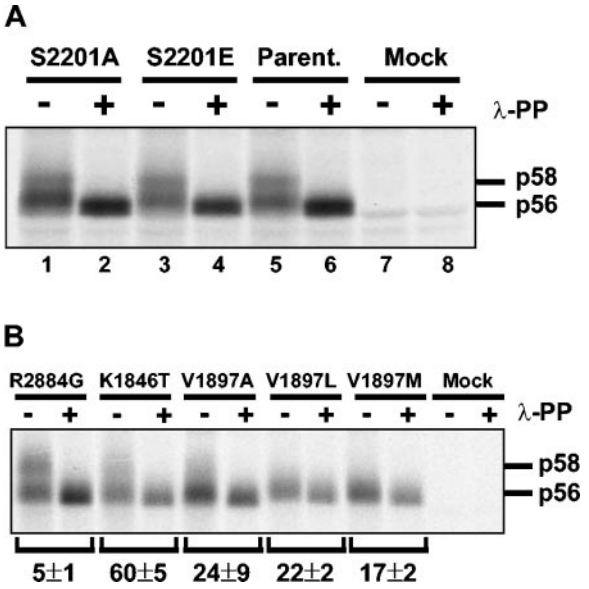

FIG. 2. Effect of adaptive mutations in NS4B, NS5A, and NS5B on hyperphosphorylation of NS5A. T7-based plasmids encoding NS3 to -5B polyproteins and carrying the mutations specified above the lanes were transfected into Huh-7 cells that stably express T7 RNA polymerase. Proteins were radiolabeled metabolically with [^{35}S]methionine and [^{35}S]cysteine, and NS5A proteins were isolated by immunoprecipitation. One-half of the immunocomplex was treated with λ-phosphatase (+), the other half was mock treated (−), and both samples were separated by 10% polyacrylamide (A) or 8% polyacrylamide (B) SDS-PAGE. Mock-transfected cells served as a negative control. The positions of p56 and p58 are indicated to the right. Values below the lanes depict replication efficiencies of the corresponding mutants as reported by Lohmann et al. (28). The parental replicon carrying the two weakly adaptive mutations in NS3 was set as 1.

NS5B on NS5A hyperphosphorylation, NS3 to -5B polyprotein fragments carrying these substitutions in combination with the weakly adaptive NS3 mutations were analyzed after radiolabeling with [^{35}S]methionine/cysteine as described above. Results in Fig. 2B show that the substitution in NS5B had no detectable impact on p58 formation. In contrast, a strong reduction of NS5A hyperphosphorylation was found with all adaptive mutations affecting residue 1897, as well as with the highly adaptive K1846T mutation. This result is consistent with the inverse correlation between the reduction of NS5A hyperphosphorylation and enhancement of RNA replication as found with the alanine substitutions in NS5A (S2201A, S2204A, and S2207A).

Currently we do not know whether hyperphosphorylation is directly involved in replication (e.g., by regulating an activity or interaction of NS5A with another viral or cellular protein) or whether hyperphosphorylation is an epiphenomenon that reflects a distinct conformation of NS5A. In the latter case, it would not be the hyperphosphorylation itself that affects RNA replication but rather the structure of NS5A and in this respect its accessibility for the cellular kinase. In agreement with earlier reports, such structural changes could be induced by substitutions within NS5A itself or by mutations affecting other HCV proteins within the RC, most importantly NS4B (25, 34).

Cluster 3 at the C terminus of NS5A involved in basal phosphorylation is dispensable for RNA replication. Basal phosphorylation of NS5A requires two regions that reside in the center (amino acids [aa] 2200 to 2250) and at the C terminus of the molecule (aa 2350 to 2419; Fig. 1A) (21). To analyze the role of phosphorylation in these regions for RNA replication, we replaced single or multiple serine residues by alanine residues and analyzed the corresponding replicons and polyprotein fragments as described above. Mutations in cluster 2 that reside at the 3′ border of the central region did not affect RNA replication (Fig. 3A). The analogous result was found with replicons in which 3 or 4 serine residues in cluster 3 were replaced at the same time by alanine residues (mutants 3-3 and 3–4). Even the simultaneous substitution of all seven serine residues reduced RNA replication only about twofold (mutant 3–7, Fig. 3A). During the course of these studies, we identified a Huh-7 cell clone that carried a stably replicating genomic

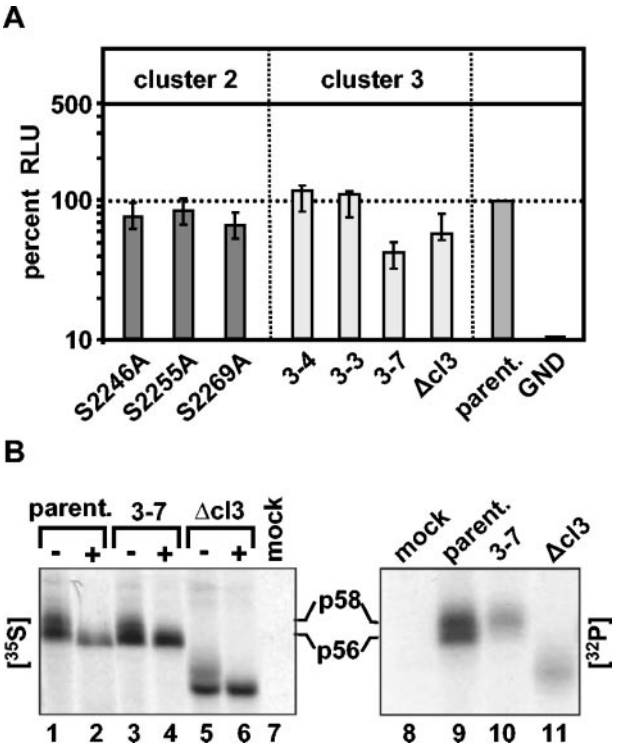

FIG. 3. Mutations at the C terminus of NS5A reduce basal phosphorylation and have only minor impact on RNA replication. (A) Substitutions affecting single serine residues in cluster 2 or multiple serine residues in cluster 3, as well as a deletion of cluster 3 (Δcl3), were analyzed in a transient replication assay as described in the legend to Fig. 1. The parental replicon (parent.), carrying two weakly adaptive mutations in NS3 and the inactive mutant (GND) served as a positive and negative control, respectively. All values were measured 48 h after electroporation and, after correction for their transfection efficiency, normalized with respect to the parental replicon, which was set 100%. (B) NS3 to -5B constructs carrying the mutations specified above the lanes were transiently expressed in Huh-7 cells by using the vaccinia virus T7 hybrid system. Proteins were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine (left panel) or [^{32}P]orthophosphate (right panel) for 6 h, and NS5A proteins were isolated from cell lysates by immunoprecipitation. In the case of the ^{35}S-labeled proteins, one-half of the samples was treated with phosphatase from bacteriophage λ (+) whereas the other half was mock treated (−) prior to SDS-PAGE (10% polyacrylamide).

replicon with an in-frame deletion in NS5A from aa 2370 to 2412 (N.A. and R.B., unpublished observations). This mutation led to a complete removal of cluster 3, suggesting that this region is dispensable for RNA replication. In order to support this conclusion, we introduced the deletion into the subgenomic reporter replicon and found that replication of this mutant (Δcl3) was only slightly reduced as compared to the parental RNA (Fig. 3A). These results clearly show that serine cluster 3 in NS5A is dispensable for RNA replication, similar to what has been described recently with a replicon isolated from HeLa cells (50).

The effect of these substitutions on NS5A phosphorylation was studied as described above. Analysis of ^{35}S-radiolabeled proteins revealed that NS5A was expressed in all cases to about the same level (Fig. 3B, left panel). The p56/p58 double band was clearly detected with the unaltered NS5A (lane 1). Both phospho variants were sensitive to treatment with phosphatase, as deduced from the disappearance of p58 and the increase in the electrophoretic mobility of phosphatase-treated p56 (lane 2). No such shift of p56 was found with the two mutants 3–7 and Δcl3 after phosphatase treatment, arguing that the mutations affected basal phosphorylation (lanes 4 and 6). This conclusion was confirmed by the analysis of the ^{32}P-labeled proteins (Fig. 3B, right panel). While a clear double band was found with the unaltered NS5A (lane 9), in the case of mutant 3–7, radiolabel was incorporated almost exclusively into the NS5A species that corresponds to p58 (compare lane 9 with lane 10). Given the higher electrophoretic mobility of the NS5A protein carrying the C-terminal deletion (Δcl3), no firm conclusions could be drawn from the labeling pattern. However, the production of a phosphatase-sensitive higher-molecular-mass species that corresponds to p58 of full-length NS5A suggests that the truncated protein is also hyperphosphorylated (compare lane 5 with lane 6). In summary, these data show that the C-terminal serine cluster of NS5A is involved in basal phosphorylation but nonessential for RNA replication.

We attempted to address the question of whether NS5A phosphorylation is required at all for RNA replication by generating a panel of replicons in which the deletion of cluster 3 was combined with single or multiple substitutions that reduce or block hyperphosphorylation. However, in all cases, NS5A phosphorylation was still detectable, although in some cases it was very low (e.g., combination of Δcl3 with S2204A; data not shown). This result suggests that a very low level of NS5A phosphorylation is sufficient for efficient RNA replication. Whether phosphorylation of NS5A is required at all for replication could not be answered by this approach.

High genetic flexibility of the C terminus of NS5A, which tolerates large insertions of heterologous sequences. The finding that multiple substitutions introduced simultaneously into the C terminus of NS5A as well as a complete deletion of cluster 3 do not affect RNA replication indicated that this region of the molecule is genetically highly flexible and may tolerate even more substantial manipulations. In fact, while this report was in preparation, Moradpour and colleagues reported that the insertion of the enhanced green fluorescent protein (eGFP) into NS5A did not abrogate replication of a subgenomic replicon (33). We therefore wanted to know whether an insertion of heterologous sequences into the C-terminal deletion identified in this study is also tolerated. In the first set of experiments, we introduced a convenient multiple cloning site carrying recognition sequences for AscI, XbaI, and PmeI with a total length of 36 nucleotides. For reasons of easy readout of RNA replication, we inserted the luciferase gene from *Renilla reniformis*, the eGFP gene from *Aequorea victoria*, or the gene coding for the red fluorescent protein (dsRed) from *Discosoma* species via AscI and PmeI restriction sites that were added to the reporter genes' termini by PCR with the appropriate primers (Fig. 4A). These insertions led to an extension of the total length of NS5A by 197 aa in the case of eGFP, 182 aa for dsRed, and 269 aa in the case of the *Renilla* luciferase. As shown in Fig. 4B, the insertion of the latter completely blocked RNA replication but did not affect activity of this reporter because high levels of *Renilla* luciferase activity were detected at 4 h posttransfection (not shown). In contrast, an about 100-fold-reduced but clearly detectable replication was found with the two RNAs that carried the insertions of eGFP or dsRed, confirming the high genetic and at least to some extent the structural flexibility of the NS5A C terminus.

Analysis of the cleavage pattern after expression of NS3 to -5B polyproteins carrying the various insertions in NS5A revealed that the heterologous elements had no obvious effect on the polyprotein processing. As shown in Fig. 4C, in all cases, we were able to detect NS4B, NS5B, and the NS5A fusion proteins. Thus, the inhibition of RNA replication by the insertion of the *Renilla* luciferase gene is not due to a defect of polyprotein processing but rather to disturbance of NS5A function.

Having established viable replicons that carry in-frame insertions of eGFP or dsRed in NS5A, we wanted to know whether these proteins can be directly visualized. Owing to low replication of these mutants in transient assays, NS5A-eGFP or NS5A-dsRed could not be detected. Therefore, we expressed the respective NS3 to -5B polyproteins by using the T7 system as described for Fig. 2A. The fluorescence analysis in Fig. 4D demonstrates functionality of the NS5A-eGFP fusion protein that displayed an endoplasmic reticulum (ER)-like subcellular distribution pattern. This pattern is similar to the membranous web that can be induced by NS4B in the absence of RNA replication (10). In contrast, no fluorescence was found with the NS5A-dsRed fusion protein. This result may be ascribed to the fact that for fluorescence, dsRed must homotetramerize, in contrast to eGFP that is functional as a monomer (2).

In summary, our data show that the C terminus of NS5A is genetically highly flexible. The fact that only insertions of the eGFP or dsRed genes were tolerated may be due to their particular structures with the N and C termini in close proximity and the majority of the polypeptide forming an almost perfect cylinder (35, 48). It is likely that the inserted proteins form a well-separated extra-domain in which the NS5A junctions are preserved in close proximity by the N and C termini of eGFP or dsRed. This would explain why these fusion proteins are still functional. Unfortunately, the crystal structure of *Renilla* luciferase is not known, and, therefore, we can only speculate about the inhibition of replication by its insertion into NS5A. Presumably, this luciferase protein has a more extended conformation which is preserved in the fusion protein as deduced from high-level *Renilla* luciferase activity but disturbs NS5A folding and thereby its function for RNA replication.

Conclusion. The study presented here extends previous assumptions that NS5A is a protein that is crucially involved in HCV RNA replication. In most cases, we observed an inverse

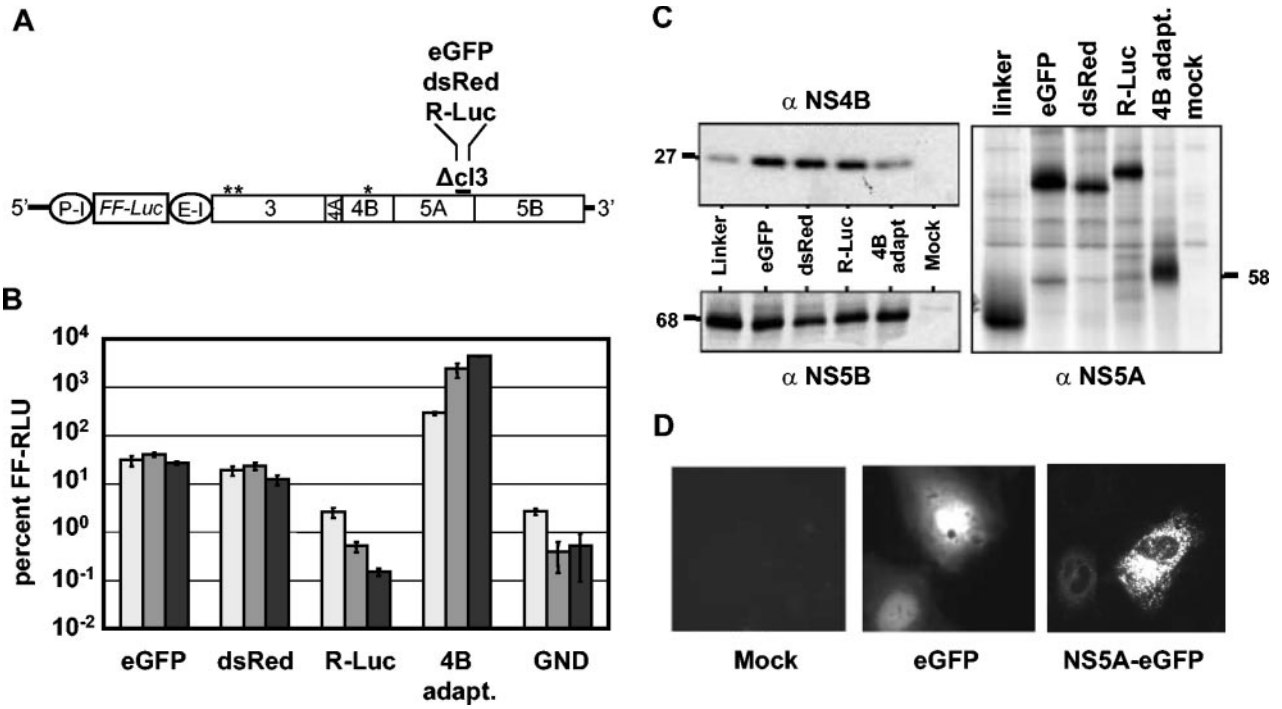

FIG. 4. Insertion of eGFP or dsRed, but not the luciferase from *R. reniformis*, into the C-terminal deletion of NS5A results in viable subgenomic replicons. (A) Schematic representation of the replicon indicating the positions of three cell culture adaptive mutations (E1202G, T1280I, and K1846T). Reporter genes were inserted into the C-terminal deletion (Δcl3). (B) Time course of a transient replication assay. Transfected Huh-7 cells were harvested 4, 24, 48, and 72 h after electroporation, and luciferase activities in the cell lysates were determined. Values obtained after 24 (light grey bars), 48 (dark grey bars), and 72 (black bars) h posttransfection after normalization for transfection efficiency by using the 4-h values are shown. The replicon carrying no insertions in NS5A and the inactive replicon (GND) served as positive and negative controls, respectively. (C) Analysis of polyprotein processing by using the vaccinia virus/T7 hybrid system. NS3 to -5B proteins carrying given insertions in NS5A were expressed in Huh-7 cells in parallel with the parental polyprotein (4B adapt), and 4 h after radiolabeling with [^{35}S]methionine and [^{35}S]cysteine, given HCV proteins were analyzed by immunoprecipitation and SDS-PAGE (10% polyacrylamide). (D) Fluorescence analysis of the NS5A-eGFP fusion protein in the context of an NS3 to -5B polyprotein. Huh-7 cells constitutively expressing T7 RNA polymerase were transfected with the expression vector only (mock), the vector containing only the eGFP gene (pTM/eGFP), or the vector containing the NS3 to -5B polyprotein with the NS5A-eGFP fusion protein. Cells were fixed 6 h after transfection with 3% paraformaldehyde and analyzed by fluorescence microscopy.

correlation between reduction of NS5A hyperphosphorylation and efficient enhancement of RNA replication, arguing that large amounts of p58 are inhibitory for high-level replication. However, in some cases, the reduction of p58 formation was very moderate, suggesting that NS5A hyperphosphorylation per se does not block RNA replication. Unfortunately, in spite of extensive mutational analysis, attempts to generate NS5A variants that are no longer phosphorylated were not successful. It is therefore unclear whether phosphorylation of NS5A is a regulatory mechanism and, if so, which step in the viral life cycle it may control. Very recently, we found that adaptive mutations in NS5A dramatically reduce the production of HCV particles (Pietschmann et al., submitted for publication). Taking into account that these mutations on one hand enhance RNA replication, but on the other hand reduce particle production, and NS5A hyperphosphorylation, it is tempting to speculate that phosphorylation of this protein is involved in regulating a switch from replication to assembly, e.g., by "arresting" the viral replication complex in an assembly-incompetent state. If this assumption is correct, a reduction of NS5A phosphorylation or hyperphosphorylation, as achieved by highly adaptive mutations, would enhance RNA replication at the expense of assembly. This model would also explain why cell culture adaptive mutations reduce or block infectivity in vivo (8). Based on these assumptions, NS5A might be a key regulatory molecule in the HCV replication cycle.

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