The hepatitis C virus NS2/3 protease is responsible for cleavage of the viral polyprotein between nonstructural proteins NS2 and NS3. We show here that mutation of three highly conserved residues in NS2 (His\textsuperscript{952}, Glu\textsuperscript{972}, and Cys\textsuperscript{993}) abrogates NS2/3 protease activity and that introduction of any of these mutations into subgenomic NS2–5B replicons results in complete inactivation of NS2/3 processing and RNA replication in both stable and transient replication assays. The effect of uncleaved NS2 on the various activities of NS3 was therefore explored. Unprocessed NS2 had no significant effect on the in vitro ATPase and helicase activities of NS3, whereas immunoprecipitation experiments demonstrated a decreased affinity of NS4A for uncleaved NS2/3 as compared with NS3. This subsequently resulted in reduced kinetics in an in vitro NS3 protease assay with the unprocessed NS2/3 protein. Interestingly, NS3 was still capable of efficient processing of the polyprotein expressed from a subgenomic replicon in HuH-7 cells in the presence of uncleaved NS2. Notably, we show that fusion with NS2 leads to the rapid degradation of NS3, whose activity is essential for RNA replication. Finally, we demonstrate that uncleaved NS2/3 degradation can be prevented by the addition of a proteasome inhibitor. We therefore propose that NS2/3 processing is a critical step in the viral life cycle and is required to permit the accumulation of sufficient NS3 for RNA replication to occur. The regulation of NS2/3 cleavage could constitute a novel mechanism of switching between viral RNA replication and other processes of the hepatitis C virus life cycle.

Hepatitis C virus (HCV)\textsuperscript{3} is the primary causative agent of parenterally transmitted and community-acquired non-A/non-B viral hepatitis and an important cause of chronic liver disease, leading to cirrhosis and hepatocellular carcinoma in humans (1–3). It is estimated that nearly 200 million individuals worldwide are currently infected with HCV. Of particular concern is that the virus establishes a chronic infection in \(<85\%\) of cases and that there are no specific and broadly effective anti-HCV compounds to date (3).

HCV is a single-stranded positive sense RNA virus of the Flaviviridae family (4, 5). It encodes a single polypeptide of \(\sim 3000\) amino acids that is cleaved co- and post-translationally into both structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (reviewed in Ref. 6). Host signal peptides are responsible for cleaving the structural proteins, whereas two virally encoded proteases (NS2/3 and NS3) process the nonstructural proteins required for viral replication. NS3 is a serine protease that mediates the cis-cleavage at the NS3/4A site as well as the trans-cleavage of NS4B, NS5A, and NS5B (7–10). NS4A forms a stable complex with the N terminus of NS3 and acts as an essential cofactor for its protease activity while also anchoring NS3 to the endoplasmic reticulum membrane, where a replication complex is thought to form (9, 11, 12). The C-terminal segment of the NS3 protein also harbors nucleoside triphosphatase and RNA helicase activities (13, 14). Although much work has focused on the elucidation of the function of both the protease and helicase domains of NS3, the role of the NS2/3 protease remains to be extensively characterized.

NS2/3 is an autocatalytic protease that is responsible for the intramolecular cleavage of NS2 and NS3 at amino acids 1026–1027 (15, 16). Encoded by NS2 and the N-terminal third of NS3, NS2/3 extends from amino acids 810 to 1206, with a minimal region required for activity beginning at amino acid 907 (17). Although NS2/3 cleavage does not require NS3 protease activity, the protease domain of NS3 cannot be substituted for another protein, suggesting that it plays a structural role in the folding of the NS2/3 enzyme (15, 18). No homology between NS2/3 and other proteases has been identified, and the catalytic mechanism of action remains unclear. Although the observation that NS2/3 activity is stimulated by zinc and inhibited by EDTA has led some groups to suggest that NS2/3 is a novel metalloprotease, others have proposed that it may function as a cysteine protease, and studies performed with classical protease inhibitors have not yielded a definite classification (17, 19, 20). Mutagenesis studies have identified His\textsuperscript{952} and Cys\textsuperscript{993} within NS2 as being essential for NS2/3 protease activity (15, 16), and in addition, mutations thought to perturb the local conformation of the cleavage site also inactivate the enzyme (21). Furthermore, molecular chaperones have been proposed to be required for efficient cleavage at the NS2/3 site (22).

Recently, a few studies have focused on the possible roles of...
NS2 after its release from NS3 (18, 23–25); however, the exact role of NS2/3 processing in viral replication remains unclear. Although NS3 3′-untranslated region (UTR) replicons have been shown to replicate efficiently in Huh-7 cells in the absence of NS2 (26), NS2/3 activity has been shown to be essential for productive replication in vivo, as demonstrated by an HCV clone devoid of NS2/3 protease activity that failed to establish productive infection in a chimpanzee (27). In this study, we further investigated the role of NS2/3 cleavage in viral replication using the replicon system. Our results demonstrate the critical importance of NS2/3 autoprocessing for RNA replication using the replicon system. Our results demonstrate the further investigated the role of NS2/3 cleavage in viral replicative infection in a chimpanzee (27). In this study, we

**Experimental Procedures**

**Strains and Constructs**—To generate an adapted NS2 3′-UTR genotype 1b replicon (pNeo2-5B), a Pmel-BarGI fragment from I389neo/NS2-3′/wt (26) was inserted into adapted replicon pFK-Neo3-5/1.1 (28). To construct NS2/3 mutants, DNA fragments representing amino acids 806–1106 were generated from this template by overlapping PCR using synthetic oligonucleotide primers for insertion of the mutations. The resulting DNA was digested with SnaBI and BarGI and then ligated back into the corresponding site of pNeo2-5B to create pNeo2-5B/WT (where WT is wild-type), pNeo2-5B/H952A, pNeo2-5B/E972A, and pNeo2-5B/C993A. For in vitro translated constructs, a 2-FLAG tag (referred to as 2xF) was generated with overlapping oligonucleotide annealing, digested, and ligated into the HindIII-BamHI fragment of pcDNA3. DNA containing NS2/3 amino acids (810–1657) and NS2/3 mutants was generated following the above method and ligated into the EcoRI-Xhol fragment of pcDNA3-2xF to generate 2xF-NS2/3 and 2xF-NS3. A DNA fragment containing NS4A was amplified by PCR from pNeo2-5B, digested, and ligated into the BsrGI-Xhol sites of 2xF-NS2/3 and 2xF-NS3 to form 2xF-NS2/3A and 2xF-NS3A, respectively. NS2/3 protease mutants were generated by inserting a BsiWI-BerGI fragment (amino acids 941–1100) from pNeo2-5B replicon constructs containing site-directed mutations into the BsiWI-BerGI site of the 2xF-NS2/3A fragment to construct 2xF-NS2/3A H952A, E972A, and C993A. For bacterial expression constructs, NS2/3 amino acids 806–1106 were generated from this template by overlapping PCR using synthetic oligonucleotide primers for insertion of the mutations. Plasmid pFK-replI-luc/NS2-3′/ET was generated by insertion of a fragment encompassing the eencephalomyocarditis virus internal ribosome entry site, NS2, and the N terminus of NS3 from plasmid I389neo/NS2-3′/wt (26) into pFK-replI-luc/ET (29) using HindIII and SfiI restriction sites. The replicon harbors adaptive mutations E1202G, T1280I, and K1846T (26) into pFK-replI-luc/ET (29) using HindIII and SfiI restriction sites. In all cases, correct insertion of mutations and fidelity of DNA sequence were verified by sequencing. Exonucleolytic dissociation was performed in 5% milk in phosphate-buffered saline with 0.2% (v/v) Tween 80. The HCV NS2/3 mutants were introduced by site-directed mutagenesis of an NS2/3 fragment into pNEB193 using a QuikChange Site-directed mutagenesis kit (Stratagene) and then ligated into pFK-replI-luc/NS2-3′/ET using BsiWI and BerGI restriction sites. In all cases, correct insertion of mutations and fidelity of DNA sequence were verified by sequencing.

**In Vitro Transcription and Purification of RNA**—Circular DNA plasmids were linearized with XbaI for the 2xF expression constructs, with ScaI for the pNeo2-5B replicon constructs, and with AscI-ScaI for the pFK-replI-luc/NS2-3′ replicon constructs and purified by phenol/chloroform extraction. DNA was transcribed with T7 RNA polymerase using a Megascript kit (Ambion Inc.) following the manufacturer’s suggested protocol; template DNA was removed by digestion with DNase I, and the RNA was purified by passing through a column using an RNaseasy mini kit (Qiagen Inc.) and dissolved in RNase-free water. The RNA concentration was determined by measuring the absorbance at 260 nm, and RNA integrity was checked by denaturing agarose gel electrophoresis.

**Cell Culture**—Huh-7 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units of penicillin, 100 μg of streptomycin, and 100 μM nonessential amino acids. A Huh-7 clone cured with selective inhibitor cells was used for transient replication assays (31).

**Electroporation of Replicons and G418 Selection**—Subconfluent Huh-7 cells were electroporated with RNA (10 μg) and selected with G418 as described previously (32). Four weeks after transfection, colonies were stained with Coomassie Blue.

**Transient Replication Assays with Luciferase Replicons**—Transient replication assays were performed as described previously (29) with several modifications. In brief, 1 μg of RNA was mixed with 400 μl of cured Huh-7 cells (105 cells/ml in Cytomix (33) containing 2 mM ATP and 5 mM glutathione), electroporated as described, immediately transfected with 5 μl of complexed DNA, and seeded into 6-well plates. Cells were harvested 4, 24, 48, and 72 h after electroporation. For luciferase activity assays, cells were scraped from the plate with 350 μl of luciferase lysis buffer (1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol (DTT)). Cleared lysate (100 μl) was mixed with 360 μl of luciferase assay buffer (25 mM glycylglycine, 15 mM MgSO4, 4 mM EGTA, 1 mM DTT, 2 mM ATP, and 0.01% CHAPS (pH 7.8)) and measured for 20 s in a Lumat LB9507 luminometer (Berthold Technologies) after the addition of 200 μl of 200 μM luciferin solution. Values obtained with cells harvested after 4 h were used to correct for transfection efficiency.

**Immunoblotting**—For Western blot analysis, 20 μg of RNA was electroporated as described above for transient replication assays, and cells were seeded into 10-cm plates. Cells were harvested by scraping them with cold PBS, and cell pellets were resuspended in phosphate-buffered saline and stored at –70°C. Protein extraction and analysis was performed by resuspension of the pellet in 5% SDS and multiple passages of the cells through the tip of a 21-gauge needle. Following quantitation using the DC protein assay (Bio-Rad), total cellular proteins were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membrane blocking and antibody dilutions were followed in 5% milk in phosphate-buffered saline with 0.2% (v/v) Tween 80. The anti-HCV NS2/3 or luciferase antibodies were used to detect expressed proteins; keratin antibodies were used to ensure expression of a 70-kDa band in all membranes used as an internal control.

**Proteasome Inhibitor Treatment**—Cells were electroporated as described above for Western blot assays. Four hours after plating, cells were treated with 10 μM MG132 (Sigma) or vehicle control (dimethyl sulfoxide) for an additional 4 or 20 h. Cells were then processed for Western blot analysis as described above.

**Immunoprecipitation of In Vitro Translated NS2/3A Mutants and NS3A/4A**—Purified RNA was translated for 90 min at 30°C in the presence of [35S]methionine/cysteine (Redivue Pro-mix, Amersham Biosciences) and electrophoresed as described. Immuno blotting and autoradiography were performed in 5% milk in phosphate-buffered saline with 0.2% (v/v) Tween 80. The anti-HCV NS2/3 or luciferase antibodies were used to detect expressed proteins; keratin antibodies were used to ensure expression of a 70-kDa band in all membranes used as an internal control.

**ATPase and Helicase Assays**—NS3 ATPase assays were performed based on the colorimetric method described by Chan et al. (34) and Kyono et al. (35). Equimolar concentrations of in vitro translated and immunoprecipitated FLAG-tagged proteins were mixed with 50 mM HEPES (pH 7.5), 2.5 mM MgCl2, and 0.04 μM poly(U), and the reaction was initiated by the addition of 2 mM ATP. The reaction was allowed to proceed at room temperature for 30 min, and the quantity of...
released phosphate was determined by the addition of 4 volumes of a malachite green/molybdate/polyvinyl alcohol reagent prepared as described by Chan et al. (14). The absorbance of the colored complex was measured at 650 nm on a SPECTROmax Plus 344 spectrophotometer (Molecular Devices, Inc.), and the amount of phosphate present was determined using a standard curve obtained using potassium dihydrogen phosphate solutions of known concentration. NS3 helicase activity was measured based on methods followed by Gallinari et al. (14) using a DNA probe developed by Pang et al. (36). Briefly, an 18-bp release strand oligonucleotide was labeled at the 5’-end using T4 polynucleotide kinase and [γ-32P]ATP (PerkinElmer Life Sciences) and purified by passing through a Sephadex G-25 column. Annealing to a partially complementary 36-bp strand was performed by heating the labeled and unlabeled strands in a 1:3 ratio to 95°C for 2 min, followed by slow equilibration to room temperature (21°C) and sonicated at 4°C in lysis buffer containing 100 mM Tris (pH 8.0), 1% Triton X-100, 5 mM EDTA, 20 mM MgCl2, and 100 mM NaCl. Unwinding was measured by preincubating immunoprecipitated proteins in 25 mM MOPS-NaOH (pH 7), 5 mM MgCl2, 2 mM DTT, and 0.1 mg/µl bovine serum albumin for 10 min with 1.25 fmol/µl labeled probe. The reaction was initiated by the addition of 4 mM ATP and allowed to proceed for 30 min at 30°C. The reaction was stopped by the addition of 2× stop buffer (50 mM EDTA (pH 8), 0.8% SDS, 0.04% Nonidet P-40, 20% glycerol, and 0.4 mg/ml bromphenol blue) and excess unlabeled release strand. The reaction products were run on a 20% polyacrylamide gel, visualized by autoradiography, and quantified by phosphorimaging.

Enzyme Expression and Purification—NS3 (1027–1206) and NS3/3 (904–1206) were expressed in Escherichia coli BL21(DE3) pLysS cells. Cells were grown at 37°C to A600 = 0.4–0.5 and, following induction with 1 mM isopropyl β-D-thiogalactopyranoside, grown at 37°C for an additional 3 h. Cells were harvested by centrifugation at 6000 g at room temperature (21°C) and sonicated at 4°C in lysis buffer containing 100 mM Tris (pH 8), 1 mM EDTA, and 100 mM NaCl. Protein was purified and folded as described by Thibault et al. (19). In brief, cell pellets were thawed at room temperature (21°C) and sonicated at 4°C in lysis buffer containing 100 mM Tris (pH 8), 1% Triton X-100, 5 mM EDTA, 20 mM MgCl2, and 5 mM DTT. Following centrifugation at 30,000 × g for 30 min at 4°C, the soluble pellet was homogenized in extraction buffer (100 mM Tris (pH 8), 6 mM guanidinium HCl, and 0.5 mM NaCl) using a glass tissue homogenizer. The supernatant was clarified at 30,000 × g for 1 h at 4°C. To purify the protein, the supernatant was mixed with nickel-nitritotriacetic acidagarose (Qiagen Inc.) for 1 h at 4°C. Beads were recovered by centrifugation and washed twice with extraction buffer containing 20 mM imidazole. Protein was then eluted with 200 mM imidazole. Fractions containing the purified protein were pooled and quantified using the Bio-Rad protein assay. NS2/3 and NS3 proteins were folded on a Superose 12 (10/300) column (Amersham Biosciences) equilibrated in 50 mM Tris (pH 8), 0.5 mM guanidine HCl, 1% N,N-dimethyldecylamide N-oxide, and 5 mM tris(2-carboxyethyl)phosphine as described by Thibault et al. (19).

NS3 Protease Kinetics—Enzymatic assays and kinetics were performed using a fluorogenic substrate and purified enzyme. Briefly, enzynatic activity was determined by monitoring the fluorescence change associated with cleavage of the fluorogenic substrate Ac-Asp-Glu-Asp-N(ε-isocyanatol-ethyl)-naphthylamine-1-sulfonic acid-Glu-Glu-thiaminobutyryl-L-lactoyl-Ser-Lys-(4-(4-dimethylaminophenylazo)benzoyl)-NH2 (Bachem Bioscience Inc., King of Prussia, PA) on a PerkinElmer Life Sciences Victor 3 fluorometer (λex = 355 nm and λem = 485 nm). Reactions were performed in black OptiPlate 96-well plates (PerkinElmer Life Sciences) at room temperature for up to 1 h in assay buffer (50 mM Tris (pH 7.4), 10% glycerol, 25 mM NaCl, 10 mM DTT, 0.1% n-dodecyl β-maltoside, and 1 mg/ml bovine serum albumin) containing 2 mM enzyme and 10 µM co-factor peptide 44A (KKGKSGVIVGRILSGLR-NH2, AnaSpec, Inc., San Jose, CA). For kinetic studies, kinetic parameters were calculated from a nonlinear least-squares fit of initial rates as a function of substrate concentration (0.5–10 µM) using GraphPad Prism Version 4.00 assuming Michaelis-Menten kinetics.

RESULTS

NS3/3 Cleavage Mutants—The catalytic activity of the NS3/3 protease has been demonstrated previously to require His952 and Cys993 (15, 16). One study has also suggested the importance of Glu972 for NS3/3 autoprocessing based on the observation that an E972Q point mutation reduces the catalytic activity of the enzyme (16). Site-directed mutagenesis was therefore used to generate point mutations in an NS3/3/4A expression construct to create H952A, E972A, and C993A single mutants (Fig. 1A). As expected, no cleavage with the H952A and C993A mutants was observed upon comparison of the processing of both the wild-type and mutant proteins after in vitro transla-
Are indicated by rated by SDS-PAGE on a 15% gel. The NS2/3, NS3, and NS2 proteins translated in vitro (Helicase Activities of NS3—\(\text{context of an uncleaved NS2/3 protein.}\) 

The various catalytic activities of NS3 were investigated in uncleaved NS2 affects the function of NS3. To test this hypothesis, the various catalytic activities of NS3 were investigated in the context of an uncleaved NS2/3 protein. 

Presence of Uncleaved NS2 Has No Effect on the ATPase and Helicase Activities of NS3—The ATPase and helicase activities of NS3 have been shown to be required for viral replication (27), and therefore, the effect of uncleaved NS2 on these processes was examined. Due to technical difficulties producing recombinant NS2/3-(904–1597) encoding both the protease and helicase domains of NS3, FLAG-tagged NS3/4A and NS2/3/4A mutants were incubated with a DNA probe, and the reaction products were visualized on an 8% nondenaturing acrylamide gel. The migration positions of the double-stranded (ds) probe and the released single-stranded (ss) probe are indicated. % unwinding refers to the amount of single-stranded probe released by the enzyme compared with the boiled control (first lane). 

Presence of Uncleaved NS2—Effect of NS2/3 mutations on autoprocessing. Wild-type (WT; lane 1) and mutant (lanes 2–4) 2xF-NS2/3 proteins were translated in vitro in the presence of \(^{35}\text{S}\), and the labeled bands were separated by SDS-PAGE on a 15% gel. The NS2/3, NS3, and NS2 proteins are indicated by arrows.
ference was in fact due to a decrease in NS3/4A complex stability and not the effect of differential cleavage of NS4A from the precursor proteins, it was determined that, in our system, NS3/4A and NS2/3/4A showed only a very slight difference in NS4A cleavage (90 and 80% cleavage, respectively) (data not shown). This therefore demonstrated that the effect seen after immunoprecipitation was due to a decrease in NS3/4A complex stability as opposed to a cleavage effect. These results suggest that the addition of NS2 causes a conformational change at the NS3 N terminus, resulting in a decrease in its affinity for NS4A.

**NS3 Protease Kinetics Are Affected by Unprocessed NS2**—To understand the effect of NS2/3 catalytic mutants on NS3 protease activity, NS3-(1027–1206) and inactive NS2/3-(904–1206) proteins were generated from genotype 1b of HCV were expressed and purified from E. coli. These constructs are shown schematically in Fig. 1D and encompass only the protease domain of NS3. Both the NS2/3 active-site mutants and NS3 proteins were recovered from inclusion bodies utilizing protocols for NS2/3 protease production (19). Following purification on a nickel column, the proteins were subsequently refolded on a gel filtration column. Although it was possible to produce soluble NS3, we chose to purify NS3 from the insoluble fraction, as was done with NS2/3, to control for any effects the folding buffers may have on NS3 protease activity. An in vitro study of NS3 protease kinetics was then performed. This involved measuring cleavage of a fluorescent substrate encoding the NS4A/NS4B cleavage site, a reaction that is dependent on the addition of an NS4A cofactor peptide. The results are summarized in Table I. The fusion of NS2 to the NS3 protease domain did not cause a reduction in the ability of NS3 to bind its peptide substrate as indicated by the similar $K_m$ values. However, a 4–5-fold decrease was seen in the catalytic constants of the enzyme in the presence of uncleaved NS2, which could be partially accounted for by the decreased stability of the NS3/4A complex as found in our immunoprecipitation experiments.

**NS3-dependent Polyprotein Processing Occurs Normally in Mutant NS2/3 Replicons**—To determine whether the decrease observed in the in vitro kinetics of NS3 in the presence of uncleaved NS2 translates into an impairment of NS3-dependent polyprotein processing, the ability of mutant NS2/3 to cleave downstream proteins was investigated in the replicon context. The pNeo2–5B/WT, pNeo2–5B/H952A, pNeo2–5B/E972A, and pNeo2–5B/C993A replicons were transfected into cured Huh-7 cells, and the expression of NS2/3, NS3, and cleaved NS5B was determined. The rabbit polyclonal antibody used in this study was raised against the protease domain of NS3 and was found to recognize full-length uncleaved NS2/3 and NS3 to similar extents using recombinant proteins (data not shown). It was also observed that, in our system, mutant NS2/3 was visualized as multiple bands, with the lower bands possibly representing proteolytic degradation fragments of the full-length protein. Fig. 6 shows that, although expression of mutant NS2/3 replicon proteins was much lower compared with that of wild-type replicon, discrete NS2/3 and NS5B could be seen, indicating the ability of uncleaved NS2/3 to correctly process the viral polyepitide despite the impaired kinetics of the NS3 protease activity seen in vitro. These results are in agreement with previously published data showing that a C993A single or H952A/C993A double mutant is still able to perform all NS3-mediated polyprotein cleavages when the HCV polyprotein is expressed in BHK-21 cells using a vaccinia system (15, 27).

**Replicon-encoded Mutant NS2/3 Is Rapidly Degraded in Huh-7 Cells**—Franck et al. (25) have recently shown that NS2 is rapidly targeted for degradation in a cell line stably expressing a full-length replicon, and therefore, the possibility that NS3 could also have a decreased half-life due to the presence of uncleaved NS2 was investigated. Adapted wild-type (pFKrepPI-luc/NS2-3/ET), non-replicating mutant NS5B (pFKrepPI-luc/NS2-3/GND), and mutant NS2/3 (pFKrepPI-luc/NS2-3/H952A) luciferase replicons were transfected into cured Huh-7 cells, and NS3 and NS2/3 levels were visualized at several time points post-electroporation. The ET replicon showed NS3 levels that increased over time, whereas the non-replicating GND construct, in addition to much lower initial expression levels, showed stable levels of NS3 for the first 24 h, followed by a decrease after 48 h (Fig. 7A). In contrast, the H952A construct showed very low levels of NS2/3 after 4 h, which decreased further after 8 h and were not detectable after 24 h. To ensure that the low levels of NS2/3 observed were not due to a decrease in the transfection efficiency or stability of the mutant RNA, luciferase levels were also visualized and were observed in similar amounts for both the ET and H952A replicon constructs after 4 h. However, it is interesting to note that the GND construct showed much lower luciferase levels compared with the ET construct, as is consistent with the levels of NS3 protein expressed. Results similar to those observed with the H952A mutant were obtained with the E972A and C993A mutants (data not shown), suggesting that the addition of NS2 has a destabilizing effect on the NS3 protein. These data strongly suggest that inactivation of the NS2/3 protease pre-
As Franck et al. (25) have also shown that NS2 can be phosphorylated at Ser977 and that mutation of this residue prevents degradation of NS2 by the proteasome, we investigated whether this amino acid could also be involved in NS2/3 degradation. A Ser-to-Ala mutation at amino acid 977 was therefore introduced into both wild-type ET and proteolytically inactive NS2/3 luciferase replicons, and NS2/3 levels were determined after electroporation into cured Huh-7 cells. As shown in Fig. 7B, the S977A mutant behaved as did the original ET construct in that NS2/3 was completely cleaved into NS2 and NS3 and the levels of NS3 increased over time. Interestingly, the H952A/S977A double mutant was found to be rapidly degraded after 4 h, as was observed with the H952A single mutant. Furthermore, to confirm that the S977A mutation did not interfere with another aspect of polyprotein processing or RNA replication, luciferase-based transient replication assays were performed, and it was found that, although the H952A/S977A double mutant failed to replicate, the S977A single mutant could replicate efficiently in Huh-7 cells (data not shown). Similar results were obtained for the E972A/S977A and C993A/S977A double mutants (data not shown). These results therefore suggest that, although the S977A mutation was previously found to be sufficient to prevent degradation of NS2, this is not the case for NS2/3, where additional factors might be involved.

Uncleaved NS2/3 Levels Are Increased by a Proteasome Inhibitor—As NS2 degradation has been shown to require the proteasomal degradation pathway (25), we investigated whether this is also the case for the uncleaved NS2/3 protein. Non-replicating GND and H952A replicon constructs were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with 10 μM MG132 or Me6SO control and harvested at the time points indicated. NS3 and NS2/3 levels were visualized by Western blot analysis using an NS3-specific antibody. The lower panel indicates a nonspecific band used as a loading control.

into cured Huh-7 cells. After attachment (time 0), cells were treated with a proteasome inhibitor (MG132) for an additional 4 and 20 h. Fig. 8 shows that the levels of NS2/3 seen with the H952A replicon were significantly increased upon the addition of MG132 after both 4 and 20 h (lanes 8 and 10) compared with untreated cells (lanes 7 and 9). However, treatment of the GND replicon cells with the proteasome inhibitor had no significant impact on the levels of cleaved NS3. These results indicate that the rapid degradation of uncleaved NS2/3 observed in our system is most likely also proteasome-mediated.

**FIG. 7. Degradation of the NS2/3 mutant compared with cleaved NS3.** Wild-type (pFK-repPI-luc/NS2-3'/ET) and mutant (pFK-repPI-luc/NS2-3'/GND and pFK-repPI-luc/NS2-3'/H952A) luciferase replicons were electroporated into cured Huh-7 cells, and the amount of NS23, NS3, or luciferase was visualized by Western blot analysis using an NS3- or firefly luciferase-specific antibody at the post-electroporation time points indicated. A, comparison of the ET, GND, and H952A constructs; B, effect of the S977A mutation on the stability of the NS23 and NS3 proteins. In A and B, the lower panels indicate a nonspecific band used as a loading control.

**FIG. 8. Effect of a proteasome inhibitor on uncleaved NS2/3 degradation.** The pFK-repPI-luc/NS2-3'/GND and pFK-repPI-luc/NS2-3'/H952A replicons were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with 10 μM MG132 or Me6SO control and harvested at the time points indicated. NS3 and NS2/3 levels were visualized by Western blot analysis using an NS3-specific antibody. The lower panel indicates a nonspecific band used as a loading control.

**DISCUSSION**

Although one study has shown the importance of HCV NS2/3 protease cleavage for viral infectivity in the chimpanzee model (27), its exact role in viral RNA replication has remained elusive due to the observation that subgenomic NS3–5B replicons replicate efficiently in Huh-7 cells (26). In this study, we explored the critical role of the HCV NS2/3 protease in viral RNA replication using catalytically inactive NS2/3 mutants. Previous studies have suggested that Gln972 could be an important residue for NS2/3 protease activity (16), and we confirmed here the importance of this residue for autoprocessing and viral replication in the context of the replicon system. This gives further evidence that Gln972 could be the third residue in a catalytic triad also involving His932 and Cys993. Indeed, it has recently been shown by Lackner et al. (46) that these three residues, in addition to being conserved in all HCV isolates, are also conserved in and of importance for the NS2/3 protease of bovine viral diarrhea virus, a pestivirus related to HCV and often used as a surrogate model for its study (47).

NS2/3 cleavage is an important and essential step in the replication of NS2–5B replicons, as shown here by protease-inactivating mutants that failed to support replication in Huh-7 cells. This confirms the importance of NS2/3 autoprocessing, as was shown in the in vivo chimpanzee model (27), while suggesting that NS2/3 cleavage plays a role in viral RNA replication itself. However, this does not eliminate the possibility that NS2/3 cleavage could have additional functions in other aspects of the viral life cycle in addition to its requirement for genome replication. Nevertheless, the results reported here further substantiate inhibition of NS2/3 cleavage as a valid target for development of anti-HCV therapies.

It is the presence of NS2 fused to the N terminus of NS3 (not the absence of the NS2 protein) that interferes with RNA replication, and we therefore explored the possible mechanisms by which uncleavable NS2/3 could have this effect. Although NS3 ATPase and helicase activities were unaffected in the context of uncleaved NS2, this protein had a 2–3-fold lower affinity for NS4A than did cleaved NS3. This could be due to the manner in which NS4A has been shown to bind NS3, forming an integral part of its N-terminal structure (40, 41). A
conformational change induced by the presence of uncleaved NS2 may leave NS4A unable to associate as tightly with the enzyme. However, the fusion of NS2 to the N terminus of NS3 does not completely abolish the interaction with NS4A. In fact, several groups have reported synthetic 3A peptides as potent inhibitors of NS2/3 autoprocessing (19, 48). Nevertheless, we found a decrease in NS3 protease kinetics in an in vitro assay dependent on the addition of a 4A peptide using purified NS2/3-(904–1206). In addition to the decreased stability of the NS2/3/A complex, 4A binding may not be able to provoke the rearrangement of the catalytic triad of NS3 necessary to stimulate protease activity.

It has been demonstrated by several groups, both by in vitro translation (16) and in cell expression systems (15, 27), that uncleavable NS2/3 causes no defect in NS3-dependent processing. Our results here are in agreement with these studies and confirm that the same is true when NS3 is expressed in Huh-7 cells as part of the HCV polyprotein in the replicon context. However, despite its ability to correctly process the viral polyprotein, this does not exclude the possibility that the NS3 protease kinetics of mutant NS2/3 may be reduced sufficiently in vivo to cause an effect on RNA replication.

Uncleaved NS2/3 is rapidly degraded when expressed in Huh-7 cells as part of an HCV replicon. In the replicon system, polyprotein translation and processing are rapid events as demonstrated by the appearance of nonstructural proteins soon after transfection (within 4 h). However, it takes significantly longer for RNA replication to occur. Once translated, transfected RNA is rapidly degraded by the cell, whereas new RNA is found to be synthesized only 24 h post-transfection (28). We therefore suggest that uncleaved NS2 may prevent RNA replication by destabilizing NS3 and causing its rapid elimination from the cells. In this case, although NS3 functions may not be disturbed by uncleaved NS2, the NS2/3 protein is not present in sufficient quantities to support RNA replication. It is of importance to note that the rapid degradation of uncleaved NS2/3 has not been observed previously, likely due to the differences inherent to the systems used. Uncleaved NS2/3 is easily detected in a vaccinia-induced expression system in BHK-21 cells (15, 27). However, in this system, large and continuous overexpression of the HCV proteins is achieved, which might saturate the degradation pathway. The replicon system used here allows the investigation of the NS2/3 protein at more physiologically relevant levels.

Cleaved NS2 has also recently been reported to be a short-lived protein in cells (25). In that case, NS2 degradation was found to be regulated in a phosphorylation-dependent manner by the protein kinase CK2. It would therefore be interesting to determine whether inhibition of the protein kinase CK2 itself could have an effect on NS2/3 stability and possibly help rescue RNA replication. Unfortunately, these experiments have proved difficult due to the observed toxicity of the curcumin CK2 inhibitor added soon after transfection (data not shown). However, a Ser-to-Ala mutation of conserved residue 977, which has been demonstrated to be sufficient to prevent proteasome-mediated degradation of NS2, was found not to be sufficient to restore levels of NS2/3 in our system. NS2/3 degradation was found here to be proteasome-dependent; however, a replication rescue experiment could not be performed due to the toxicity of the proteasome inhibitor after a 24-h incubation. It is therefore likely that NS2/3 behaves differently from cleaved NS2 and that there are either other residues or additional mechanisms involved in the regulation of its degradation. For example, association of NS3 with NS4A is essential for NS3 membrane localization and stability (11), and as NS4A has been shown here to have a decreased affinity for NS2/3, this could also be a contributing factor to the short half-life observed. Further study is required to determine the exact mechanisms involved in NS2/3 degradation and how this process could be regulated.

Bovine viral diarrhea virus NS2/3 cleavage plays a crucial role in the generation of different bovine viral diarrhea virus strains. In this pestivirus, uncleaved NS2/3 is present in noncytopathic strains causing persistent infection, whereas discrete NS3 is present in cytopathic strains required to cause disease (49, 50). For this virus, viral RNA levels have been shown to correlate with cleaved NS3 protein (46); however, uncleaved NS2/3 is required for viral infectivity (51). It has recently been suggested that bovine viral diarrhea virus NS2/3 is an autoprotease whose temporal regulation is involved in modulating the different stages of RNA replication and virus morphogenesis (46). It is possible that HCV NS2/3 could perform a similar regulatory role. By causing the degradation of NS3, uncleaved NS2/3 could potentially constitute a switch between synthesis of viral RNA and the later events of the viral life cycle, such as virion packaging and release.

It has previously been reported that NS5B levels are decreased by the presence of a cellular ubiquitin-like protein and that this may function to regulate viral RNA replication (52). The controlled cleavage and degradation of NS2/3 may therefore constitute an additional level of regulation for the virus. Although the mechanism of NS2/3 degradation has yet to be elucidated, it is likely that cellular proteins are involved. Cellular factors have also been proposed to be required for efficient NS2/3 protease activity (20, 22), and the availability of these factors may modulate the degree of NS2/3 cleavage over the course of infection. In addition, the NS2/3 cleavage products themselves could potentially be involved in the regulation of these processes through their actions on host cellular proteins. The exact role of NS2 after cleavage has not yet been firmly established, although NS2 has been shown to inhibit cellular gene transcription (24). NS3 is known to have several functions in modulating cell signaling events, as demonstrated by its cleavage of the Toll-like receptor-3 adaptor protein TRIF (53) and disruption of RIG-1 (retinoic acid-inducible gene-1) signaling (54, 55), preventing interferon regulatory factor-3 activation and the host cell interferon response (56). Regulation of NS2/3 cleavage and degradation are therefore both possible mechanisms the virus could use to control the stages of its own life cycle, and further study is required to fully understand these events.

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