

## HEPATITIS C VIRUS NS2/3 PROCESSING IS REQUIRED FOR NS3 STABILITY AND VIRAL RNA REPLICATION\*.

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Running Title: HCV NS2/3 processing in viral RNA replication

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The hepatitis C virus NS2/3 protease is responsible for cleavage of the viral polyprotein between non-structural proteins NS2 and NS3. We show here that mutation of three highly conserved residues in NS2, H952, E972 and C993, 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 to NS3. This subsequently resulted in reduced kinetics in an *in vitro* NS3 protease assay by 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. Importantly, 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 HCV life cycle.

Hepatitis C virus (HCV)<sup>1</sup> 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 approximately 85% of cases and 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 approximately 3000 amino acids in length that is cleaved co and post-translationally into both structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (reviewed in ref. 6). Host signal peptidases are responsible for cleaving the structural proteins, while two virally encoded proteases (NS2/3 and NS3) process the non-structural 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 ER membrane where a replication complex is thought to form (9,11,12). The C-terminal segment of the NS3 protein also harbours nucleoside triphosphatase and RNA helicase

activities (13,14). Although much work has focused on the elucidation of the function of both 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 810-1206 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 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 NS2/3 is a novel metalloprotease, others have proposed 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 amino acids His 952 and Cys 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' 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 fails to establish a productive infection in a chimpanzee (27). In this study, we further investigated the role of NS2/3 cleavage in viral replication by using the replicon system. Our results demonstrate the critical importance of NS2/3 autoprocessing for RNA replication. The ability of uncleaved NS2/3 to perform the various NS3 catalytic functions was

therefore examined in order to determine the mechanism by which unprocessed NS2/3 could interfere with viral RNA replication. We show that the protease, ATPase and helicase functions of NS3 are not significantly affected by uncleaved NS2 but that the presence of NS2 leads to a rapid degradation of the NS3 protein, possibly constituting for the virus a novel mechanism of regulating viral RNA replication.

## Experimental Procedures

**Strains and constructs.** To generate an adapted NS2-3'UTR genotype 1b replicon (pNeo/2-5b), a PmeI-BsrGI fragment from I389neo/NS2-3'/wt (26) was inserted into adapted replicon pFKNeo/3-3'/5.1 (28). To construct NS2/3 mutants, DNA fragments representing aa 806-1106 were generated from this template by overlapping PCRs using synthetic oligonucleotide primers for the insertion of the mutations. The resulting DNA was digested with SnaBI and BsrGI and then ligated back into the corresponding site of pNeo/2-5B to create pNeo/2-5B WT, H952A, E972A and C993A. For *in vitro* translation constructs, a 2x FLAG tag was generated with overlapping oligonucleotide annealing, digested, and ligated to the HindIII-BamHI fragment of pcDNA3. DNA containing NS2/3 (aa810-1657) and NS3 (aa 1027-1657) was amplified from the replicon constructs and ligated to the EcoRI-XhoI fragment of pcDNA3 2xF to generate 2xF NS2/3 and 2xF NS3. A DNA fragment containing NS4A was amplified with PCR from pNeo/2-5B, digested, and ligated to the BsrGI-XhoI sites 2xF NS2/3 and 2xF NS3 to form 2xF NS2/3/4A and 2xF NS3/4A. NS2/3 protease mutants were generated by inserting a BsiWI-BsrGI fragment (aa 947-1100) from pNeo/2-5B replicon constructs containing the NS2/3 mutations, into the BsiWI-BsrGI site of the 2xF NS2/3/4A construct to form 2xF NS2/3/4A H952A, E972A and C993A. For bacterial expression constructs, the NS2/3(904-1206) and NS3(1027-1206) sequences were amplified from pNeo/2-5B. An N-terminal His6 tag was included in the synthetic oligonucleotide primer to facilitate downstream purification. The DNA was then inserted into a pET11d vector via XhoI-BamHI sites that were included in the oligonucleotide primers used in the amplification. Plasmid pFK repPI-luc/NS2-3'/ET (referred to in this report as

PI-luc/NS2-3'/ET) was generated by insertion of a fragment encompassing part of the EMCV-IRES, NS2 and the amino terminus of NS3 from plasmid I389neo/NS2-3'/wt (26) into pFK rep PI-luc/ET (29), using Hind III and a Sfi I restriction sites. The replicon harbours adaptive mutations E1202G, T1280I and K1846T, referring to the amino acid position of the polyprotein from the Con1-isolate (EMBL-database accession number AJ242654). Plasmid pFK repPI-luc/NS2-3'/GND, which is replication deficient due to a aspartic acid to asparagine substitution in the active centre of the NS5B-polymerase at position 2737 of the polyprotein was generated by replacing the NS5B region in pFK repPI-luc/NS2-3'/ET with a corresponding fragment from plasmid pFK I341 sp PI luc EI3420-9605/GND (30) using a Xho I restriction site within NS5A and a Spe I restriction site directly adjacent to the 3'NTR. Mutation H952A was introduced by site-directed mutagenesis in a PCR-reaction, the PCR-fragment containing the mutation was introduced into pFK repPI-luc/NS2-3'/ET using BsiWI and BsrGI restriction sites to obtain pFK repPI-luc/NS2-3'/H952A. E972A, C993A, S977A as well as double mutations were introduced by site-directed mutagenesis of an NS2/3 fragment in pNEB193 (QuickChange Site-Directed Mutagenesis kit, Stratagene) and then ligated into pFK repPI-luc/NS2-3'/ET using BsiWI and BsrGI restriction sites. In all cases, correct insertion of mutations and fidelity of DNA sequence was verified by sequencing.

#### ***In vitro* transcription and purification of RNA**

Circular DNA plasmids were linearized with XbaI for the 2xF expression constructs, ScaI for the pNeo/2-5B replicon constructs and AseI/ScaI for PI-luc/NS2-3' replicon constructs and purified using phenol/chloroform extraction. DNA was transcribed with T7 RNA polymerase (Ambion Megascript kit, Ambion) following the manufacturers suggested protocol, template DNA was removed by digestion with DNase 1 and the RNA was purified by passing through a column (RNeasy mini kit, Qiagen) and dissolved in RNase-free water. The RNA concentration was determined by measuring the optical density at 260nm and RNA integrity was checked by denaturing agarose gel electrophoresis.

**Cell culture** Huh-7 cells were grown in Dulbecco's modified Eagles medium (Gibco-BRL,

Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 100 U penicillin, 100 µg streptomycin as well as 100µM non-essential amino acids. A Huh-7 clone cured with a selective inhibitor cells was used for transient replication assays (31).

#### **Electroporation of replicons and G418 selection**

Sub-confluent Huh-7 cells were electroporated with RNA (10µg) and selected with G418 as described previously (32). Four weeks after transfection, colonies were stained with Coomassie Blue stain.

#### **Transient replication assays with luciferase replicons**

Transient replication assays were performed as described previously (29) with several modifications. In brief, 1µg RNA was mixed with 400µl cured Huh-7 cells (10<sup>7</sup> cells/ml in Cytomix (33) containing 2mM ATP and 5mM glutathione), electroporated as described, immediately transferred to 12ml complete DMEM 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 350µl luciferase lysis buffer (1% Triton X-100, 25mM glycylglycine pH 7.8, 15mM MgSO<sub>4</sub>, 4mM EGTA, 1mM DTT). Cleared lysate (100µl) was mixed with 360µl luciferase assay buffer (25mM glycylglycine, 15 mM MgSO<sub>4</sub>, 4mM EGTA, 1mM DTT, 2mM ATP, 15mM K<sub>2</sub>P0<sub>4</sub>; pH 7.8) and was measured for 20s in a luminometer (Lumat LB9507, Berthold Technologies) after addition of 200µl of a 200µM luciferin solution. Values obtained with cells harvested after 4h were used to correct for transfection efficiency.

**Immunoblotting** For western blot analysis, 20µg RNA was electroporated as described above for transient replication assays and cells were seeded into 10cm plates. Cells were harvested by scraping the cells of the plate in phosphate buffered saline (PBS), centrifuged and lysis 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 (DC Protein Assay, Bio-Rad), total cell proteins were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membrane blocking and antibody dilutions were performed in 5% milk in PBS with 0.2% (vol/vol) Tween 20 for NS3 or luciferase (Chemicon International) detection and in 2% milk in PBS with 0.5% Tween

20 for NS5B analysis. For antibody detection, appropriate species-specific HRP-conjugated secondary antibodies (anti-rabbit from Amersham Biosciences, anti-mouse from Jackson ImmunoResearch laboratories) and Western Lightning Chemiluminescence Reagents (Perkin Elmer) were used. To ensure equal loading of lanes, a non-specific band present on all membranes was used as an internal control.

**Proteasome inhibitor treatment** Cells were electroporated as described above for western blot assays. 4 hours after plating, cells were treated with 10 $\mu$ M MG132 (Sigma) or vehicle control (dimethyl sulphoxide) for an additional 4 or 20 hours. Cells were then processed for western blot analysis as described above.

**Immunoprecipitation of *in vitro* translated NS2/3/4A mutants and NS3/4A** Purified RNA was translated for 90 minutes at 30°C in the presence of [<sup>35</sup>S] methionine and cysteine (Redivue Pro-mix, Amersham), using a rabbit reticulocyte lysate (RRL) system (Promega) following manufacturers suggested protocol. Lysates were then incubated with Ez view Red ANTI-FLAG M2 Affinity gel (Sigma) at 4°C for 2hrs in IP buffer containing 20mM Hepes-KOH pH 7.7, 150mM NaCl, 10% glycerol and 0.5% Triton X-100. Following centrifugation at 8200xg for 30 seconds, beads were washed three times with 1ml cold IP buffer. Immunoprecipitated protein was eluted from the beads with 3X Flag peptide (Sigma, final concentration 150ng/ $\mu$ l). Radiolabelled proteins were visualized by SDS-PAGE followed by autoradiography. Relative band intensity was measured using a FUJI X BAS 2000 phosphorimager.

**ATPase and Helicase Assays** NS3 ATPase assays were performed based on the colourimetric method described by Chan *et al.* and Kyono *et al.* (34,35). Equi-molar concentrations of *in vitro* translated, immunoprecipitated FLAG-tagged proteins were mixed with 50mM Hepes pH 7.5, 2.5mM MgCl<sub>2</sub>, 0.04 $\mu$ g/ $\mu$ l poly(U) and the reaction initiated by the addition of 2mM ATP. The reaction was allowed to proceed at room temperature for 30 minutes 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.* (34). The absorbance of the coloured complex was measured

at 630nm on a SPECTROmax Plus 384 spectrophotometer (Molecular Devices) and the amount of phosphate present was determined by using a standard curve obtained using potassium dihydrogen phosphate solutions of known concentration. NS3 helicase activity was measured based on methods used by Gallinari *et al.* (14) and a DNA probe developed by Pang *et al.* (36). Briefly, an 18bp release strand oligonucleotide was labelled at the 5' end using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P] ATP (Perkin Elmer) and purified by passing through a G-25 sephadex column. Annealing to a partially complementary 36bp strand was performed by heating the labelled and unlabelled strands in a 1:3 ratio to 95°C for 2 min followed by slow equilibration to room temperature in 10mM Tris pH 8, 1mM EDTA and 100mM NaCl. Unwinding was measured by preincubating immunoprecipitated proteins in 25mM MOPS-NaOH pH 7, 3mM MgCl<sub>2</sub>, 2mM DTT and 0.1  $\mu$ g/ $\mu$ l BSA for 10min with 1.25 fmol/ $\mu$ l labelled probe. The reaction was initiated by the addition of 4mM ATP and allowed to proceed for 30 min at 30°C. The reaction was stopped by the addition of 2X stop buffer (50mM EDTA pH 8, 0.8% SDS, 0.04% NP-40, 20% glycerol, 0.4mg/ml bromophenol blue) and excess cold 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 NS2/3(904-1206) were expressed in *E. coli* BL21(DE3)pLysS cells. Cells were grown at 37°C to an OD<sub>600</sub> of 0.4-0.5 and following induction with 1 mM IPTG were grown at 37°C for a further 3 hours. Cells were harvested at 6000x g and the pellet was stored at -80°C. Protein was purified and folded as described in Thibeault *et al.* (19). In brief, cells pellets were thawed at room temperature (21°C) and sonicated at 4°C in a lysis buffer containing 100mM Tris, pH8.0, 1% Triton X-100, 5mM EDTA, 20mM MgCl<sub>2</sub>, 5mM DTT. Following centrifugation at 30,000x g for 30 minutes at 4°C, the insoluble pellet was homogenized in extraction buffer (100mM Tris, pH 8.0, 6M guanidine HCl, 0.5M NaCl) using a glass tissue homogenizer. The supernatant was clarified at 30,000 x g for 1 hour at 4°C. To purify the protein, the supernatant was mixed with Ni-NTA agarose (Qiagen) for 1 hour at 4°C. Beads

were recovered by centrifugation and washed twice with extraction buffer containing 20mM imidazole. Protein was then eluted with 200mM imidazole. Fractions containing the purified protein were pooled and quantified using the Bio-Rad Protein Assay (Bio-Rad). NS2/3 and NS3 proteins were folded on a superose 12 (10/300) column (Amersham Biosciences) equilibrated in 50mM Tris, pH 8.0, 0.5M arginine HCl, 1% LDAO, 5mM TCEP as per Thibeault *et al.* (19).

**NS3 protease kinetics** Enzymatic assays and kinetics were performed using a fluorogenic substrate and purified enzyme. Briefly, enzyme activity was determined by monitoring the fluorescence change associated with the cleavage of the fluorogenic substrate Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-L-Lactoyl-Ser-Lys(DABCYL)-NH<sub>2</sub> (Bachem Bioscience Inc. King of Prussia, PA) on a Perkin Elmer Victor 3 Fluorometer ( $\lambda_{ex}$ = 355nm,  $\lambda_{em}$ =485nm). Reactions were performed in black optiplate 96-well plates (Perkin Elmer), at room temperature for up to 1 hour in assay buffer (50mM Tris, pH 7.4, 10% glycerol, 25mM NaCl, 10mMDTT, 0.1% n-dodecyl- $\beta$ -D-maltoside, 1mg/ml BSA) containing 2nM enzyme and 10 $\mu$ M cofactor peptide 4A (KKKGSVVIVGRIILSGR-NH<sub>2</sub>, 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 (0.5-16 $\mu$ M) using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA), assuming Michaelis-Menton kinetics.

## Results

**NS2/3 cleavage mutants.** The catalytic activity of the NS2/3 protease has been previously demonstrated to require amino acids histidine 952 and cysteine 993 (15,16). One study has also suggested the importance of glutamic acid 972 for NS2/3 autoprocessing from 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 a NS2/3/4A expression construct to create H952A, E972A and C993A single mutants (Figure 1A). When processing of both the wild-type and mutant proteins were compared after *in vitro* translation in rabbit reticulocyte lysate, as

expected, no cleavage is observed with the H952A and C993A mutants (Figure 2). In addition, the complete absence of NS2 and NS3 cleavage products clearly indicates that glutamic acid 972 is also a critical residue necessary for NS2/3 protease activity.

**Cleavage at the NS2/3 site is required for replication of subgenomic replicons.** To further investigate the role of NS2/3 protease activity on the replicative capacity of HCV RNA, a series of NS2/3 mutants were generated in the context of an adapted NS2-3'UTR genotype 1b neomycin replicon (Figure 1B). *In vitro* synthesized RNA from replicons containing mutations affecting NS2/3 catalytic activity (H952A, E972A, C993A) were electroporated into Huh-7 cells and colonies selected using G418. Following selection, many colonies were visible with the wild-type replicon, while NS2/3 protease deficient mutants failed to produce any visible colonies (Figure 3A). In addition, these same mutations were introduced into a luciferase reporter replicon (Figure 1C) and also completely inhibited any replication in a transient transfection experiment (Figure 3B). Adapted wild-type (PI-luc/NS2-3'/ET) replicons showed a 10 fold increase of luciferase levels at 72h compared to 4h, while the H952A, E972A and C993A mutants showed a sharp decrease in luciferase activity over the course of the experiment. This decrease is similar to what we observed with the non-replicating GND replicon that contains an inactivating mutation in NS5B. This data suggests that efficient processing of NS2 and NS3 is a critical step in the viral RNA replication process. As replicons that do not contain NS2 are fully capable of replicating, these results indicate that the presence of NS2 still attached to NS3 could itself be interfering with the replication process. A likely mechanism would be that uncleaved NS2 is affecting 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 protease and helicase

domains of NS3, FLAG-tagged NS3/4A and NS2/3/4A H952A, E972A or C993A constructs (Figure 1A) were used to generate RNA that was *in vitro* translated in rabbit reticulocyte lysate in presence of [<sup>35</sup>S] methionine and cysteine. Upon anti-FLAG immunoprecipitation, followed by elution with FLAG peptide, the proteins were separated by SDS-PAGE and their relative concentrations determined. NS4A was included in the constructs since it has been proposed by several groups that in addition to functioning as a cofactor for NS3 protease activity, NS4A may have a role in modulating NS3 helicase activity, either positively (36-38) or negatively (14,39). Equi-molar quantities of wild-type NS3/4A and the catalytically inactive NS2/3/4A mutants were evaluated for NS3 helicase activity by measurement of their ability to unwind a radiolabelled dsDNA probe. Figure 4 shows that no significant difference in the helicase activity of NS3 could be measured in the presence or absence of NS2. Similarly, there was no significant change in the ATPase activity of the enzymes when approximately equimolar amounts of each enzyme were assayed (data not shown). To confirm that these results were due to the expressed proteins and not caused by a contaminating protein co-precipitated from the RRL, an NS3 protein containing a K1236N helicase inactivating mutation was also subjected to the above assays and showed activities similar to background (data not shown). NS3 is therefore a fully functional helicase in the presence of NS2 *in vitro*.

**The ability of NS4A to associate with NS3 is decreased by the presence of uncleaved NS2.** It has been shown by x-ray crystallography that NS4A binds to the extreme N-terminus of NS3 (40,41) and that this interaction is necessary for the efficient cleavage by NS3 of the downstream polyprotein (42-44). We speculated therefore that the inability of NS2/3 to autoprocess NS2 and NS3 could leave NS4A unable to correctly associate with NS3. Indeed, Nedderman *et al.* demonstrated that the generation of an authentic N-terminus on NS3 is important for the formation of a stable complex between NS3 and NS4A (45). To test this, N-terminal FLAG-tagged NS2/3/4A mutants and NS3/4A were *in vitro* translated and immunoprecipitated under conditions shown to allow NS3/4A complex formation (39). The labelled proteins were then analyzed by SDS-

PAGE and quantified by phosphorimager to determine the relative amount of NS4A pulled down with either the NS2/3 mutants or NS3. Figure 5 shows that NS3 is able to co-precipitate more NS4A than equal molar amounts of the uncleaved NS2/3 proteins (compare lane 2 with lanes 3, 4 and 5). A phosphorimager was used to quantify the relative intensities of the NS2/3, NS3 and NS4 bands and a 2 to 3 fold decrease in NS4A binding to NS2/3 was determined as compared with NS3. To confirm that this difference is in fact due to a decrease in NS3/4A complex stability and not the effect of differential cleavage of 4A from the precursor proteins, it was determined that in our system, NS3/4A and NS2/3/4A show only a very slight difference in 4A cleavage (90% and 80% cleavage respectively) (data not shown). This therefore demonstrated that the effect seen after immunoprecipitation is in majority due to a decrease in NS3-NS4A complex stability as opposed to a cleavage effect. These results suggest that the addition of NS2 causes a conformational change at the NS3 amino terminus resulting in a decrease of 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 derived from the 1b genotype of HCV were expressed and purified from *E. coli*. These constructs are shown schematically in Figure 1D and encompass only the protease domain of NS3. Utilizing protocols for NS2/3 protease production (19), both the NS2/3 active site mutants and NS3 proteins were recovered from inclusion bodies. 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 which is dependent on the addition of an NS4A cofactor peptide. The results are summarized in Table 1. The fusion of NS2 to the NS3 protease domain does 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 to 5 fold decrease is 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-NS4A complex as found in our immunoprecipitation experiments.

**NS3 dependent polyprotein processing occurs normally in NS2/3 mutant replicons.** To determine whether the decrease observed in the *in vitro* kinetics of NS3 in 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. pNeo/2-5B WT, H952A, E972A or C993A replicons were transfected into cured Huh-7 cells and the expression of NS2/3, NS3 as well as cleaved NS5B determined. The rabbit polyclonal antibody used in this study was raised against the protease domain of NS3 and has been found to recognize full-length uncleaved NS2/3 and NS3 to similar extents using recombinant proteins (data not shown). It has also been observed that in our system, mutant NS2/3 is visualized as multiple bands, the lower bands possibly representing proteolytic degradation fragments of the full length protein. Figure 6 shows that although expression of NS2/3 mutant replicon proteins is much lower compared to wild-type, discrete NS2/3 as well as NS5B can be seen, indicating the ability of uncleaved NS2/3 to correctly process the viral polypeptide 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.** Recently, Franck *et al.*, have shown that NS2 is rapidly targeted for degradation in a cell line stably expressing a full-length replicon (25) 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 (PI-luc/NS2-3'/ET), as well as a non-replicative NS5B mutant (PI-luc/NS2-3'/GND) and H952A NS2/3 luciferase replicons were transfected into cured Huh-7 cells and NS3 and NS2/3 levels were

visualized at several time points post-electroporation. As seen in Figure 7A, the ET replicon shows NS3 levels that increase over time, whereas the non-replicating GND construct, in addition to much lower initial expression levels, shows stable levels of NS3 for the first 24 hours, followed by a decrease in levels after 48 hours. In contrast, the H952A construct shows very low levels of NS2/3 after 4 hours which decreases further after 8 hours and is not detectable after 24 hours. 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 are shown in similar amounts for both ET and H952A replicon constructs after 4 hours. However, it is interesting to note that the GND construct shows much lower luciferase levels compared to ET, as is consistent with the levels of NS3 protein expressed. As similar results to those observed with the H952A mutant were obtained with E972A and C993A mutants (data not shown), this suggests that the addition of NS2 has a destabilizing effect on the NS3 protein. This data strongly suggests that inactivation of the NS2/3 protease prevents accumulation of the NS3 protein which is required to drive HCV RNA replication.

As Franck *et al.* have also shown that NS2 can be phosphorylated at serine 977 and that mutation of this residue prevents degradation of NS2 by the proteasome (25), we investigated whether this amino acid could also be involved in NS2/3 degradation. A serine to alanine mutation of amino acid 977 was therefore introduced into both wild-type ET and NS2/3 proteolytically inactive luciferase replicons and NS2/3 levels were determined after electroporation into cured Huh-7 cells. As can be seen in Figure 7B, the S977A mutant behaves as does the original ET construct in that NS2/3 is completely cleaved into NS2 and NS3 and levels of NS3 increase over time. Interestingly, the H952A/S977A double mutant was found to be rapidly degraded after 4 hours as was observed with the H952A single mutant. Furthermore, to confirm the S977A mutation does 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 E972A/S977A and C993A/S977A double mutants (data not shown). These results therefore suggest that although an 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-replicative GND and H952A replicon constructs were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with a proteasome inhibitor (MG132) for an additional 4 and 20 hours. Figure 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 hours (lanes 8 and 10), as compared to 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.

## 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 NS3-5B subgenomic replicons replicate efficiently in Huh-7 cells (26). In this report, we explored the critical role of the HCV NS2/3 protease in viral RNA replication using NS2/3 catalytically inactive mutants. Previous studies have suggested that E972 could be an important residue for NS2/3 protease activity (16) and we confirm here the importance of this residue for autoprocessing and viral replication in the context of the replicon system. This gives further evidence that E972 could be the third residue in a catalytic triad also involving H952 and C993. 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 found to be conserved and

of importance for the NS2/3 protease of the bovine viral diarrhoea virus (BVDV), 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 fail 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, and 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 be having this effect. Although NS3 ATPase and helicase activities are unaffected in the context of uncleaved NS2, this protein has two to three fold lower affinity for NS4A than cleaved NS3 does. This could be due to the manner in which NS4A has been shown to bind NS3, forming an integral part of its amino-terminus 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 amino terminus of NS3 does not completely abolish the interaction with NS4A. In fact, several groups have reported synthetic 4A peptides as potent inhibitors of NS2/3 autoprocessing (19,48). Nevertheless, we find 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 NS2/3-4A 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 non-structural proteins soon after transfection (within 4h). However, it takes significantly longer for RNA replication to occur. Transfected RNA, once translated, is rapidly degraded by the cell, while new RNA is found to be synthesized only 24 hours post-transfection (28). We therefore suggest that uncleaved NS2 may be preventing 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 previously observed 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 a 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 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 serine to alanine mutation of conserved residue 977 that 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 hour incubation. It is likely that NS2/3 therefore behaves differently than 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 is 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 in order 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 BVDV strains. In this pestivirus, uncleaved NS2/3 is present in non-cytopathic strains causing persistent infection, while 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 BVDV 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 cell 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 retinoic acid-inducible gene 1 (RIG-1) signaling (54,55), preventing IRF-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 in order to fully understand these events.

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## Footnotes

\* This work was supported by a grant EOP62492 from the Canadian Institutes of Health Research to A.P. and by start-up funds from the McGill Cancer Centre. A.P. is a recipient of a Canada Research Chair in Molecular Oncology. S.W. was supported by a Fonds de la Recherche en Santé du Québec Fellowship (Masters) and a McGill-Canadian Institutes of Health Research Chemical Biology Training Grant.

<sup>1</sup> Abbreviations used are: HCV, hepatitis C virus; DMEM, Dulbecco's modified Eagles medium; PBS, phosphate buffered saline; RRL, rabbit reticulocyte lysate; BVDV, bovine viral diarrhea virus.

## Figure Legends

**Figure 1: Schematic representation of the HCV 1b constructs used in this study.** A) *In vitro* translation expression constructs where a 2X tag (Flag) was added to the N-terminus of full length NS3/4A and NS2/3/4A. B) pNeo/2-5B replicon construct encompassing the 5' HCV NTR (5'), a neomycin resistance gene (N), non-structural proteins (NS2-5B) driven by an EMCV IRES (EI) and the 3' HCV NTR (3'). C) Luciferase replicon PI-luc/NS2-3' contains the HCV 5' NTR (5'), the gene encoding firefly-luciferase (L) under translational control of the Poliovirus-IRES (PI), the EMCV-IRES (EI), non-structural proteins NS2 to NS5B and the HCV 3'NTR (3'). D) Recombinant expression constructs where the protease domain of NS3 is preceded by a 6-histidine tag (His), as well as the same construct including a truncated form of NS2. Numbers under constructs refer to the amino acid residue of the beginning and end of each expressed protein while the asterisk (\*) indicates either wild-type sequence or mutations at amino acids H952, E972, C993 or S977 in NS2.

**Figure 2: Effect of NS2/3 mutations on autoprocessing.** 2xF NS2/3 WT (lane 1) and mutant (lanes 2, 3 and 4) proteins were *in vitro* translated in presence of [<sup>35</sup>S] and the labelled bands separated by SDS-PAGE on a 15% gel. NS2/3, NS3 and NS2 proteins are indicated by arrows on the right.

**Figure 3: Effect of NS2/3 protease mutants on the replication of subgenomic NS2-5B replicons.** A) pNeo/2-5B replicon constructs expressing either wild-type or mutant NS2/3 were electroporated into Huh-7 cells. Following 4 weeks selection with G418, colonies were visualized by Coomassie Blue staining. B) Indicated PI-luc/NS2-3' luciferase replicon constructs were electroporated into cured Huh-7 cells, harvested at the indicated time points and luciferase activity measured. To correct for differences in transfection efficiency, values are reported as a percentage of the counts obtained 4 hours post-transfection (set at 100%).

**Figure 4: NS3 helicase activity in presence of uncleaved NS2.** Increasing amounts (1x, 2x and 3x concentrations) of equi-molar, *in vitro* translated/ immunoprecipitated NS3/4A and NS2/3/4A mutants were incubated with a DNA probe and the reaction products visualized on an 8% non-denaturing acrylamide gel. The migration of the double-stranded (ds) probe and the released single-strand (ss) are indicated. Percent unwinding refers to the amount of single-stranded probe released by the enzyme as compared to the boiled control (lane 1).

**Figure 5: Effect of uncleaved NS2 on the ability of NS3 to associate with NS4A.** FLAG- tagged NS3/4A and NS2/3/4A mutants were *in vitro* translated in presence of [<sup>35</sup>S] and immunoprecipitated using anti-Flag beads. The immunoprecipitated proteins were run on a 15% SDS-PAGE gel. Size of

NS2/3, NS3 and NS4A proteins are indicated by arrows on the right. Lane 1 shows translation/IP of an NS3 construct not encoding NS4A as a control.

**Figure 6: Effect of NS2/3 mutants on polyprotein processing.** Wild-type and NS2/3 mutant pNeo/2-5B replicons were electroporated into cured Huh-7 cells. 75µg of total cell protein obtained after 4 (lanes 1,3,5,7) and 6 (lanes 2,4,6,8) hours were separated by SDS-PAGE and subjected to western blot analysis using NS3 and NS5B specific antibodies. The lower panel indicates a non-specific band used as a loading control.

**Figure 7: Degradation of NS2/3 mutant compared to cleaved NS3.** Wild-type ET, or mutant PI-luc/NS2-3' luciferase replicons were electroporated into cured Huh-7 cells and the amount of NS2/3, NS3 or luciferase visualized by western blot analysis using an NS3 or firefly luciferase specific antibody at the time points post-electroporation indicated. A) Comparison between ET, GND and H952A constructs. B) Effect of an S977A mutation on stability of NS2/3 and NS3 proteins. In each case, the lower panel indicates a non-specific band used as a loading control.

**Figure 8: Effect of a proteasome inhibitor on uncleaved NS2/3 degradation.** GND and H952A mutant PI-luc/NS2-3' replicons were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with 10µM MG132 or DMSO 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 non-specific band used as a loading control.

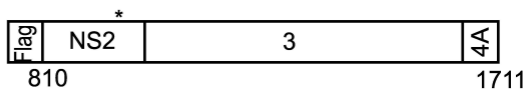
TABLE 1: Comparison of NS3 protease kinetics in presence or absence of NS2

<b>Construct</b>	<b><math>K_{cat}</math> (<math>s^{-1}</math>)</b>	<b><math>K_m</math> (<math>\mu M</math>)</b>	<b><math>K_{cat}/K_m</math> (<math>M^{-1} s^{-1}</math>)</b>
NS3 (1027-1206)	0.55	11.9	46218.5
NS2/3 (904-1206) H952A	0.11	9.3	11828.0
NS2/3 (904-1206) E972A	0.09	10.9	8256.9
NS2/3 (904-1206) C993A	0.08	8.8	9090.9

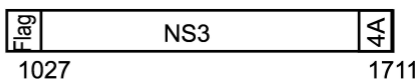
Figure 1

**A**

2xF NS2/3/4A

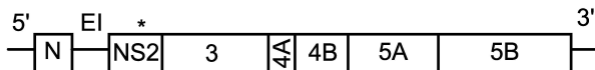


2xF NS3/4A



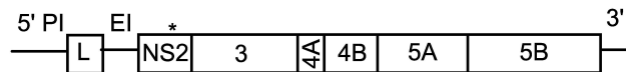
**B**

pNeo/2-5B



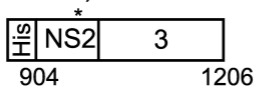
**C**

PI-luc/NS2-3'



**D**

NS2/3(904-1206)



NS3(1027-1206)

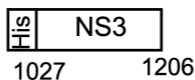


Figure 2

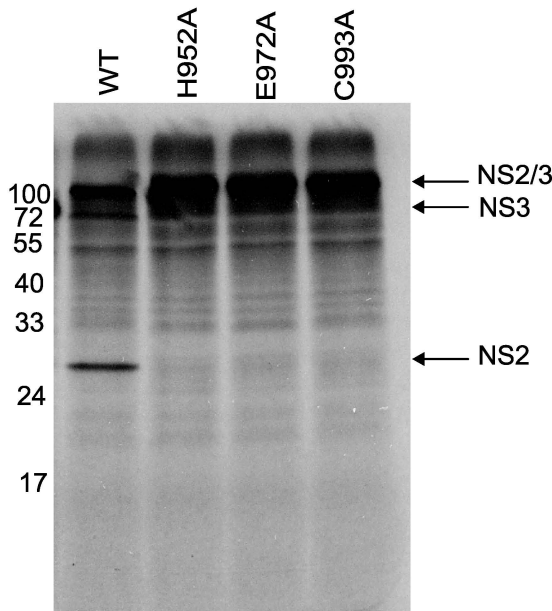
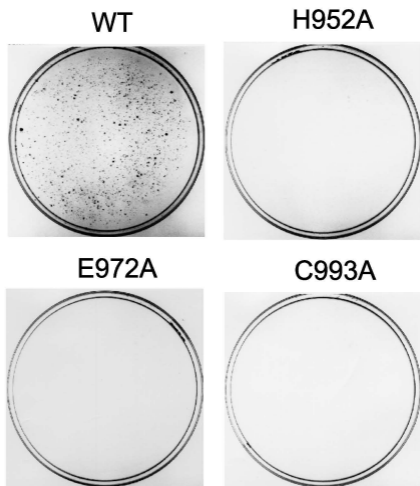


Figure 3

A



B

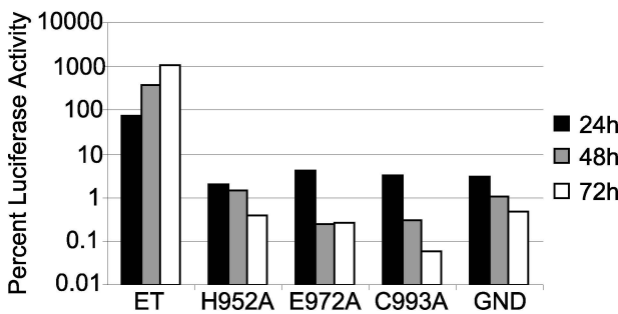


Figure 4

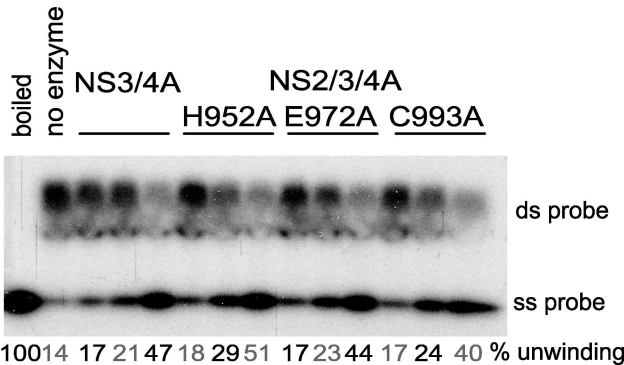


Figure 5

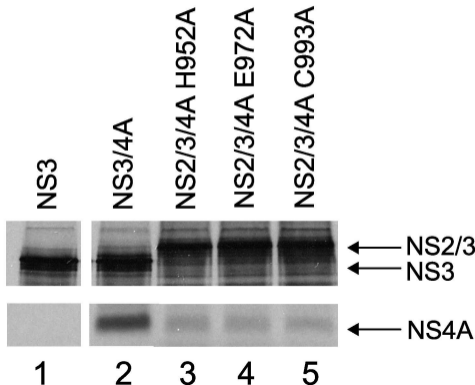


Figure 6

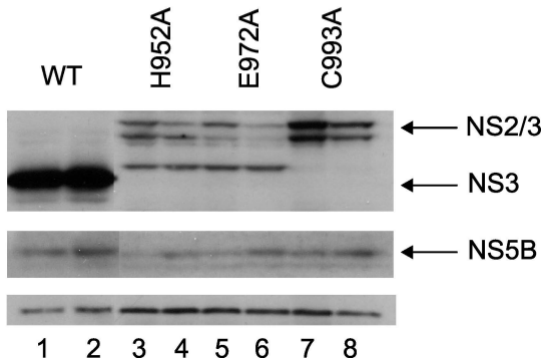


Figure 7

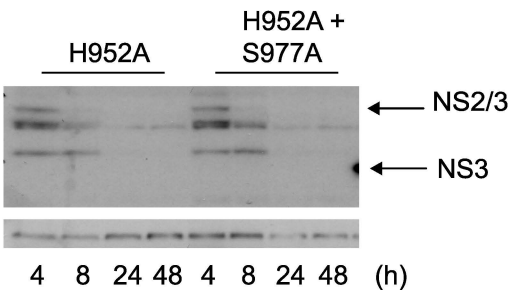
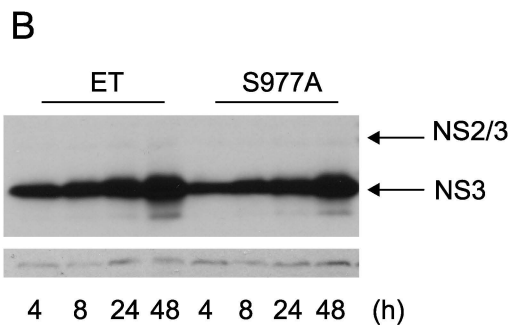
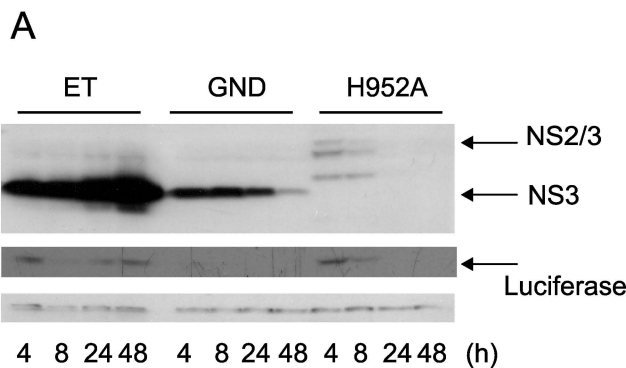


Figure 8

