Short Communication

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Insertion and deletion analyses identify regions of non-structural protein 5A of *Hepatitis C virus* that are dispensable for viral genome replication

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Hepatitis C virus (HCV) non-structural protein 5A (NS5A) plays an essential role in viral genome replication. A series of transposon-mediated insertion mutants and deletion mutants of NS5A was used to examine the colony-forming ability of HCV subgenomic replicons encoding the mutant proteins. The results reveal that two regions of NS5A can tolerate insertions: one spanning residues 240–314, which contain the interferon sensitivity-determining region (ISDR), and the other spanning residues 349–417 at the carboxy terminus. The majority of these sites also tolerated insertion of enhanced green fluorescent protein. Furthermore, replicons encoding NS5A with deletions in ISDR or in the carboxy-terminal regions were replication-competent, indicating that these regions of NS5A are not necessary for replication. Taken together, the results suggest that the central region spanning the ISDR and the carboxy-terminal region of the molecule are dispensable for the functions of NS5A in viral genome replication.

Hepatitis C virus (HCV) is an enveloped virus belonging to the genus *Hepacivirus* within the family *Flaviviridae*. The positive-sense, ~9.6 kb RNA genome is translated to produce a polyprotein, which is processed proteolytically to generate the mature structural and non-structural (NS) proteins (Major & Feinstone, 1997). The NS proteins are involved in viral genome replication, whereas the structural proteins participate in virion assembly and release (Bartenschlager & Lohmann, 2000; Rosenberg, 2001). Development of cell lines supporting replication of subgenomic replicons of HCV has revealed that adaptive mutations within the NS proteins are required for efficient replication of the replicons derived from genotype 1a and 1b genomes (Lohmann *et al.*, 1999; Blight *et al.*, 2000, 2003; Krieger *et al.*, 2001; Yi & Lemon, 2004), although a similarly constructed replicon derived from a genotype 2a virus replicates efficiently with few or no adaptive mutations (Kato *et al.*, 2003).

The NS5A protein is multifunctional. It contains an interferon sensitivity-determining region (ISDR) spanning aa 237–276 and may play a role in resistance to alpha interferon (Polyak *et al.*, 1999). It contains an amphipathic α -helix and a zinc-binding domain at the amino terminus, which are required for replication (Brass *et al.*, 2002; Elazar *et al.*, 2003; Tellinghuisen *et al.*, 2004, 2005). In addition, the phosphorylation pattern of NS5A plays an important role in HCV genome replication (Evans *et al.*, 2004b; Appel *et al.*, 2005). It also interacts with a multitude of cellular proteins and alters the host cells to support viral RNA replication (Gale

et al., 1998; Chung *et al.*, 2000; Park *et al.*, 2002; Evans *et al.*, 2004a; Gao *et al.*, 2004).

Transposon-mediated insertion mutagenesis (Goryshin & Reznikoff, 1998) is a powerful tool that allows insertion of short peptides into proteins encoded in cloned DNA to evaluate permissive sites in the protein. It can also be used to assess regions of the protein that are dispensable for its functions. In the present study, we used an Ez-Tn5 In-Frame Linker Insertion kit (Epicentre Biotechnologies) to introduce 57 bp in-frame insertions (corresponding to 19 aa in the protein) randomly into a plasmid encoding the NS5A protein. As the amphipathic α -helix and the zinc-binding domain, which are required for replication, are located in the amino-terminal region, this region of the protein was excluded for insertion mutagenesis. We took advantage of the presence of a unique *Mlu*I site (corresponding to aa 92 in NS5A) for subsequent manipulation of the NS5A coding region. To facilitate subcloning of NS5A, an *Mlu*I site was engineered between aa 431 and 432 in NS5A (Fig. 1a) within the parental subgenomic replicon Con1S/G-Neo (Blight *et al.*, 2000), henceforth referred to as Con1. This manipulation, which resulted in insertion of two residues (Thr and Arg) in NS5A at this position, did not affect colony-forming ability of the new replicon, Con1-*Mlu*I (Fig. 1b). Subsequently, the entire NS5A coding region of the new replicon was subcloned into the pGEM3 vector and used as template for transposon-mediated insertion mutagenesis as described previously (Das & Pattnaik, 2005). A panel of

Fig. 1. Effect of insertion mutations in NS5A on colony-forming ability of the HCV subgenomic replicon. (a) The Con1 subgenomic replicon (top) with the 5' untranslated region (UTR), 3' UTR and HCV NS proteins is shown. Neo, Neomycin phosphotransferase gene; I_E , the internal ribosome entry site of encephalomyocarditis virus. The NS5A coding region with insertion of 19 aa at various locations (upward arrows) is shown at the bottom. Numbers on top of the rectangular boxes represent the amino acid positions of NS5A, whereas the numbers in parentheses correspond to NS5A sequence relative to the viral polyprotein in the replicon. The engineered *MluI* site (at residue 431) in NS5A is shown in grey. The target for insertion mutagenesis (residues 92–431) is shaded in grey. The ISDR is also highlighted. Transposon-mediated insertion mutants are named as TIM followed by the amino acid at which insertion occurred. (b) Colony-forming ability of various insertion mutants. Huh7.5 cells were electroporated (270 V, 950 μF) with *in vitro* transcripts by using a GenePulser II (Bio-Rad). The cells were seeded in six-well plates. At 48 h post-electroporation, the cells were maintained in medium containing 1 mg G418 ml^{-1} for 3 weeks, with medium being changed at 3–4-day intervals. Drug-resistant cell colonies were stained in 1.25 % crystal violet in 25 % ethanol, washed and counted. Data shown are means of three independent experiments, with SD represented by error bars. (c) One microgram of total RNA from cells harbouring the parental Con1-*MluI* or mutant replicons was subjected to RT-PCR and the products were analysed in agarose gel and visualized by staining with ethidium bromide. The product generated from the parental replicon is expected to be 645 bp, whereas those from the mutant replicons are expected to be larger by 57 bp. DNA size markers in base pairs (bp) are shown on the left. (d) Western blot analysis of NS5A proteins from cells harbouring various replicons. Total proteins from cells were separated by SDS-PAGE, transferred to a membrane and probed with anti-NS5A (top) and anti-actin (bottom) antibody. Protein size markers (in kDa) are shown on the left.

mutants with an insertion between residues 92 and 431 of NS5A was identified (Fig. 1a) and subcloned individually into the subgenomic replicon Con1-*MluI*.

RNA derived from plasmids encoding various mutant replicons was electroporated into Huh-7.5 cells and the colony-forming ability of the replicons was examined by selection with G418 as described previously (Blight *et al.*, 2000) for 3 weeks. As shown in Fig. 1(b), insertion of 19 aa within the amino-terminal region of the NS5A protein (residues 96–227) and within residues 327–339 was lethal for replication. However, G418-resistant cell colonies, indicative of replication, could be generated by replicons encoding NS5A

proteins with insertions at the carboxy-terminal region (residues 349–417), as well as the central region of the molecule (residues 240–314) spanning the ISDR (Fig. 1b), albeit with different efficiencies.

The observation that replicons with NS5A proteins containing insertions within the central region of the molecule spanning the ISDR could establish G418-resistant cell colonies is interesting. This contrasts with studies reported recently (Moradpour *et al.*, 2004) identifying only two sites near the carboxy terminus that are permissible for insertion: one after residue 384 and the other after residue 418. It is possible that analysis of only 14 individual drug-resistant

cell colonies from cells electroporated with a pool of insertion mutant-bearing replicons may have led to detection of only the mutant replicons with significantly higher efficiency of colony formation (Moradpour *et al.*, 2004). This interpretation is compatible with our observation that insertions at the carboxy-terminal region of NS5A result in much higher efficiency of colony formation compared with that resulting from insertions in the central region.

RT-PCR analyses of RNA from various replicon-bearing cells maintained for at least 16 weeks in the presence of the drug showed that the products generated with RNA obtained from various mutant replicon-bearing cells were predictably larger than an expected product of 645 bp from the parental replicon-bearing cells (Fig. 1c), indicating that insertions were present in the replicons. Western blot analysis (Fig. 1d) of total cell extracts from mutant replicon-bearing cells also detected NS5A proteins that were slightly larger than NS5A obtained from the parental replicon-containing cells. It should be noted that NS5A generated from the TIM396 replicon possessed a slightly reduced electrophoretic mobility compared with the other insertion mutants. The reason(s) for this is not clear, but it could be due to hyperphosphorylation or other modification of this protein.

As we were able to generate replicons containing mutant NS5A with small (19 aa) insertions at several different sites in NS5A, we examined whether insertion of enhanced green fluorescent protein (eGFP) at these sites would also be tolerated. By using the unique *NotI* site present within the insertion sequences, we inserted full-length eGFP sequences in-frame with the NS5A protein in various replicons.

Replicons with eGFP insertions within ISDR (TIM240-eGFP and TIM271-eGFP) were, in general, less efficient at establishing G418-resistant cell colonies than replicons (TIM349-eGFP, TIM389-eGFP, TIM396-eGFP, TIM410-eGFP and TIM417-eGFP) with insertions at the carboxy-terminal region of NS5A (Fig. 2a). Furthermore, replicons with insertions of eGFP were five- to 15-fold less efficient at establishing drug-resistant cells than the corresponding replicons with insertions of 19 aa. Although the majority of the sites tolerated insertion of eGFP, such insertion at site 314 was lethal. This site, at which a small insertion (19 aa) led to a viable replicon (TIM314), is in close proximity to several other sites spanning residues 327–348, where the small insertions were also lethal. It is possible that this region may be involved in specific interaction with a cellular or viral component necessary for replication. Insertion of eGFP at residue 314 may have blocked this interaction due to steric hindrance, resulting in a lethal phenotype.

Drug-resistant Huh-7.5 cells harbouring replicons encoding NS5A–eGFP fusion proteins were examined further by fluorescence microscopy. Bright epifluorescence of eGFP was observed in the majority of the cells (Fig. 2b). The epifluorescence pattern is punctate and perinuclear and is characteristically similar to the distribution of NS5A protein in cells supporting subgenomic HCV replication (El-Hage & Luo, 2003; Shi *et al.*, 2003; Moradpour *et al.*, 2004). To confirm that the eGFP signal observed in these cells was due to the NS5A–eGFP fusion protein, cell extracts from the cells were examined for the presence of the fusion protein by Western blot analyses. Results showed that both anti-NS5A antibody (Fig. 2c) and anti-eGFP antibody (Fig. 2d) detected

Fig. 2. Replicons encoding the NS5A–eGFP fusion protein are functional. (a) Mean colony-forming efficiencies with SD of replicons containing 19 aa or eGFP insertions in NS5A from three independent experiments are shown. (b) Fluorescence microscopy of drug-resistant Huh7.5 cells harbouring replicons encoding NS5A–eGFP fusion proteins. Images of eGFP epifluorescence, as well as 4,6-diamidino-2-phenylindole (DAPI)-stained nuclei, in the cells are shown. (c, d) Western blotting of total cell proteins from cells harbouring the replicons with anti-NS5A antibody (c) and anti-eGFP antibody (d).

a protein with a molecular mass of ~ 84 kDa, the predicted size of the NS5A–eGFP fusion protein, in cells harbouring these replicons. Taken together, the results demonstrate that the epifluorescence observed in cells bearing these replicons is due to the NS5A–eGFP protein and suggest that the fusion protein is processed appropriately and is stable.

As insertions at several sites in NS5A were permissible, we examined whether these regions of the protein could be deleted without affecting its function in replication. Accordingly, we generated a series of deletion mutants (Fig. 3a) by taking advantage of the unique *NotI* site present in the insertion mutants. Although the replicons encoding the deletion mutants established drug-resistant cell colonies, their efficiency of colony formation was significantly lower (Fig. 3a) than that of the parental replicon or insertion-mutant replicons. Mutant replicons from which residues 240–417 or 271–417 of NS5A were deleted were non-functional in replication. Western blot analysis (Fig. 3b) of cell extracts from four deletion-mutant replicon-bearing cells revealed the presence of deleted NS5A proteins, although some deletion mutants possessed anomalous

electrophoretic mobility, which could be due to the extent of phosphorylation of the proteins. RT-PCR analysis (data not shown) of RNA from the cells bearing these replicons and subsequent sequencing of the products confirmed that each of the mutant replicons still retained the introduced deletions.

Our results presented here show that all of the mutants with insertions at the amino-terminal region of the protein (spanning residues 92–227) were non-functional in replication, suggesting that this region of NS5A may contain important domains involved in replication. The amino-terminal domain of NS5A appears to interact with several cellular proteins (Gale *et al.*, 1998; Chung *et al.*, 2000; Park *et al.*, 2002; Waris *et al.*, 2003; Evans *et al.*, 2004a; Gao *et al.*, 2004) that may be necessary for replication. In addition, an amphipathic α -helix and a zinc-binding site located at the amino terminus are required for replication (Brass *et al.*, 2002; Elazar *et al.*, 2003; Tellinghuisen *et al.*, 2004, 2005). The amino-terminal region of the protein, spanning residues 1–213 (domain I; Tellinghuisen *et al.*, 2004), is suggested to be globular and is proposed to be involved in homodimer formation for interaction with the membrane during replication (Tellinghuisen *et al.*, 2005). Insertions within this domain may affect dimer formation and thereby inhibit the replication functions of the protein. In contrast, the central region, spanning residues 240–314 [which include part of the low-complexity sequence I (LCS I) and part of domain II; Tellinghuisen *et al.*, 2004], and the carboxy-terminal region, spanning residues 349–417 (which include part of LCS II and part of domain III; Tellinghuisen *et al.*, 2004), may be flexible and can accept insertions without adversely affecting the replication functions of NS5A. In this regard, it is interesting to note that adaptive mutations resulting in deletion of the ISDR (Blight *et al.*, 2000) and the carboxy-terminal sequences (residues 399–441) have been demonstrated (Zhu *et al.*, 2003). Our results with mutants containing insertions between residues 349 and 417 suggest that this region of NS5A may not possess important domains involved in replication. This interpretation is supported by our observation that deletion of residues 349–410 of NS5A did not abrogate replication functions of the protein.

In conclusion, our studies reported here demonstrate that the region spanning the ISDR, as well as the carboxy-terminal region of NS5A, can tolerate insertion of eGFP, whereas the amino terminus of the protein cannot tolerate even small insertions. Furthermore, deletions in the region spanning the ISDR and also in the carboxy-terminal regions of NS5A led to proteins that were functional in replication, albeit with reduced activity. With the recent establishment of full-length cDNA clones of the viral genome that produce infectious HCV in cultured cells (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005), it will be very useful to incorporate eGFP into the NS5A protein and examine replication of the virus by live-cell imaging. In addition, the panel of non-functional NS5A insertion mutants that we have generated will be useful in studies to address the role of

Fig. 3. Deletion mutants of NS5A and their activity in replication. (a) Schematic of deletion mutants, with the residues deleted from NS5A shown on the left. The efficiency of colony formation (mean and SD from three experiments) for replicons encoding each of the mutant proteins is shown on the right. (b) Western blot analysis of cell extracts from cells bearing replicons encoding some of the deletion-mutant proteins using anti-NS5A (top), anti-NS3 (middle) and anti-actin (bottom) antibody.

specific cellular and viral protein interactions with the NS5A protein that mediate viral RNA replication.

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