

Short Communication

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Tagging of NS5A expressed from a functional hepatitis C virus replicon

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Knowledge of how hepatitis C virus (HCV) proteins associate with components of the host cell to form a functional replication complex is still limited. To address this issue, HCV replicon constructs were generated where either green fluorescent protein (GFP) or the *Propionibacterium shermanii* transcarboxylase domain (PSTCD) was introduced into the NS5A coding region. Insertion of both GFP and PSTCD was tolerated well, allowing formation of stable replicon-containing cell lines that contained viral protein and transcript levels that were comparable to those of an unmodified parental replicon. Cell lines generated from the GFP-tagged NS5A replicon allowed live-cell visualization of the location of NS5A. Cell lines generated from the PSTCD-tagged replicons allowed rapid and efficient precipitation of the PSTCD-tagged NS5A, as well as other HCV non-structural proteins, using streptavidin-coated magnetic beads. Both replicons represent useful tools that offer different but complementary ways of examining replication-complex formation in cells.

Hepatitis C virus (HCV) is an enveloped RNA virus responsible for significant global morbidity and mortality (Lavanchy *et al.*, 1999). In common with other positive-strand RNA viruses, the six non-structural proteins (NS2–NS5B) are likely to form part of a macromolecular complex responsible for replication of the RNA genome. Whilst structural information pertaining to several of the NS proteins is now available (O'Farrell *et al.*, 2003; Tellinghuisen *et al.*, 2005; Yao *et al.*, 1999), very little is known about the structure and composition of the RNA replication complex. Identifying both the viral and cellular components of this complex will prove important, not only in providing an insight into how replication of the viral genome is coordinated, but also in the rational design of therapeutics for treating HCV infection.

Progress towards this goal will require the development of robust protocols for isolation of functional RNA replication complexes. This could potentially be achieved by affinity purification of either the viral RNA or the NS proteins. In this regard, RNA replication complexes were recently isolated from replicon-harbouring cells by a two-step purification process: following hybridization with biotin- and digoxigenin-tagged oligonucleotides, complexes were

purified by sequential avidin–agarose and anti-digoxigenin precipitation steps (Waris *et al.*, 2004). This allowed the isolation of complexes that contained all of the NS proteins and were capable of *in vitro* RNA synthesis. In a separate study, it was shown that functional HCV replicons could be generated in which either a small epitope tag or the coding sequence for enhanced green fluorescent protein (EGFP) was inserted in frame close to the C terminus of NS5A. The NS5A–EGFP fusion protein could be directly visualized and was used to demonstrate that NS5A co-localized with both NS3 and the sites of viral RNA synthesis (Moradpour *et al.*, 2004; Appel *et al.*, 2005). Based on these studies, we speculated that it might be possible to introduce a novel tag into the C-terminal end of NS5A that would result in biotinylation of the NS5A fusion protein and thus permit efficient and rapid purification of NS5A and associated proteins on immobilized avidin. One candidate protein was a domain of the 1·3S subunit of *Propionibacterium shermanii* transcarboxylase (PSTCD). This 123 aa protein is metabolically biotinylated by biotin ligase in both prokaryotic and eukaryotic cells (Parrott & Barry, 2000) and has been used successfully as an affinity tag for the purification of recombinant proteins.

To facilitate the cloning of novel tags into the replicon, we identified a unique *Bsa*BI restriction site within the FK5.1 culture-adapted subgenomic replicon, situated 60 nt from the end of the NS5A-coding sequence (Fig. 1a). This site was chosen because it allowed positioning of additional coding sequences within NS5A only 8 aa C-terminal to the

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Fig. 1. Functional subgenomic replicons with insertions at the C terminus of NS5A. (a) Construction of modified replicons. The coding sequence for EGFP, PSTCD or Δ PSTCD, flanked by flexible hinge regions, was cloned into the indicated location within NS5A as described in the text. (b) Colony-formation assay. T7 transcripts were electroporated into Huh-7 cells as described previously (McCormick *et al.*, 2004), seeded into six-well plates and selected with G418 (1 mg ml^{-1}) 48 h post-transfection. Colony numbers were counted 2 weeks later and the results of three independent experiments are presented graphically relative to the FK5.1 replicon. (c) Northern blot analysis. RNA extraction, separation and Northern blot analysis were performed as described previously (McCormick *et al.*, 2004). The probes were directed to the NS5B-coding region (nt 6266–7100 of pFK-1389neo/NS3-3'/5.1; upper panel) and GAPDH (lower panel). (d) Western blot analysis. The indicated cell lines were lysed in Glasgow lysis buffer [GLB: 10 mM PIPES/NaOH (pH 7.2), 120 mM KCl, 30 mM NaCl, 5 mM MgCl_2 , 1% Triton X-100, 10% glycerol; Bentham *et al.*, 2003], supplemented with $2 \times$ Complete protease inhibitor cocktail (Roche), 1 mM Na_3VO_4 and 1 mM NaF. Samples ($5 \mu\text{g}$ protein) were analysed by Western blotting with sheep polyclonal antisera to NS5A (Macdonald *et al.*, 2003) or NS3 (Aoubala *et al.*, 2001), HRP-conjugated streptavidin (ExtrAvidin; Sigma) or monoclonal anti-GFP (Clontech) or anti-GAPDH (Biosdesign) antibodies as indicated. Bound antibody was detected with the appropriate HRP-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence.

previously described insertion point for EGFP (Moradpour *et al.*, 2004). First, we needed to confirm that the replicon could tolerate insertions at this location. Therefore, the EGFP-coding region flanked by short hinge regions was amplified by PCR (primer sequences available upon request) and ligated into the *Bsa*BI site of pLRM(wt) (Macdonald *et al.*, 2005), a plasmid containing an *Ns5I–Ns5I* fragment (nt 3682–7122) of pFK5.1neo (Krieger *et al.*, 2001). The modified *Ns5I–Ns5I* fragment was then reintroduced into pFK5.1neo, generating pFK5.1neo^{EGFP} (Fig. 1a). Subsequently, the EGFP-coding region was excised by *Clal* digestion and replaced by the coding sequences for either the full-length PSTCD or a 70 aa N-terminally truncated version (Δ PSTCD, also efficiently biotinylated) (Parrott & Barry, 2000), generating pFK5.1neo^{PSTCD} and pFK5.1neo ^{Δ PSTCD} (Fig. 1a).

To establish whether the modified replicon constructs were functional, *in vitro*-transcribed RNAs were electroporated into Huh-7 cells as described previously (McCormick *et al.*, 2004). The parental pFK5.1neo and a replication-deficient replicon (GDD \rightarrow GND mutation within NS5B) served as controls. After G418 selection, the numbers of colonies formed following transfection of RNA derived from pFK5.1neo^{EGFP}, pFK5.1neo^{PSTCD} and pFK5.1neo ^{Δ PSTCD} were found to be almost identical to those obtained from pFK5.1neo (Fig. 1b). As expected, no colonies were observed in cells transfected with the GND mutant-derived transcripts. The fact that these insertions within NS5A at aa 2398 had negligible impact on colony formation was unexpected, as previous reports (Moradpour *et al.*, 2004; Appel *et al.*, 2005) described a reduction in colony formation of 25- and 2500-fold upon insertion of GFP into NS5A at sites near to the site chosen by us (aa 2390 and 2356, respectively). Overall, this variation between constructs may reflect a greater propensity for the virus replication complex to accept insertions closer to the C terminus of NS5A. Alternatively, the inclusion of hinge regions on either side of our constructs may help to relieve any conformational constraints required for NS5A function. Structural analysis of both GFP (Ormo *et al.*, 1996) and the PSTCD (Reddy *et al.*, 2000) have demonstrated that the N and C termini are close to each other. This is perhaps also important in the success of this approach, as the insertion is less likely to disrupt the structure of NS5A.

Polyclonal cell lines containing all four replicon constructs were then examined by Northern (Fig. 1c) and Western (Fig. 1d) blotting to establish whether viral transcript and protein levels were altered due to the presence of the inserts within NS5A. Transcript levels were similar for all the replicons (Fig. 1c); however, levels of HCV protein expression were reduced in cells harbouring the NS5A–GFP replicon in comparison with wild-type FK5.1 and the two PSTCD-containing replicons (Fig. 1d). This suggested that the stability of the polyprotein containing the GFP insertion might be reduced, although the integrity of the NS5A–EGFP fusion protein was confirmed by Western blotting with an

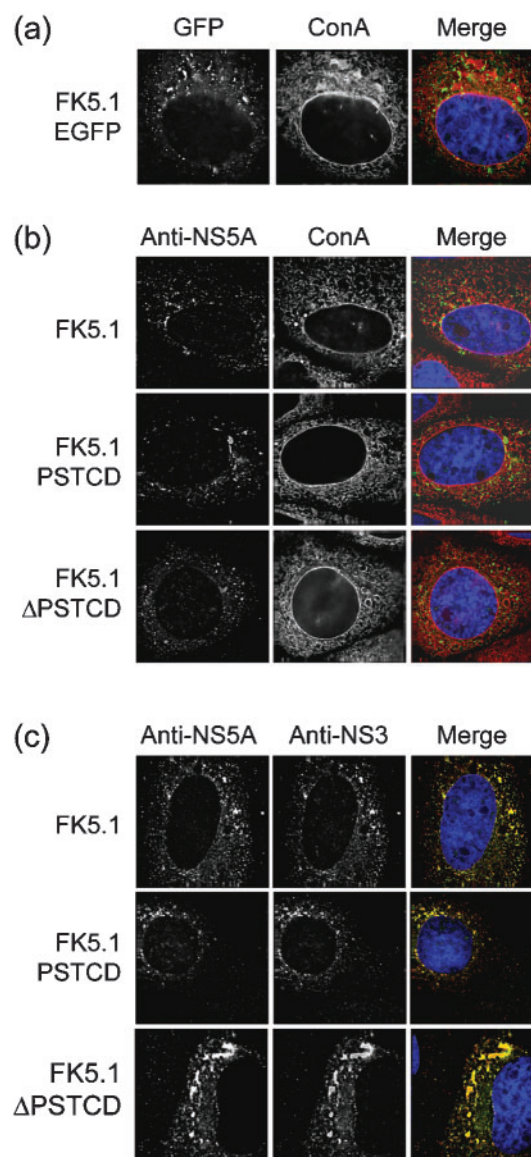


Fig. 2. Subcellular localization of HCV non-structural proteins in cells harbouring subgenomic replicons with EGFP or PSTCD insertions. The indicated cells (2×10^5) on glass coverslips in 12-well dishes were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Incubation with primary (1:50 dilution of rabbit anti-NS5A or sheep anti-NS3 polyclonal sera) and secondary (1:100 dilution of Alexa Fluor 488 nm-conjugated goat anti-rabbit or 1:200 dilution of Alexa Fluor 594 nm-conjugated donkey anti-sheep; Molecular Probes) antibodies was performed in PBS/10% fetal calf serum for 1 h at room temperature. The endoplasmic reticulum was stained with Texas red-conjugated concanavalin A (ConA, 1:500; Molecular Probes) and nuclei were labelled with Hoechst 33258 dye (1:10000 in PBS). Image capture and analysis were performed as described previously (Griffin *et al.*, 2004). (a) EGFP replicon cells; (b) cells stained for NS5A and endoplasmic reticulum; (c) cells stained for NS5A and NS3.

anti-GFP antibody. The NS5A–PSTCD fusion proteins migrated at their predicted molecular masses and, moreover, no obvious truncated forms of the NS5A fusion proteins were observed, indicating that the inserts were not lost during selection and maintenance of the cell lines. Both the basal and hyperphosphorylated forms of NS5A were observed – this is best visualized in Fig. 3(a), lane 10. Western blotting with ExtrAvidin–horseradish peroxidase (HRP) demonstrated that the NS5A–PSTCD and NS5A– Δ PSTCD fusion proteins were the predominant cellular biotinylated proteins, although, as expected, there were a number of other biotinylated proteins, including one that co-migrated with NS5A–PSTCD.

Having established the presence of an NS5A–EGFP fusion protein, we confirmed the functionality of the EGFP moiety by observing direct GFP fluorescence in cells harbouring the FK–GFP replicon (Fig. 2a), but not in any of the other replicon cell lines (data not shown). Furthermore, consistent with previous reports, fluorescence activity was restricted to the cytoplasm of the cell, with distinct punctate staining focused around the perinuclear area. To confirm that fusion of NS5A with the PSTCD domain did not affect the subcellular distribution of NS5A or the HCV RNA replication complex, cells harbouring either the wild-type FK5.1 or the two PSTCD-containing replicons were examined by co-immunofluorescence staining using a rabbit anti-NS5A serum and either Texas red-conjugated concanavalin A (Fig. 2b) or a sheep anti-NS3 serum (Fig. 2c). This analysis confirmed that the distribution of the NS5A–PSTCD and NS5A– Δ PSTCD fusion proteins was identical to that of wild-type NS5A. Fig. 2(c) confirms that, as for wild-type NS5A, the NS5A–PSTCD and NS5A– Δ PSTCD fusion proteins co-localized precisely with NS3.

Previous reports have demonstrated that there is co-association between NS5A and the other non-structural proteins (Dimitrova *et al.*, 2003; Shirota *et al.*, 2002). As the PSTCD system provided an ideal method to confirm this observation, lysates were prepared from naïve Huh-7 cells or lines harbouring the FK5.1, FK5.1–PSTCD or FK5.1– Δ PSTCD replicons and biotinylated proteins were isolated from these cell lysates by using streptavidin-coated magnetic (SM) beads. As shown in Fig. 3(a), wild-type NS5A did not bind to SM beads (Fig. 3a, lanes 4–6). However, both NS5A–PSTCD and NS5A– Δ PSTCD bound very efficiently and were quantitatively depleted from the lysates by incubation with SM beads. Neither protein was present in the flow-through (Fig. 3a, lanes 8 and 11), but they were eluted from the SM beads following extensive washing and incubation in reducing SDS-PAGE loading buffer (Fig. 3a, lanes 9 and 12). The NS5A–PSTCD fusion proteins were purified very efficiently by the SM beads, indicating that the PSTCD moiety is an efficient substrate for biotinylation within Huh-7 cells. The efficiency of both biotinylation and recognition by immobilized streptavidin is probably due to the conformation of the PSTCD moiety – the biotinylated residue (lysine 89) is present on an exposed loop on the opposite face of the native 1-3S protein to the N and C termini and is thus likely to be highly exposed within the NS5A–PSTCD fusion protein. In addition, unlike other biotinylated proteins that have been used as affinity tags, such as the biotin carboxyl carrier protein from *Escherichia coli* acetyl-CoA carboxylase, the biotin moiety does not interact with the protein itself (Reddy *et al.*, 2000) and is thus free for interaction with streptavidin.

Western blot analysis was then used to determine whether NS3 and NS5B associated with NS5A–PSTCD and

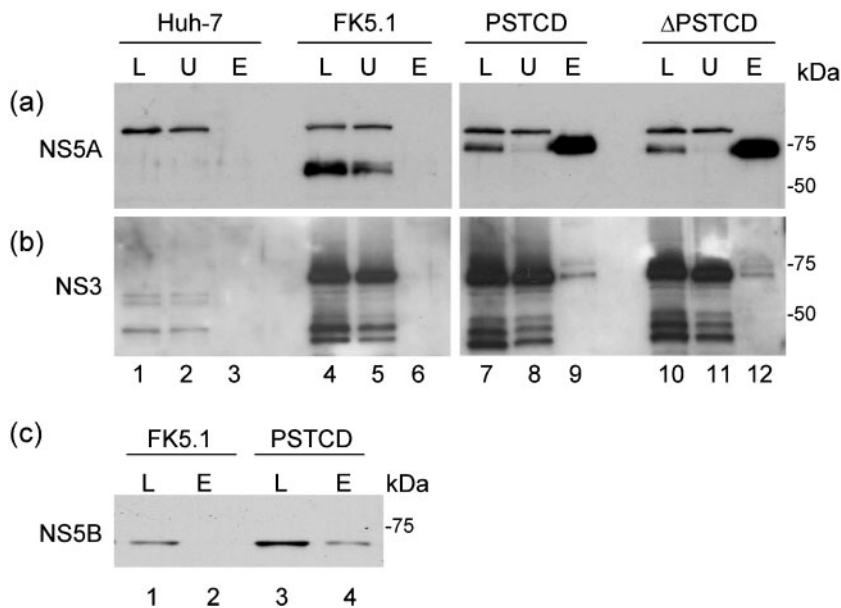


Fig. 3. Use of the PSTCD system to isolate NS5A and associated proteins. Lysates (500 μ g protein) from each of the indicated cell lines were incubated for 1 h at 4 °C on a rotating blood mixer with 20 μ l SM beads (New England BioLabs) pre-washed with GLB. Bound proteins were washed three times with GLB supplemented with 0.5 M KCl, once with GLB and eluted in 1 \times reducing sample buffer prior to SDS-PAGE and Western blot analysis. Lane L represents 5 μ g total lysates and lane U a corresponding amount of the unbound (flow-through) fractions. Lanes E represents 10% of the material captured on SM beads. Blots were probed with sheep polyclonal antisera to NS5A (a), NS3 (b) or NS5B (c).

NS5A- Δ PSTCD. This analysis revealed that the majority of NS3 was in fact not associated with NS5A and remained in the flow-through (Fig. 3b, lanes 8 and 11). Furthermore, NS3 did not bind non-specifically to the SM beads (Fig. 3b; compare lanes 4 and 6). However, a small proportion of NS3 bound to the SM beads from lysates containing either NS5A-PSTCD or NS5A- Δ PSTCD (Fig. 3b; lanes 9 and 12). NS5B was more problematic, as our initial experiments demonstrated that NS5B bound non-specifically to the SM beads (data not shown). This problem was circumvented by performing the binding incubation in the presence of 0.5 M KCl. Under these conditions, NS5B only bound to the SM beads in the presence of either NS5A-PSTCD or NS5A- Δ PSTCD (Fig. 3c). It is interesting that, under the conditions used in this study, only a small proportion of the other non-structural proteins (NS3 and NS5B) co-purified with the NS5A-PSTCD fusion protein. There are a number of possible reasons for this. Firstly, it may be that this reflects the low proportion of the non-structural proteins in replicon cells that form active RNA replication complexes at any one time. Secondly, it may be that the replication complex is labile and not maintained under the assay conditions; in this regard, it is pertinent to note that the cells were disrupted with non-ionic detergent (Triton X-100). As the replication complex is membrane-bound, and indeed requires the presence of membrane-associating motifs within both NS5A and NS5B (Dubuisson *et al.*, 2002), it is likely that disrupting membranes with detergent might also destabilize the replication complex. However, HCV RNA replication has been reported to be associated with a detergent-resistant membrane fraction (Shi *et al.*, 2003) and as such might be expected to be stable in non-ionic detergent.

The PSTCD system has several advantages over more 'conventional' antibody-based purification strategies for analysis of the HCV replication complex. The high affinity ($K_d = 10^{-15}$ M) and specificity of the biotin-avidin interaction mean that complexes can be purified rapidly and efficiently by using readily available reagents. In addition, it does not rely on the use of costly antibodies that are often of variable quality and efficacy and may interfere directly with the composition of the complex. Although we were able to reduce non-specific binding of NS5B to the SM beads with high salt, the use of monomeric avidin coupled with an elution step involving competition with free biotin (not possible with streptavidin) might be a less harsh way of overcoming the background of non-specific binding. Such experiments are under way.

In conclusion, our study confirms that the C terminus of NS5A can tolerate the insertion of large protein domains, consistent with this region of the protein being dispensable for RNA replication. The PSTCD system will be amenable to a range of future studies, including purification and characterization of replication complexes. Coupled with mutagenesis of the replicon, the use of the baculovirus delivery system that we have described previously and recent advances in establishing the complete HCV replication cycle in

cultured cells (Wakita *et al.*, 2005), this should facilitate a detailed analysis of the cellular factors required for HCV RNA replication.

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