Analysis of hepatitis C virus core/NS5A protein co-localization using novel cell culture systems expressing core–NS2 and NS5A of genotypes 1–7

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Hepatitis C virus (HCV) is an important human pathogen infecting hepatocytes. With the advent of infectious cell culture systems, the HCV particle assembly and release processes are finally being uncovered. The HCV core and NS5A proteins co-localize on cytoplasmic lipid droplets (cLDs) or on the endoplasmic reticulum (ER) at different stages of particle assembly. Current knowledge on assembly and release is primarily based on studies in genotype 2a cell culture systems; however, given the high genetic heterogeneity of HCV, variations might exist among genotypes. Here, we developed novel HCV strain JFH1-based recombinants expressing core–NS2 and NS5A of genotypes 1–7, and analysed core and NS5A co-localization in infected cells. Huh7.5 cells were transfected with RNA of core–NS2/NS5A recombinants and putative adaptive mutations were analysed by reverse genetics. Adapted core–NS2/NS5A recombinants produced infectivity titres of \(10^{2.5} – 10^{4.5}\) f.f.u. ml\(^{-1}\). Co-localization analysis demonstrated that the core and NS5A proteins from all genotypes co-localized extensively, and there was no significant difference in protein co-localization among genotypes. In addition, we found that the core and NS5A proteins were highly associated with cLDs at 12 h post-infection but became mostly ER associated at later stages. Finally, we found that different genotypes showed varying levels of core/cLD co-localization, with a possible effect on viral assembly/release. In summary, we developed a panel of HCV genotype 1–7 core–NS2/NS5A recombinants producing infectious virus, and an immunostaining protocol detecting the core and NS5A proteins from seven different genotypes. These systems will allow, for the first time, investigation of core/NS5A interactions during assembly and release of HCV particles of all major genotypes.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of severe liver disease such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Alter & Seeff, 2000). Due to its high degree of genetic diversity, HCV has been classified into seven major genotypes and several subtypes based on sequence homology (Bukh et al., 1993; Simmonds et al., 1993, 2005). The virus particle contains a positive-sense ssRNA genome, which encodes a single ORF flanked by 3′- and 3′-untranslated regions (UTRs). The ORF encodes a polyprotein that is processed by viral and cellular proteases to produce structural proteins (core, E1 and E2), p7 and non-structural proteins (NS2, NS3, NS4A/B and NS5A/B) (Gottwein & Bukh, 2008). The entire viral life cycle of HCV could not be studied in vitro until 2005, when the first cell culture system based on the genotype 2a strain JFH1 was developed (Wakita et al., 2005). J6/JFH1 recombinants, produced by replacing the core–NS2 region of JFH1 with the corresponding region derived from the genotype 2a J6CF isolate (Yanagi et al., 1999), produced higher infectivity titres and did not rely on culture adaptation (Gottwein et al., 2007; Lindenbach et al., 2005; Pietschmann et al., 2006). Several other JFH1-based recombinants, carrying the core–NS2 region from the seven major genotypes, were subsequently developed (Gottwein et al., 2007, 2009; Jensen et al., 2008; Pedersen et al., 2013; Pietschmann et al., 2006; Scheel et al., 2008, 2011a; Yi et al., 2007). These constructs have been useful for studying the effect of neutralizing antibodies and antiviral drugs against diverse HCV genotypes, and for functional studies of core–NS2 interactions (Gottwein et al., 2009, 2011; Griffin et al., 2008; Pedersen et al., 2013; Prentoe et al., 2011). In addition, we developed a panel of recombinants carrying the NS5A region from genotypes...
1–7 in the J6/JFH1 backbone, with the purpose of analysing the effect of newly developed NS5A inhibitors on different strains of HCV and for functional genotype-specific studies of NS5A (Scheel et al., 2011b, 2012). However, at the onset of this study, efficient culture systems using recombinants combining strain-specific structural proteins and NS5A were only available for genotype 2a isolates (Scheel et al., 2011b; Wakita et al., 2005; Zhong et al., 2005).

The availability of genotype 2a HCV cell culture systems has allowed the study of essential steps of the viral replication cycle, including viral assembly and release (Bartenschlager et al., 2011; Lindenbach, 2013). To produce infectious viruses, newly synthesized HCV genomes need to be delivered to the site of assembly, probably the surface of cytoplasmic lipid droplets (cLDs) or the cytosolic side of the endoplasmic reticulum (ER), and packaged into the nucleocapsid (Appel et al., 2008; Miyanari et al., 2007; Shavinyskaya et al., 2007). The core and NS5A proteins play essential roles in the early stage of virion assembly (Masaki et al., 2008) and are among the better-understood proteins involved in particle formation. The core protein is the main constituent of the viral nucleocapsid, which packages the genomic RNA for particle release. NS5A is a phosphoprotein involved in the formation of the viral replication complex and has been shown to possess RNA-binding capacity (Huang et al., 2005; Tellinghuisen et al., 2005), suggesting that it could be involved in the transport of viral genomic RNA to the assembly site (Miyanari et al., 2007). After synthesis, core protein is mobilized to the surface of cLDs, where it is thought to initiate particle assembly (McLauchlan et al., 2002; Miyanari et al., 2007). The extent of core protein accumulation on the surface of cLDs is inversely correlated with the efficiency of particle assembly (Boulant et al., 2007; Shavinyskaya et al., 2007), probably reflecting a transient localization of core protein on cLDs prior to its transfer to the ER where the assembly process can proceed (Boson et al., 2011). The core and NS5A proteins co-localize extensively in the cytoplasm and their interaction is critical for the production of viral particles (Masaki et al., 2008; Miyanari et al., 2007). Moreover, both localize on the surface of cLDs and the extent of core/NS5A co-localization on cLDs is also inversely correlated with the efficiency of virion production (Appel et al., 2008). NS2 is a major player in the subsequent stages of assembly (Jirasko et al., 2008; Ma et al., 2011; Popescu et al., 2011). The nascent viral particle subsequently enters the ER lumen, where it associates with E1/E2 through a not fully defined mechanism and interacts with very-low-density lipoproteins to form lipoviroparticles (Gastaminza et al., 2008; Huang et al., 2007). Mature virions are then released through the secretory pathway via the Golgi network. Several aspects of these processes remain unclear, and our knowledge of the factors implicated and their interaction is incomplete. Moreover, these processes have been only elucidated in experiments with genotype 2a viruses, and the genetic divergence between HCV isolates could result in variation in the assembly and release pathways among different genotypes.

Here, we developed novel cell culture-adapted recombinants with genotype 1–7-specific core–NS2/NS5A in the JFH1 backbone. We demonstrated that core and NS5A proteins from all recombinants could be visualized by immunofluorescence microscopy, and displayed similar co-localization patterns irrespective of the HCV genotype. The developed culture systems are suitable for investigation of the assembly of HCV particles in the context of different genotypes, since they carry isolate-specific versions of most viral proteins involved in particle production and release.

### RESULTS

#### Development of HCV recombinants carrying genotype-specific core–NS2 and NS5A regions

Most functional studies of HCV in the context of the complete viral life cycle have relied on genotype 2a cell culture systems. To enable genotype-specific studies of interactions between NS5A and the structural HCV proteins for all seven major HCV genotypes, we developed core–NS2/NS5A recombinants containing the core–NS2 and NS5A regions from isolate-specific versions of genotypes 1a, 1b, 3a, 4a, 5a, 6a and 7a (Bukh et al., 2010; Gottwein et al., 2009; Sakai et al., 2007). Thus, we replaced the complete NS5A of the following JFH1-based core–NS2 recombinants that also carried culture-adaptive mutations with the corresponding strain-specific NS5A consensus sequence: H77C/JFH1V787A,Q1247I (1a), TN/JFH1R1408W (1a), J4/JFH1P866L,Q1496L (1b), S52/JFH1P878Q,K1398Q (3a), ED43/JFH1T827A,T977S (4a), SA13/JFH1S923K,N1118R (5a), HK6a/JFH1S349S,N417T (6a) and QC69/JFH1L878P (7a) (all numbering is according to the H77 reference polyprotein, GenBank accession no. AF009606) (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008, 2011a). Such core–NS2/NS5A recombinants could potentially produce higher titres compared with previously developed genotype-specific core–NS2 and/or NS5A recombinants (Gottwein et al., 2009; Scheel et al., 2011b), since efficient virus production could depend on genotype-specific interactions between NS5A and core.

In vitro-transcribed RNA corresponding to the HCV genome of core–NS2/NS5A genotype 1–7 recombinants was transfected into Huh7.5 hepatoma cells in parallel with RNA of the genotype 2a recombinant, J6/JFH1 (Lindenbach et al., 2005). One day after transfection, for most recombinants 10–30 % of cells expressed HCV core protein, as determined by immunostaining. However, for the ED43(4a), SA13(5a) and QC69(7a) recombinants, only 1–5 % of cells were positive (Fig. 1). J6/JFH1 infected most of the culture after 3–6 days, while the same was observed for S52(3a), HK6a(6a) and QC69(7a) after 6–12 days, and for H77C(1a), TN(1a), J4(1b), ED43(4a) and SA13(5a) after 18–39 days (Fig. 1). Only the QC69 recombinant
produced infectivity titres above $10^3$ f.f.u. ml$^{-1}$ during the first 10 days after transfection, although delayed around 5 days compared with J6/JFH1 (Fig. 2). Since mutations adapting the previously developed core–NS2 recombinants could potentially compensate for interactions with JFH1 NS5A and could thus be a disadvantage for core–NS2/NS5A recombinants with strain-specific NS5A, we tested ED43(4a) and SA13(5a) core–NS2/NS5A without such mutations. However, this led to no spread of infection for ED43(4a) and slower spread of infection for SA13(5a) (Fig. 1).
Fig. 2. Infectivity titres after transfection of Huh7.5 cells with JFH1-based recombinants expressing genotype–specific core–NS2/NS5A. Infectivity was measured in supernatants from the first 10 days after transfection with RNA transcripts of recombinants of isolates (genotypes): (a) TN(1a), (b) H77C(1a), J4(1b), S52(3a), (c) SA13(5a), QC69(7a) and (d) HK6a(6a). The ED43 recombinants with and without Y1644H/E2267G mutations spread slowly to the majority of culture cells (22–27 and 17 days, respectively), and are therefore not shown. For comparison, J6/JFH1 was included in each separate experiment as a control. Coloured boxes indicate the core–NS2 and NS5A isolate of the given recombinant. Numbering of mutations is according to the H77 reference polyprotein (GenBank accession no. AF009606). All recombinants were passaged to naïve Huh7.5 cells to obtain full ORF sequences (Tables S1–S7). The lower limit of detection in the experiments shown was up to $10^{-2.3}$ f.f.u. ml$^{-1}$; titres below this level are shown as $<2.3 \log_{10}$ (f.f.u. ml$^{-1}$). Error bars indicate SEM of triplicate titre determinations.
After passage to naïve Huh7.5 cells, infectivity titres above $10^3$ f.f.u. ml$^{-1}$ were observed for all recombinants, except for J4(1b) and ED43(4a). Viral RNA was extracted from supernatants with peak HCV infectivity titres and sequenced to obtain the near full-length genomic sequence, including the entire ORF. Putative adaptive mutations were identified in all recombinants, except QC69, that apparently did not depend on additional adaptation (Tables S1–S7, available with the online version of this paper).

**Generation of cell-culture-adapted core–NS2/NS5A recombinants by reverse genetic analysis**

Selected mutations observed for the core–NS2/NS5A recombinants were tested in reverse genetic studies for H77C(1a), TN(1a), J4(1b), S52(3a), SA13(5a) and HK6a(6a) (Figs 1 and 2). While the SA13(5a)A1021G,K1118R,R1978G,C2419R recombinant immediately produced titres comparable to J6/JFH1 in transfection cultures, the H77C(1a)V787A,C1185S,Q1247L,TN(1a)I1312V,R1408W, S52(3a)I787S,K1398Q,C2419R and HK6a(6a)F349S,N417T,I2268N mutants produced titres around $10^7$ f.f.u. ml$^{-1}$, and J4(1b)F886L,C1185S,Q1496L produced titres around $10^{2.5}$ f.f.u. ml$^{-1}$ (Table 1). Since the ED43(4a) core–NS2/NS5A recombinant did not adapt to efficient titre production during three serial passages to naïve cells, mutations identified in recovered virus (Table S5) were not tested in reverse genetic studies during three passages to naïve cells, mutations identified in reverse recovered cultures. We instead inserted the Y1644H and E2267G substitutions previously shown to adapt a J6/JFH1-based recombinant with previously developed J6/JFH1-based NS5A recombinants (Scheel et al., 2011a,b). The ED43(4a)T827A,T977S,Y1644H,E2267G recombinant produced titres around $10^3$ f.f.u. ml$^{-1}$ (Table 1) 22 days after transfection. After passage of the final genotype 1–7 core–NS2/NS5A recombinants to naïve Huh7.5 cells, peak HCV RNA titres were around $10^7$ IU ml$^{-1}$ (Table 1). Peak HCV infectivity titres were around $10^4$ f.f.u. ml$^{-1}$ for S52(3a), SA13(5a) and QC69(7a), $10^{3.5}$ f.f.u. ml$^{-1}$ for H77C(1a) and HK6a(6a), and below $10^3$ f.f.u. ml$^{-1}$ for TN(1a), J4(1b) and ED43(4a) (Table 1). No additional mutations were identified in the recovered viruses by sequencing of the complete ORF, except for the TN(1a) and ED43(4a) recombinants (Tables S1–S7). Thus, we developed a genotype-specific panel of core–NS2/NS5A recombinants producing infectious virus, which could be useful for functional studies. Except for the SA13(5a) and QC69(7a) recombinants, virus production was lower for these recombinants compared with previously developed J6/JFH1-based NS5A recombinants (Scheel et al., 2011b), and JFH1-based core–NS2 recombinants (Gottwein et al., 2009; Scheel et al., 2011a).

**Validation of labelling efficiency of core and NS5A proteins from HCV genotypes 1–7 in infected Huh7.5 cells**

Proper evaluation of co-localization of core and NS5A from the different HCV recombinants relies on comparable efficiency of protein labelling and signal intensity across genotypes. Therefore, we initially performed single-staining of core and NS5A from Huh7.5 cells infected with each core–NS2/NS5A recombinant to verify reactivity of primary antibodies against different genotypes. Supernatants containing passaged H77C(1a)V787A,C1185S,Q1247L,TN(1a)I1312V,R1408W, J4(1b)F886L,C1185S,Q1496L, J6/JFH1, S52(3a)I787S,K1398Q,C2419R, ED43(4a)T827A,T977S,Y1644H,E2267G, SA13(5a)A1021G,K1118R,R1978G,C2419R, HK6a(6a)F349S,N417T,I2268N and QC69(7a)F349S,N417T,I2268N recombinants (Table 1) were used to infect naïve Huh7.5 cells. Cells were fixed at 48 h post-infection and stained using primary antibodies

**Table 1. Characteristics of culture-adapted core–NS2/NS5A HCV recombinants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Isolate</th>
<th>Engineered mutations*</th>
<th>Peak titres†</th>
<th>Pearson’s coefficient‡</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Transfection</td>
<td>Viral passage</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>log$_{10}$(f.f.u. ml$^{-1}$)</td>
<td>log$_{10}$(f.f.u. ml$^{-1}$)</td>
<td>log$_{10}$(IU ml$^{-1}$)</td>
</tr>
<tr>
<td>1a</td>
<td>H77C</td>
<td>V787A, C1185S, Q1247L</td>
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<td>3.5</td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>I1312V, R1408W</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>1b</td>
<td>J4</td>
<td>F886L, C1185S, Q1496L</td>
<td>2.5</td>
<td>1.8</td>
</tr>
<tr>
<td>2a</td>
<td>J6/JFH1</td>
<td></td>
<td>4.2</td>
<td>5.0</td>
</tr>
<tr>
<td>3a</td>
<td>S52</td>
<td>I787S, K1398Q, C2419R</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>4a</td>
<td>ED43</td>
<td>T827A, T977S, Y1644H, E2267G</td>
<td>2.6</td>
<td>2.3</td>
</tr>
<tr>
<td>5a</td>
<td>SA13</td>
<td>A1021G, K1118R, R1978G, C2419R</td>
<td>4.5</td>
<td>3.8</td>
</tr>
<tr>
<td>6a</td>
<td>HK6a</td>
<td>F349S, N417T, I2268N</td>
<td>3.0</td>
<td>3.3</td>
</tr>
<tr>
<td>7a</td>
<td>QC69</td>
<td>L878P</td>
<td>3.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*Mutations are numbered according to the H77 reference polyprotein (GenBank accession no. AF009606). Sequencing results of virus recovered from supernatants are shown in Tables S1–S7. Mutations introduced in this study are shown in bold.

†Representative infectivity (f.f.u.) and RNA (IU) titres observed in supernatants from transfection and passages to naive cells are indicated.

‡Pearson’s coefficients represent mean values calculated using image stacks from three to five infected cells per sample, after image alignment and three-dimensional (3D) deconvolution.

§J6/JFH1 carries the core–NS2 region from J6CF and the NS5A gene from JFH1. Titre data shown are from Gottwein et al. (2009).
anti-NS5A–mlG1 (9E10) and anti-core–mlG2a (1851). In single-staining, both antibodies had good reactivity against all genotypes (Fig. S1). However, in double-staining protocols assessing core/NS5A co-localization, several genotypes showed a markedly reduced labelling of core protein (data not shown). Three anti-core antibodies (anti-core–mlG2a 1851, 1868 and C7-50), binding to different epitopes, were subsequently used in 1:1:1 concentration ratio for double-staining with anti-NS5A, and showed efficient and consistent labelling across the genotype panel. Comparison of double-staining with anti-NS5A and either one or three anti-core antibodies was performed on the J6/JFH1 and SA13(5a) recombinants (Fig. S2). Since there was no detectable increase in background using three instead of one antibody, triple core staining was used in all subsequent experiments. To verify the specificity of our staining protocol and exclude the possibility of cross-talk between channels, cells infected with passaged viruses were fixed at 48 h post-infection, stained with either primary anti-core or anti-NS5A antibodies, and with both Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies. All samples exhibited highly specific staining, with fluorescence detected exclusively in the correct channel (Fig. 3). Thus, we developed an efficient and specific labelling strategy for double-staining of core and NS5A proteins from the seven major HCV genotypes.

**Co-localization analysis of genotype 1–7 core and NS5A proteins in infected Huh7.5 cells**

To validate our analysis protocol, we initially single-stained J6/JFH1-infected cells with either anti-NS5A or anti-core primary antibodies, followed by double-staining with secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 546. Panels show maximum intensity projections (MIPs) of two-channel stacks from cells infected with different genotypes and subtypes. In the headings, core and NS5A indicate the primary antibody used to stain each set of columns, while Alexa488 and Alexa546 indicate the acquisition channel shown in each column. Images were deconvolved and enhanced for publication. Control, stained uninfected cells.

**Fig. 3.** Cross-talk controls for single-staining of core or NS5A protein on Huh7.5 cells infected with genotype 1–7 core–NS2/NS5A recombinants and the original JFH1 virus. Cells fixed at 48 h post-infection were stained with either anti-NS5A–mlG2a or three anti-core–mlG1 antibodies. Secondary staining was performed using both anti-mlG2a–Alexa Fluor 488 and anti-mlG1–Alexa Fluor 546. Panels show maximum intensity projections (MIPs) of two-channel stacks from cells infected with different genotypes and subtypes. In the headings, core and NS5A indicate the primary antibody used to stain each set of columns, while Alexa488 and Alexa546 indicate the acquisition channel shown in each column. Images were deconvolved and enhanced for publication. Control, stained uninfected cells.
Alexa Fluor 546 fluorophores. Images obtained for double-stained cells showed a near-perfect signal overlap (Fig. 4a), and Pearson’s coefficients of 0.96 and 0.94 for NS5A and core protein, respectively, close to the theoretical value of 1 (Fig. 4c). Conversely, cells infected with J6/JFH1 and processed using anti-NS5A and anti-core antibodies together, following our standard double-staining protocol, displayed a partial overlap of NS5A and core signals (Fig. 4b) and a Pearson’s coefficient of 0.77 (Fig. 4c), similar to what was previously reported for the J6/JFH1 recombinant Jc1 (Appel et al., 2008). These data indicated that our image post-processing and analysis protocol correctly detected and interpreted the proteins fluorescent signals.

To investigate whether there are differences in the core/NS5A association among HCV genotypes, we performed co-localization analysis of the two proteins using the novel core–NS2/NS5A genotype 1–7 recombinants. The original JFH1 virus (Wakita et al., 2005) was included in the analysis as a control for cLD-associated distribution of core. Supernatants containing passaged viruses (Table 1) were used to infect Huh7.5 cells, which were stained at 48 h post-infection. Analysis of core and NS5A staining showed a partial overlap of core and NS5A signals in all samples (Fig. 4b), and comparable signal intensities for the different constructs. Core–NS2/NS5A recombinants showed both proteins dispersed within the cytoplasm in discrete aggregates, distributed in a reticular manner suggestive of localization on the ER. Conversely, JFH1 virus displayed the typical condensed distribution of core protein, indicative of association with the surface of cLds. Co-localization analysis of core and NS5A proteins from core–NS2/NS5A recombinant viruses produced Pearson’s coefficients between 0.73 and 0.82 (Fig. 4c, Table 1) and showed no significant difference in core/NS5A co-localization among genotype-specific recombinants (one-way ANOVA test; $P=0.66$). In contrast, JFH1 showed a significantly lower co-localization coefficient compared with the core–NS2/NS5A constructs (one-way ANOVA test; $P<0.05$). These results indicated that the core and NS5A proteins from efficient recombinants representing the seven major HCV genotypes have similar distributions during the viral life cycle.

**Analysis of core and cLD association in Huh7.5 cells infected with core–NS2/NS5A genotype recombinants**

cLds play an important role in the life cycle of HCV, functioning as scaffold for the recruitment of core proteins and initiation of new virion assembly. JFH1 core protein is known to localize almost exclusively on the surface of cLds (Miyanari et al., 2007), whereas core protein from the Jc1 recombinant is distributed mostly on the surface of the ER (Boson et al., 2011). To assess whether other HCV genotypes display differences in the core/cLD distribution, we performed co-localization analysis on cells triple-stained for core, NS5A and cLds. Huh7.5 cells were infected using recombinant viruses with high infectivity [J6/JFH1(2a), SA13(5a)], intermediate infectivity [S52(3a)] or low infectivity [TN(1a), JFH1(2a)]. Protein and cLD distribution was assessed at 48 h post-infection using our optimized protocol, adjusted to accommodate lipid staining with boron-dipyrromethene (BODIPY) (Fig. 5a).

Co-localization analysis of core and NS5A proteins from core–NS2/NS5A recombinant viruses produced Pearson’s coefficients between 0.68 and 0.76, comparable to our previous experiments (Fig. 5b). There was no significant difference among co-localization coefficients obtained from genotype-specific recombinants or compared with JFH1, despite the fact that the latter produced a markedly lower coefficient (one-way ANOVA test; $P=0.54$ and $P=0.50$).

To quantify the level of association between core and cLds, we evaluated the percentage of threshold core signal that colocalized with threshold cLDs signal (as implemented in the IMARIS software). This should give a more objective estimate of core/cLD association compared with the commonly used count of core-positive cLds and result in a better representation of cells with intermediate association levels. JFH1 showed a significantly higher level of core/cLD co-localization (37.5%), compared with co-localization levels produced by the core–NS2/NS5A recombinants (3.7–10.4%) (one-way ANOVA test; $P<0.001$) (Fig. 5c). Genotype-specific recombinants with low [TN(1a)] or intermediate [S52(3a)] infectivity titres displayed relatively higher co-localization levels compared with high infectivity viruses [J6/JFH1(2a), SA13(5a)]. However, only TN(1a) and SA13(5a) were significantly different in the co-localization of core and cLD signal (one-way ANOVA test; $P<0.05$). These results showed that core–NS2/NS5 recombinants representing four major HCV genotypes have comparable association levels of core and cLds overall. However, our data also suggested that genotype-specific alterations of core/cLD association might be correlated with differences in viral assembly or release.

**Time-lapse analysis of core/NS5A and core/cLD co-localization in Huh7.5 cells infected with core–NS2/NS5A genotype recombinants**

All analyses performed so far were conducted on cells fixed and stained at 48 h after infection. To investigate whether different HCV genotypes display changes in the distribution of core/NS5A/cLds at different time points after infection, we performed a time-lapse experiment on cells infected with TN(1a), J6/JFH1(2a) and SA13(5a) genotype-specific recombinants. Huh7.5 cells were infected in triplicate, and fixed at 12, 48 and 72 h post-infection. All time points were then simultaneously stained for core, NS5A and cLds using the three-colour staining protocol introduced previously. Overall, we could not observe major differences in protein or cLD distribution among the analysed genotypes (Fig. 6a). Interestingly, all three core–NS2/NS5A recombinants showed very condensed signals of NS5A and especially core protein at 12 h, almost perfectly overlapping with the cLD signal. Later time points showed more diffuse signals of both proteins, compatible with ER
Fig. 4. Co-localization analysis of core and NS5A proteins on Huh7.5 cells infected with genotype 1–7 core–NS2/NS5A HCV recombinants and the original JFH1 virus. (a) Double-staining controls of core and NS5A proteins were infected using J6/JFH1, fixed at 48 h post-infection and stained using either anti-core–mlgG1 or anti-NS5A–mlgG2a antibodies. Secondary staining was performed using either anti-mlgG1 or anti-mlgG2a antibodies, each conjugated to Alexa Fluor 488 (green) and Alexa Fluor 546 (red). (b) Cells infected with core–NS2/NS5A recombinants or JFH1 were fixed at 48 h post-infection and double stained with anti-NS5A–mlgG2a and anti-core–mlgG1 antibodies. Secondary staining was carried out using antibodies anti-mlgG2a–Alexa Fluor 488 (green, NS5A) and anti-mlgG1–Alexa Fluor 546 (red, core). Pictures show MIPs of two-channel image stacks of 40–50 slices each. White squared areas are shown enlarged to enhance details. Images were deconvolved and enhanced for publication. (c) Distribution of Pearson's coefficients of core/NS5A co-localization among core–NS2/NS5A recombinants and the original JFH1 virus. The 'J6/JFH1 core' and 'J6/JFH1 NS5A' bars represent co-localization controls where a single protein, either core or NS5A, was stained with two colours. Error bars represent SEM obtained from three to five infected cells from independent infections. *P<0.05.
Co-localization analysis of core/NS5A/cLDs on Huh7.5 cells infected with JFH1 virus or core–NS2/NS5A HCV recombinants of genotypes 1a, 2a, 3a and 5a. (a) Infected cells were fixed at 48 h post-infection and double stained with anti-NS5A–mlgG2a and anti-core–mlgG1 antibodies for 24 h at 4 °C. Secondary staining was carried out using BODIPY 505/515 (green, cLDs) and antibodies anti-mlgG2a–Alexa Fluor 633 (blue, NS5A) and anti-mlgG1–Alexa Fluor 546 (red, core) for 2 h at room temperature. Pictures show MIPs of representative three-channel image stacks, made up of 50–60 slices each. White squared areas are shown enlarged to enhance details. Images were deconvolved and enhanced for publication. (b) Distribution of Pearson's coefficients of core/NS5A co-localization among genotype-specific recombinants and JFH1. Error bars represent SD obtained from four infected cells from independent infections. (c) Amount of core protein co-localized with cLDs among genotype-specific recombinants and JFH1. Values are calculated as the percentage of thresholded core signal that co-localizes with thresholded cLD signal. Error bars represent SD obtained from four to six infected cells from independent infections. *P<0.05; ***P<0.001.
association. Despite a general increase in the size of cLDs, the 48 and 72 h time points did not display important differences in signal distribution, either within the same genotype or among genotypes. Analysis of Pearson’s coefficients for core/NS5A co-localization showed no significant differences among genotype-specific constructs at any time point (two-way ANOVA test; \( P > 0.32 \)), whereas all three recombinants showed a significant difference between 12 and 72 h (two-way ANOVA test; \( P < 0.001 \)) (Fig. 6b). Conversely, evaluation of core/cLD association indicated that SA13(5a) had significantly less core localized on cLDs than TN(1a) at 12 and 48 h post-infection (two-way ANOVA test; \( P < 0.001 \)), whereas the difference became non-significant at 72 h (Fig. 6d). All three genotypes, however, displayed significantly more core protein localized on cLDs at 12 h (two-way ANOVA test; \( P < 0.001 \)) (Fig. 6c). Taken together, these data indicated that in the early stage of the viral life cycle new particle assembly of core–NS2/NS5A recombinant viruses is restricted to the surface of cLDs, while virion assembly is later transferred to the ER surface. Our results are also consistent with a correlation between genotype-specific levels core/cLD association and efficiency of particle assembly/release, as suggested by the comparison between SA13(5a) and TN(1a) recombinants.

**DISCUSSION**

In order to be able to study the genotype specific interaction of the NS5A protein with the structural proteins of HCV, we developed infectious genotype 1–7 core–NS2/NS5A HCV recombinants of genotypes 1a, 2a and 5a. (a) Infected cells were fixed at 12, 48 or 72 h post-infection and double-stained with anti-NS5A–mlgG2a and anti-core–mlgG1 antibodies for 24 h at 4 °C. Secondary staining was carried out using BODIPY 505/515 (green, cLDs) and antibodies anti-mlgG2a–Alexa Fluor 633 (blue, NS5A) and anti-mlgG1–Alexa Fluor 546 (red, core) for 2 h at room temperature. Pictures show merged MIPs of representative three-channel image stacks from each time point, made up of 50–60 slices each. White squared areas are shown enlarged to enhance details. Images were deconvolved and enhanced for publication. (b) Distribution of Pearson’s coefficients of core/NS5A co-localization for genotype-specific recombinants at the indicated time points post-infection. Error bars represent SD obtained from four to eight infected cells from independent infections. (c, d) Comparison of core/cLD association over time for each genotype-specific recombinant (c) and comparison of core/cLD association among different genotypes over time (d). Values are calculated as the percentage of thresholded core signal that co-localizes with thresholded cLD signal. Error bars represent SD obtained from four to eight infected cells from independent infections. *\( P < 0.05 \); ***\( P < 0.001 \).
S52(3a), SA13(5a) and QC69(7a) were the most efficient (Gottwein et al., 2009). Apparently, adding a genotype-specific NS5A gene caused a general fitness reduction. While J6/JFH1-based NS5A recombinants can be of particular advantage for specific inhibitor and replication studies, since only the NS5A gene differs (Scheel et al., 2011b), the novel core–NS2/NS5A recombinants will be useful for NS5A interaction studies as shown here and possibly for the development of full-length genotype-specific culture systems. In addition, these systems could be useful to assess the efficacy of novel combination therapies involving NS5A and p7 inhibitors or NS5A inhibitors combined with neutralizing antibodies.

Most of the recombinants relied on adaptation for efficient growth, in analogy to what has been reported for inter-genotypic JFH1-based core–NS2 and J6/JFH1-based NS5A recombinants (Gottwein et al., 2007, 2009; Jensen et al., 2008; Kaul et al., 2007; Scheel et al., 2008, 2011a, b; Yi et al., 2007). The R1978G mutation conferring adaptation to SA13(5a) was observed previously for the J6/JFH1-based NS5A SA13(5a) recombinant, and was demonstrated to increase the amount of processed NS5A (Scheel et al., 2011b). The S52(3a) and SA13(5a) recombinants both acquired C2419R, located within the cleavage site at the C-terminus of NS5A. The neighbouring mutation V2418L, also in the NS5A/NS5B cleavage site, was shown to slow down cleavage kinetics, thereby reducing the dependence on cyclophilin A (Kaul et al., 2009), and it is possible that C2419R has a similar function. Adaptive mutations in NS5A domain II improved growth of the ED43(4a) and HK6a(6a) core–NS2/NS5A recombinants; the HK6a(6a) core–NS2/NS5A recombinant carried the same mutation observed in the J6/JFH1-based NS5A HK6a(6a) recombinant (Scheel et al., 2011b). Such mutations could mediate cross-genotypic interactions with other HCV proteins interacting with NS5A (Jirasko et al., 2010; Ma et al., 2011). The H77C(1a) and J4(1b) core–NS2/NS5A recombinants both acquired the C1185S mutation close to the active site of the NS3/4A protease (Kim et al., 1996; Love et al., 1996), possibly mediating NS5A cleavage compatibility. Furthermore, mutation of the adjacent residue 1187 was shown to influence NS5A phosphorylation (Lindenbach et al., 2007). I1312V improved growth kinetics for the TN(1a) core–NS2/NS5A recombinant. This mutation has been shown to adapt JFH1 and core–NS2 recombinants of genotypes 1a, 1b, 3a and 5a, suggesting a more general effect in increasing viral fitness in cell culture (Gottwein et al., 2009; Jensen et al., 2008; Kaul et al., 2007; Scheel et al., 2008, 2011a).

Several studies have previously investigated co-localization of different HCV proteins and host cell components; most, if not all, of these studies have, however, limited their analyses to genotype 2a culture systems (Appel et al., 2008; Camus et al., 2013; Herker et al., 2010; Jirasko et al., 2010; Masaki et al., 2008; Miyanari et al., 2007; Shavinskaya et al., 2007). In particular, the core and NS5A proteins have been shown to co-localize extensively in cells infected with genotype 2a viruses, and their interaction was confirmed to be essential for production of infectious particles using biochemical assays (Appel et al., 2008; Masaki et al., 2008).

In order to analyse the distribution of core and NS5A in genotypes different from 2a, we optimized a protocol for efficiently and selectively staining core and NS5A of genotypes 1–7. Despite the low variability of the core protein among HCV genotypes, three combined anti-core antibodies were necessary to achieve comparable staining across all recombinants. The epitope for the 9E10 NS5A antibody used here overlaps with aa 414–428, which is 100 % conserved among the isolates analysed here (Scheel et al., 2012). This probably explains why this antibody efficiently reacted to all isolates in the current study, but not to J6 NS5A, which has a single amino acid change in this region (Scheel et al., 2012). Analysis of the distribution of core and NS5A signal intensities permitted the quantification of protein co-localization and the comparison of core/NS5A distribution among different genotypes. Core and NS5A proteins co-localized to a similar extent in all recombinants, showing no significant differences between proteins of different origin. This finding indicates that the differences in viability among genotype-specific recombinants were not caused by differences in the core/NS5A association. This in turn suggests that, in spite of high sequence variation among the prototype isolates of genotypes 1–7, the interaction between core and NS5A is conserved and seems to be essential for HCV. Interestingly, infection with JFH1 virus resulted in significantly lower core/NS5A co-localization coefficients compared with genotype-specific core–NS2/NS5A recombinants. Such difference can be explained considering that while JFH1 core is mostly associated with cLDs, JFH1 NS5A is largely localized on the ER, thus resulting in reduced correlation of the two signals (Boson et al., 2011; Miyanari et al., 2007).

The marked difference in core/NS5A co-localization between JFH1 and genotype-specific recombinants also indirectly suggested that the proteins from the novel recombinants were mostly ER-associated, rather than cLD-associated. Moreover, the core and NS5A proteins were highly co-localized and distributed within the cytoplasm in a reticular framework, compatible with association to the ER. We previously demonstrated co-localization of cLDs and core signals for the core–NS2 recombinants of genotypes 1–7 that were used to generate the core–NS2/NS5A recombinants (Gottwein et al., 2009). In that report, 5–15 % of core protein co-localized with cLDs, with slight variations between genotypes. However, we did not observe a correlation between variations in core/cLD co-localization and virus production. Here, we analysed core/NS5A/cLD distribution in cells infected with four core–NS2/NS5A genotype-specific recombinants and with the original JFH1 virus. Core/NS5A co-localization coefficients obtained from triple staining was comparable to what we observed previously, strengthening our conclusion that different HCV genotypes have a similar core/NS5A distribution within the cytoplasm. In addition, we found that 3–10 % of core protein from the novel recombinants co-localized with cLDs, in line with what we

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had observed in our previous study. These figures were significantly different from the core/cLD co-localization obtained from JFH1 virus (approximately 40%), supporting our conclusion that proteins from the core–NS2/NS5A recombinants are mostly ER associated. Interestingly, there seemed to be an inverse correlation between the level of core/cLD association and the infectivity titres of our novel recombinants, as had been shown for JFH1 (Appel et al., 2008; Shavinskaya et al., 2007). Although the measured differences were relatively small, the two recombinants with the highest (SA13) and lowest (TN) infectivity titres produced significantly different levels of core/cLD co-localization. Since reduced accumulation of core and NS5A proteins on cLDs is indicative of a more efficient assembly of viral particles, it is possible that variations in core/NS5A/cLD association could confer genotype-specific differences affecting particle assembly or release.

Finally, to further investigate the differences in the dynamics of particle assembly of different HCV genotypes, we performed a time-lapse analysis of core/NS5A/cLD co-localization using genotype-specific recombinants TN(1a), J6/JFH1(2a) and SA13(5a). All three viruses produced comparable and increasing levels of core/NS5A co-localization over time, supporting our previous finding that the protein distribution is independent of the infecting genotype. Interestingly, core protein from all three genotype-specific recombinants was highly associated (>50%) with cLDs at 12 h post-infection, whereas the association decreased to <10% at later time points. These data support the hypothesis that the cLDs might serve as initial storage areas for the accumulation of core before the actual assembly of viral particles is initiated and transferred to the surface of the ER. Furthermore, the low-titre TN(1a) recombinant showed the highest levels of core/cLD association at all time points, although the differences were significant only compared with SA13(5a) at 12 and 48 h. These data further support our hypothesis that the presence of genotype-specific differences in the dynamics of core/cLD association might affect particle assembly/release and in turn viral production and infectivity.

In summary, we developed a panel of replication-competent HCV recombinants carrying core–NS2 and NS5A regions of genotypes 1–7, permitting studies of the interaction of NS5A with HCV structural proteins, p7 and NS2 for all major genotypes. Moreover, we optimized a sensitive assay capable of detecting core and NS5A proteins from seven different genotypes and quantitatively determined their cellular co-localization. We found that core and NS5A co-localized extensively in all analysed genotypes, and there was no significant difference in protein co-localization among genotypes. In addition, we found that core and NS5A had a high degree of association with cLDs at 12 h post-infection but became mostly ER associated at later stages. Finally, we found that different genotypes showed varying levels of core/cLD co-localization, with a possible effect on viral assembly/release. These novel systems will be useful in functional studies analysing interaction between core, NS5A and other viral proteins involved in virion production, such as NS2 and p7, across HCV genotypes.

**METHODS**

**HCV sources and construction of recombinants.** As a backbone for the novel JFH1-based core–NS2/NS5A genotype recombinants we used the core–NS2 recombinants H77C/JFH1V797A,Q947L (Scheel et al., 2008), TN/JFH1R1408W (Scheel et al., 2011a), J6/JFH1V866L,Q596L (Gottwein et al., 2009), S52/JFH1V878L,K196Q (Gottwein et al., 2009), ED43/JFH1T927A,T977S (Scheel et al., 2008), SA13/JFH1I1021G,K1318R (Jensen et al., 2008), HK6a/JFH1F2495,N417T (Gottwein et al., 2009) and QC69/JFH1L873P (Gottwein et al., 2009). HCV NSSA consensus sequences of the same prototype isolates (Bukh et al., 2010; Sakai et al., 2007) were obtained as described previously (Scheel et al., 2011b). The J6/JFH1 recombinant has been described previously (Lindenbach et al., 2005). The complete NSSA gene was inserted into the HCV core–NS2 recombinants by three-piece fusion-PCR, restriction endonuclease digest and standard cloning procedures. Mutations in adapted core–NS2/NS5A recombinants were introduced by site-directed mutagenesis. The complete HCV sequence of all final plasmid preparations was confirmed (Macrogen).

**Cell culture, transfections and infections.** Huh7.5 human hepatoma cells (a gift from C. M. Rice, Rockefeller University, NY, USA) were handled as described previously (Gottwein et al., 2007). For transfection, RNA was in vitro transcribed using T7 RNA polymerase (Promega) on 10 µg DNA plasmid linearized with XbaI (NEB) and treated with mung bean nuclease (NEB), following the manufacturers’ recommendations. Transfection was performed using 2.5 µg RNA with 5 µl Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) on 4×10^5 cells plated the day before in six-well plates (Nunc). For infection, culture-derived sterile-filtered supernatants, stored at −80 °C, were inoculated onto 4×10^5 cells plated the day before in six-well plates. Infections for co-localization studies were performed at a low m.o.i. (0.01–0.05) on 2×10^4 cells plated the day before in eight-well slides (Nunc).

**Evaluation of infection and viral titres.** In core–NS2/NS5A recombinant development cultures, infected cells were monitored by immunofluorescence for HCV core. Primary antibody was mouse anti-HCV core protein mAb (B2; Anogen) and secondary antibody was Alexa Fluor 594 goat anti-mouse IgG (H+L) (Invitrogen). HCV RNA titres were determined by a TaqMan real-time PCR assay on the 5′ UTR (Gottwein et al., 2007). Infectivity titres were determined by inoculating 100 µl of triplicate sample dilutions on 6×10^4 cells per well plated out the day before on poly-D-lysine-coated 96-well plates (Nunc). At 48 h after infection, cells were fixed and immunostained for HCV protein expression using a previously established protocol (Lindenbach et al., 2005). Primary antibody was HCV NS3 antibody (H23; Abcam), recognizing the NS3 helicase domain. Secondary antibody was ECL anti-mouse IgG HRP-linked whole antibody (GE Healthcare Amersham). Staining was developed using DAB substrate kit (Dako). The number of f.f.u. was determined on an Immunospot Series 5 UV Analyser (CTL Europe) with customized software (Gottwein et al., 2010) or manually by counting under a microscope. For automatic counting, the mean f.f.u. count of six negative control wells was subtracted from f.f.u. counts in experimental wells. Lower limit of quantification was set to 3 s.d above the negative mean, corresponding to 10^5–10^6 f.f.u. ml⁻¹. Counts of up to 200 f.f.u. per well were in the linear range of a test dilution series and were comparable to manual counts.

**Sequence determination of culture-derived HCV.** RNA extraction, reverse transcription-PCR and direct sequence analysis were as
described previously (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008). Primers specific for the NSSA region were as given by Scheel et al. (2011b). All sequencing was performed by Macrogen. Sequence analysis was performed with SEQUENCHER (Gene Codes Corporation). HCV reference sequences were retrieved from the European HCV database and the Los Alamos HCV sequence database.

Immunostaining of HCV proteins for cellular co-localization studies. Cells were washed three times with PBS 48 h post-infection, fixed with 4 % parafomaldehyde and 0.02 % glutaraldehyde for 10 min at room temperature, and washed three times with PBS. Permeabilization, blocking and quenching were performed in a single step by incubating fixed cells with PBS containing 0.5 % Saponin, 10 % goat serum, 3 % BSA, 0.02 % skimmed milk and 0.1 M glycine for 30 min at room temperature. After washing with PBS, primary antibodies diluted in PBS containing 3 % BSA and 0.02 % skimmed milk were added to the cells and incubated at 4 °C for 24 h. NSSA was detected using the clone 9E10 mouse IgG2a mAb (kindly provided by C. M. Rice) at 1:200 dilution; core protein was detected using either the single mouse IgG2a mAb clone 1851 Monotope (Virostat) at 1:100 dilution or a mix of three mouse IgG1 mAbs at a 1:100 dilution: clones 1851 and 1868 Monotope (Virostat) and clone C7-50 (Enzo Life Sciences). Cells were then washed three times with PBS and incubated for 2 h at room temperature with secondary antibodies diluted 1:1000 in PBS containing Hoechst 33258 (Sigma). Single-staining experiments were conducted using Alexa Fluor 488-conjugated goat anti-mIgG antibody (Invitrogen); double-staining was performed using Alexa Fluor 488-conjugated goat anti-mIgG2a and Alexa Fluor 546-conjugated goat anti-mIgG1 antibodies; triple-staining experiments were carried out using BODIPY 505/515 at a 1:500 dilution, and Alexa Fluor 546-conjugated goat anti-mIgG1 and Alexa Fluor 633-conjugated goat anti-mIgG2a. Dual staining of NSSA or core alone was carried out using Alexa Fluor 488 and Alexa Fluor 546 conjugated to goat anti-mIgG1 (for core) or goat anti-mIgG2a (for NSSA). Finally, cells were washed with PBS and mounted using Fluoromount-G (Southern Biotech).

Image acquisition and analysis. Images were acquired avoiding pixel saturation using a Leica TCS SP2 laser scanning microscope, equipped with a Plan-Apochromat × 63 objective (numerical aperture 1.4). To ensure collection of all sample information during acquisition, images were recorded following or exceeding Nyquist sampling parameters, as calculated using the online Nyquist Calculator tool (http://www.svi.nl/NyquistCalculator). Thus, acquisition was performed using × 6 electronic zoom with 1024 × 1024 pixel resolution, resulting in a voxel size of 39 nm for the x and y dimensions and 122 nm for the z dimension. To avoid the occurrence of fluorescence cross-talk, images from each channel were acquired sequentially. For 3D deconvolution, image stacks of 40–50 slices per channel were acquired for each cell. AUTOQUANT software (MediaCybernetics) was used for image alignment and 3D deconvolution. Signal co-localization was evaluated by Pearson’s coefficient using IMARIS software (Bitplane). This coefficient represents the ratio between the channel covariance and the product of their SDs, quantifying the linear correlation of the two channels independent of their intensities. Localization of core on cLDs was evaluated by measuring the percentage of total core signal intensity above a set threshold that co-localized with signal from cLDs above its threshold, using IMARIS software. This value gives a measure of the co-occurrence of the two signals taking into account their intensities, but not their linear correlation. Statistical analyses were performed using Graphpad PRISM software.

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