Natural selection of adaptive mutations in non-structural genes increases trans-encapsidation of hepatitis C virus replicons lacking envelope protein genes

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A trans-packaging system for hepatitis C virus (HCV) replicons lacking envelope glycoproteins was developed. The replicons were efficiently encapsidated into infectious particles after expression in trans of homologous HCV envelope proteins under the control of an adenoviral vector. Interestingly, expression in trans of core or core, p7 and NS2 with envelope proteins did not enhance trans-encapsidation. Expression of heterologous envelope proteins, in the presence or absence of heterologous core, p7 and NS2, did not rescue single-round infectious particle production. To increase the titre of homologous, single-round infectious particles in our system, successive cycles of trans-encapsidation and infection were performed. Four cycles resulted in a 100-fold increase in the yield of particles. Sequence analysis revealed a total of 16 potential adaptive mutations in two independent experiments. Except for a core mutation in one experiment, all the mutations were located in non-structural regions mainly in NS5A (four in domain III and two near the junction with the NS5B gene). Reverse genetics studies suggested that D2437A and S2443T adaptive mutations, which are located at the NS5A-B cleavage site did not affect viral replication, but enhanced the single-round infectious particles assembly only in trans-encapsidation model. In conclusion, our trans-encapsidation system enables the production of HCV single-round infectious particles. This system is adaptable and can positively select variants. The adapted variants promote trans-encapsidation and should constitute a valuable tool in the development of replicon-based HCV vaccines.

INTRODUCTION

Hepatitis C virus (HCV) is an important human pathogen; worldwide, over 170 million people are chronically infected. Indeed, 75–80% of HCV-infected patients develop chronic infection, which may lead to cirrhosis and, ultimately, hepatocellular carcinoma. HCV is a positive-strand RNA virus from the genus Hepacivirus in the family Flaviviridae (Choo et al., 1991). Its RNA genome is 9600 nt long and features two untranslated regions at the 5’ and 3’ ends (5’ UTR and 3’ UTR) (Kato et al., 1990). The genome encodes a large polyprotein (with approximately 3010 aa) that is co- and post-translationally cleaved by cellular and viral proteases to form structural proteins (core, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Tang & Grisé, 2009).

Following the discovery of HCV in 1989, the investigation of viral replication and pathogenesis has notably been hampered by the lack of an appropriate viral culture system (Gottwein & Bukh, 2008). Recently, full-genome replicons capable of producing infectious virus particles in cell culture (HCVcc) have been developed. These include the genotype 2a JFH1 strain and the derived inter- and intragenotypic chimeras (Gottwein et al., 2009; Pietschmann et al., 2006; Wakita et al., 2005). Viral particle release from some of these cell culture systems was initially quite low. However, infectivity titres were improved by successive infections of...
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naı¨ve cells to obtain culture-adapted variants (Delgrange et al., 2007; Kato et al., 2007; Kaul et al., 2007; Russell et al., 2008).

Although the flaviviridae genome is translated in cis, it has been shown for some viruses that some proteins could be complemented in trans. In particular, it was reported that envelope glycoprotein E₃₃ in classical swine fever virus (a pestivirus) can be complemented in trans by an SK6 cellular cell line that constitutively expresses this protein (Widjojoatmodjo et al., 2000). Pigs vaccinated with these defective viral particles were protected against a lethal challenge with the virulent Brescia strain. This observation opened up new perspectives in the development of modified, live-attenuated vaccines. The first trans-complementation studies in HCV used NS5A to rescue lethal mutations in replicons (Appel et al., 2005; Tong & Malcolm, 2006). Following the development of HCVcc, several groups have used trans-complementation to rescue lethal mutations or deletions in core, p7, NS2, NS4B and NS5A proteins (Appel et al., 2008; Brohm et al., 2009; Jirasko et al., 2008; Jones et al., 2009; Miyanari et al., 2007). Furthermore, trans-encapsidation of HCV subgenomic replicon RNA or replicons lacking envelope-encoding genes has been achieved with viral structure proteins (Adair et al., 2009; Ishii et al., 2008; Steinmann et al., 2008).

In the present study, we developed and optimized a trans-encapsidation system based on HCV envelope glycoproteins for the production of single-round infectious particles. To complement replicons lacking E1E2 glycoproteins, we used an adenoviral system to transduce homologous or heterologous envelope proteins expressed alone or in combination with core or core, p7 and NS2. We then increased the yield of viral particles in this trans-encapsidation system by applying successive cycles of trans-encapsidation and infection. Our data agree with previous trans-encapsidation studies of structural proteins and also demonstrate that the trans-encapsidation system is adaptable in cell culture, as replicons or full-length genomes. Adaptation of this trans-encapsidation system enabled us to obtain high titres of single-round infectious particles and should be a useful tool in vaccine development.

RESULTS

Production of single-round infectious viral particles

In order to establish whether HCV JFH1 replicons lacking the E1E2 genes can be complemented in trans, two replicons were used (JFH1-LucΔE1E2 and JFH1-PuroΔE1E2, expressing Renilla luciferase and puromycin acetyltransferase proteins, respectively) (Fig. 1a). Expression of NS3 protein by JFH1-LucΔE1E2 and JFH1-PuroΔE1E2 replicons was confirmed by indirect immunofluorescence (Fig. 1b).

We also built an efficient system for envelope protein expression in trans. Previous work has shown that adenoviral systems can efficiently express proteins in hepatoma cell lines (Huh-7 cells, in particular) (Dimitrova et al., 2003; Eyre et al., 2009). Hence, cDNA encoding the 21 carboxy-terminal residues of core, E1 and E2 from the JFH1 strain was used to generate adenovirus constructs expressing genotype 2a E1E2 (Ad-E1E2-2a). Indirect immunofluorescence (Fig. 1c) and Western blot analysis (Fig. 2b) confirmed high levels of E1E2 expression after the transduction of Huh-7 cells with Ad-E1E2-2a.

Subsequently, Huh-7 cells were electroporated with JFH1-LucΔE1E2 RNA and transduced 24 h later with Ad-E1E2-2a or, as a negative control, empty adenovirus (Ad-control). Non-transduced, full-length JFH1-Luc virus (JFH1-Luc-WT) was used as a positive control. Seventy-two hours post-transduction, supernatants were harvested and infectious virus release was measured, after infection of naive Huh-7 cells, by quantifying luciferase activity, intracellular HCV RNA and f.f.u. (Fig. 1d, e, f, respectively).

According to the luciferase activity results (Fig. 1d), infectious particle production by JFH1-LucΔE1E2 trans-complemented with Ad-E1E2-2a was similar to that seen with JFH1-Luc-WT. Similar results were obtained after quantification of HCV RNA in infected cells using primers in the NS3 region to avoid quantification of the RNA expressed from adenoviruses (Fig. 1e). Surprisingly, whereas measure of luciferase activities suggested that the production of trans-complemented particles was similar to that of authentic virions we observed a 2 log₁₀ difference when measuring infectious titres (Fig. 1f). This may be due to specificity differences between the two assays. As expected, no infectious viral particles were detected after trans-encapsidation with Ad-control.

We also worked with Huh-7 cells stably replicating a JFH1-PuroΔE1E2 genome. We hypothesized that trans-encapsidation would be more efficient under these conditions since the cells replicating ΔE1E2 replicon were selected by puromycin addition and thus all cells transduced contained the replicon. As demonstrated by quantification of intracellular HCV RNA and f.f.u. in cells inoculated with the supernatant from transduced cells, JFH1-PuroΔE1E2 could also be complemented by expression of E1E2 in trans to produce single-round infectious particles. However, the yield of trans-complemented infectious particles was 3 log₁₀ lower than for the JFH1-Puro-WT (Fig. 1f). Likewise, intracellular HCV RNA quantification was 1.6 log₁₀ lower than for the JFH1-Puro-WT (5.32 log₁₀ IU µg⁻¹ and 3.76 log₁₀ IU µg⁻¹ RNA for JFH1-Puro-WT and JFH1-PuroΔE1E2 transduced with Ad-E1E2-2a, respectively; Fig. 1e). Our data show that single-round infectious particles can be produced by complementing ΔE1E2 replicons in trans with envelope proteins expressed from Ad-E1E2-2a.

To confirm that infection by trans-complemented particles obtained from transduced JFH1-PuroΔE1E2 stable cells, depends on the presence of E1E2 at the virion surface, we infected naive Huh-7 cells with the supernatant from transduced cells in the presence of an E2-specific neutralizing
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mAb. As shown in Fig. 1(g), the presence of 30 μg ml⁻¹ of anti-E2 mAb decreased the infectious titre by 88.4%. We did not detect any focus formation at a mAb concentration of 60 μg ml⁻¹. The fact that an E2-specific antibody inhibits the infection of single-round infectious particles strongly suggests that the particles’ entry into Huh-7 cells is mediated by glycoproteins.

Expression of C-E1E2 or C-E1E2-P7-NS2, rather than E1E2 alone, does not enhance the production of single-round infectious particles

Although previous results had shown that replicons and envelope proteins were efficiently expressed, viral particle production for JFH1-ΔE1E2 replicons complemented with Ad-E1E2-2a was relatively low. Hence, we decided to look at whether the expression of other viral proteins in trans could influence the yield of single-round infectious particles. Additional adenoviruses expressing C-E1E2 (Ad-5⁵′-E2-2a) or C-E1E2-P7-NS2 (Ad-5⁵′-NS2-2a) from JFH1 cDNA were produced (Fig. 2a). After transduction of naïve Huh-7 cells with Ad-5⁵′-E2-2a or Ad-5⁵′-NS2-2a, high levels of E2 expression were detected by Western blot analysis (Fig. 2b). Huh-7 cells containing JFH1-LucΔE1E2 or JFH1-PuroΔE1E2 replicons were transduced with these adenoviruses or Ad-control. Seventy-two hours later, supernatants were harvested and single-round infectious particle production was measured, by quantifying luciferase activity, intracellular HCV RNA and f.f.u., after infection of naïve Huh-7 cells. As shown in Fig. 2(c), the production of single-round infectious particles, as measured by the viral titre, was not improved by the expression of core-E2 or core-NS2, rather than E1E2 alone (1.81, 1.30 and 2.22 log₁₀ f.f.u. ml⁻¹), respectively, after trans-encapsulation of the JFH1-LucΔE1E2 replicon; 1.37, 1.13 and...
2a and Ad-E1E2-2a, respectively) or JFH1-Puro-D
JFH1-Luc-D
JFH1-Puro-WT. (c) Huh-7 cells containing JFH1-Puro
dependent on intracellular HCV RNA assays after trans-
complemented particles produced with Ad-5'-E2-2a or Ad-5'-NS2-2a, relative to Ad-
E1E2-2a (Fig S1b). Thus, our results suggest that the
concomitant expression of core or core, p7, NS2 with
E1E2 envelope proteins does not facilitate the trans-
encapsidation of ΔE1E2 replicons.

Trans-encapsidation with heterologous envelope proteins does not product single-round infectious viruses

We continued our investigation of the mechanism of trans-
encapsidation of JFH1-ΔE1E2 replicons by determining
whether single-round infectious particle production could
be restored by the expression of heterologous envelope proteins. To this end, we produced additional adenoviruses: Ad-E1E2-1a, Ad-E1E2-1b, Ad-E1E2-3a and Ad-
E1E2-4 expressing envelope proteins from genotype 1a, 1b, 3a and 4 (the UKN1A2-1, UKN1B2-16, UKN3A-1.28 and UKN4-21.16 strains, respectively) and Ad-5'-NS2-1b expressing C-E1E2-P7-NS2 from genotype 1b (the Lex strain). Importantly, these envelope proteins are known to
be functional, since they enable the production of infectious retroviral pseudoparticles (Lavillette et al., 2005). Expression of E2 by the different constructs was
monitored by Western blot analysis; high expression levels
were detected (Fig. 3a). Huh-7 cells containing JFH1-
LucΔE1E2 or JFH1-PuroΔE1E2 replicons were transduced with these adenoviruses or Ad-control and the production
of trans-complemented infectious particles was assayed as
described above. Luciferase activity values obtained after
inoculation with the supernatant from JFH1-LucΔE1E2
replicating cells transduced with Ad-E1E2-1a, Ad-E1E2-1b, Ad-E1E2-3a and Ad-E1E2-4 were close to those obtained with JFH1-LucΔE1E2 replicating cells transduced with Ad-
control (Fig S2b). Similar results were obtained after
measuring HCV RNA levels in infected cells (Fig S2a). On
the basis of an f.f.u. assay, we did not observe any
production of single-round infectious particles after trans-
encapsidation of JFH1-LucΔE1E2 or JFH1-PuroΔE1E2
(Fig. 3b). This set of results suggests that heterologous envelope proteins cannot trans-complement JFH1-ΔE1E2
replicons. Luciferase activity obtained after trans-encapsidation tests with Ad-5'-NS2-1b was similar to the
background level for the assay (Fig S2b). Furthermore,
results obtained after intracellular HCV RNA quantification
were also similar to control values (Fig S2a). No single-round infectious particles were found in the super-
natants, as shown by the f.f.u. assay results for JFH1-
LucΔE1E2 and JFH1-PuroΔE1E2 (Fig. 3b). Thus, expression in trans of genotype 1b core, p7 and NS2 with E1E2
does not enable the production of single-round infectious particles.
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D3 three times to obtain additional JFH1-Puro dation, infection and selection steps were repeated a further the aforementioned adenoviruses (m.o.i.
Puro http://vir.sgmjournals.org 1001 trans
Fig. 3. (a) Detection of E2 infection with JFH1-Puro
complemented particles produced from JFH1-Puro
trans-
Hence, we decided to perform successive infections with
production and are selected after successive infections.
especially in non-structural proteins, enhance HCVcc
It has been shown that cell culture-adaptive mutations,
particles
expression by Western blot analysis after transduction of Huh-7
cells with Ad-E1E2-1a, Ad-E1E2-1b, Ad-E1E2-3a, Ad-E1E2-4, Ad-5'-NS2-1b or Ad-control. (b) Huh-7 cells containing JFH1-
PuroΔE1E2 or JFH1-LucΔE1E2 replicons were transduced with
the aforementioned adenoviruses (m.o.i. = 10). Supernatants were
harvested 72 h later and placed in contact with naive Huh-7 cells
for 4 h. As a control, naive Huh-7 cells were infected with JFH1-
Puro-WT or JFH1-Luc-WT. f.f.u. (b) assays were performed 72 h
post-infection. Data represent the mean ± SD of three independent
experiments. NT, Non-transduced.

Successive cycles of trans-encapsidation and infection with JFH1-PuroΔE1E2 improve the production of trans-encapsidated infectious particles

It has been shown that cell culture-adaptive mutations, especially in non-structural proteins, enhance HCVcc production and are selected after successive infections. Hence, we decided to perform successive infections with trans-complemented particles. Cells infected with trans-complemented particles produced from JFH1-PuroΔE1E2 (the P0 population) were selected by puromycin addition, in order to obtain a population containing the JFH1-
PuroΔE1E2 replicon (designated P1). These trans-encapsidation, infection and selection steps were repeated a further three times to obtain additional JFH1-PuroΔE1E2 cell populations (designated P2, P3 and P4). We performed two independent experiments (series 1 and 2). Virion production was measured in supernatants collected after transduction with Ad-E1E2-2a by assaying core release. As shown in Fig. 4(a), we observed a gradual increase in core release with each cycle. We observed a 1 log10 increase in core release after trans-encapsulation by the P4 JFH1-
PuroΔE1E2 cell population, relative to the P0 population.

In agreement with these results, we also observed a gradual increase in intracellular HCV RNA after inoculation of naive Huh-7 cells with the supernatants of the transduced populations (Fig. 4b). Most importantly, we observed a 2 log10 increase when comparing viral titres for P4 and P0 (Fig. 4c). This set of results demonstrates that cell culture adaptation of trans-encapsidation systems can improve the yield of single-round infectious particles.

Identification of conserved adaptive mutations selected during successive trans-encapsidation

As is seen with JFH1-derived full-length genomes, we reasoned that the higher yields of trans-complemented particles might be due to the selection of adaptive mutations. We therefore sequenced JFH1-PuroΔE1E2 replicons from populations P0, P1, P2, P3 and P4 in series 1 and 2. The results for series 1 revealed seven conserved mutations in P2 (D21G in core, Q1012R in NS2, T1681S in NS4A as well as L2270T, S2341P, C2432R and D2437A in NS5A) and two additional mutations in P4 (S2443T, Y2475H in NS5B). For series 2, we found seven different, conserved mutations in P3 (W864R in NS5, S1215T and R1373Q in NS3, as well as S2047T, D2254G, D2292E and K2350E in NS5A). Hence, with the exception of D21G in core, all the selected mutations were located in non-structural genes (Fig. 5a). It is noteworthy that eight of the mutations were in NS5A (four in domain III and two near the junction with the NS5B gene; Fig. 5b); this finding suggests that NS5A has an essential role in the trans-encapsidation mechanism.

To characterize these mutations, we first assessed their impact on HCV RNA replication by measuring intracellular HCV RNA in the P0–P4 populations of JFH1-PuroΔE1E2 replicons. As shown in Fig. 5(c), intracellular RNA levels in P0–P4 were similar and did not differ greatly from the value found with JFH1-Puro-WT. This suggests that these mutations do not affect genomic replication.

D2437A and S2443T mutations improved the production of trans-encapsidated virions

Various mutations that enhance infectious virus production were described close to the C terminus of NS5A (Fig. 6a). In contrast, mutations close to the N terminus of NS5B were not known (Han et al., 2009; Jiang & Luo, 2012; Kang et al., 2009; Kaul et al., 2007; Russell et al., 2008; Scheel et al., 2008; Takeda et al., 2012).

In order to examine the role of the two mutations near the NS5A-B cleavage site on HCV RNA replication and particles production, the D2437A and S2443T mutations
Viral production is enhanced by successive trans-encapsidations of JFH1-PuroΔE1E2 by homologous envelope proteins. (a) Huh-7 cells stably expressing JFH1-PuroΔE1E2 were transduced with Ad-E1E2-2a (m.o.i. = 10). Supernatants were harvested 72 h later and placed in contact with naïve Huh-7 cells for 4 h. Puromycin was added 72 h post-infection. A new population (P1) stably expressing JFH1-PuroΔE1E2 was obtained. Trans-encapsidation, infection and selection were repeated three more times to obtain P2, P3 and P4. Two independent experiments were performed (series 1 and 2). These two series of four populations were transduced with Ad-E1E2-2a (m.o.i. = 10). Supernatants were harvested 72 h later and HCV core protein was quantified. (b) Intracellular RNA (b) and f.f.u. (c) assays were performed 72 h post-infection. Data represent the mean ± SD of three independent experiments.

Finally, the mutations were introduced into pJFH1-Puro-WT and stable cells lines replicating JFH1-Puro-D2437A and JFH1-Puro-S2443T mutants were produced. Interestingly, the virus titres obtained were comparable to those of JFH1-Puro-WT, thus demonstrating that the D2437A and S2443T mutations have no impact on infectious virus production of JFH1-Puro-WT in cis (Fig. 6e).

**DISCUSSION**

Trans-encapsidation of HCV genomes lacking at least the E1E2 encoding region has recently been reported by several groups, with expression of the corresponding proteins by helper viruses, transient or stable transfections or baculovirus transduction (Adair *et al.*, 2009; Ishii *et al.*, 2008; Steinmann *et al.*, 2008). Moreover, it has been suggested that this type of defective genome can be found in the blood of infected patients and be trans-encapsidated in vivo into infectious particles by helper WT viruses (Pacini *et al.*, 2009; Sugiyama *et al.*, 2009). Here, we built, characterized and optimized a system for producing single-round infectious particles by trans-encapsidation of JFH1-ΔE1E2 replicons with homologous envelope proteins produced by adenoviral vectors. Importantly, in agreement with previous reports (Adair *et al.*, 2009; Ishii *et al.*, 2008; Steinmann *et al.*, 2008), we demonstrated that these particles, like HCVcc particles, can be neutralized by an anti-E2 neutralizing antibody and are therefore likely to enter target cells in an envelope protein-dependent manner. We observed that production of infectious viral particles after trans-encapsidation of either JFH1-LucΔE1E2 or JFH1-PuroΔE1E2 replicons was 100-fold less efficient than with a full-length genome.
Surprisingly, the use of JFH1-PuroΔE1E2 replicon did not enhance trans-encapsidation whereas, contrary to JFH1-LucΔE1E2, all cells transduced with Ad-E1E2-2a contained the replicon. This finding could be explained by the different structure of the packaged genomes that could be encapsidated with different efficiency.

Interestingly, we observed that the concomitant expression of core or core, p7, NS2 with E1E2 did not facilitate the mechanism of trans-encapsidation. Indeed, transduction of cells replicating ΔE1E2 replicons with Ad-5'-E2-2a or Ad-5'-NS2-2a resulted in lower levels of trans-encapsidation than with Ad-E1E2-2a, despite the likely presence of higher levels of core or core, p7, NS2 expression, respectively. One possible explanation for this observation is that the processing of the polyproteins expressed by Ad-5'-E2-2a and Ad-5'-NS2-2a constructs into functional E1E2 is lower than with Ad-E1E2-2a. Also, Adair et al. observed that expression of NS2 in cis with replicons greatly enhanced replicon transmission to naïve recipient cells, when compared with the provision of NS2 in trans (Adair et al., 2009). Moreover, Pacini et al. observed that the best results were obtained after complementation of a ΔE1E2 replicon with core, E1, E2 and p7 expressed in trans (Pacini et al., 2009). The latter authors highlighted significant cross-talk between core and NS2, which should apparently be expressed by two separate constructs for efficient trans-encapsidation. However, we observed that trans-encapsidation of ΔE1E2 replicons with either Ad-E1E2-2a or Ad-5'-NS2-2a was achievable. One could also imagine that the amounts of core, p7 and NS2 expressed in cis and in trans were far higher than that of NS5A, which plays a key role in HCV assembly and in our trans-encapsidation system (see below).

To investigate whether heterologous trans-encapsidation was possible, we generated adenoviral constructs expressing E1E2 from strains UKN1A2-1, UKN1B2-16, UKN3A-1,28 and UKN4-21,16 (genotypes 1a, 1b, 3a and 4, respectively). We observed that these envelope proteins did not enable the production of infectious viral particles from ΔE1E2 replicons. These data confirm the results obtained by Li et al., who demonstrated that envelope proteins from genotype 2a strains JFH1 and J6 could rescue ΔE1E2 replicons, whereas H77 and Con1 (genotypes 1a and 1b, respectively) envelope proteins could not (Li et al., 2011). In contrast, Steinmann et al. reported that an NS5A-NS5B JFH1 replicon can be rescued by a helper virus encoding Con1-derived genome segments fused with JFH1 at a junction within the NS2 coding region (Steinmann et al., 2008). Overall, these results suggest that, as for intergenotypic chimeras (Morel et al., 2011), trans-encapsidation is possible only when core, E1, E2, p7 and NS2 are from the same genotype. Surprisingly, we did not observe trans-encapsidation after transduction with Ad-5'-NS2-1b. This could mean that the co-expression of heterologous and homologous core, p7 and NS2 proteins may perturb the assembly process.

Similarly to other groups, our infectious titres of trans-complemented viral particles were relatively low. In the literature, several strategies have been used to increase single-round particle infectious titres: (i) subcloning of a packaging cell line stably expressing the structural proteins (Steinmann et al., 2008), (ii) improvement of the transduction efficiency (Adair et al., 2009) and (iii) introduction of adaptive mutations (Adair et al., 2009; Li et al., 2011). In our present study, we took advantage of the ability to select cells infected with trans-complemented particles through expression of the puromycin resistance gene. This allowed us to perform successive cycles of trans-encapsidation and select adaptive mutations that improve the trans-encapsidation efficiency. After four cycles, we observed that several adaptive mutations had been selected and that single-round particle infectious titres were tenfold higher. It is tempting to speculate that additional cycles could select for other mutations and further increase infectious titres still. It is noteworthy that the majority of selected mutations appeared in NS5A; our results suggest that the mutations increase the efficiency of HCV assembly or release. These findings are in agreement with recent work illustrating the key role of NS5A in HCV assembly (Appel et al., 2008; Tellinghuisen et al., 2008) and suggest that NS5A also plays a key role in the trans-encapsidation mechanism. In particular, several mutations were located in NS5A’s domain III, which is known to interact with core protein to promote virion assembly (Masaki et al., 2008). Thus, some of the mutations selected in our study could facilitate the interaction of NS5A with core and enhance the process of trans-encapsidation. Therefore, the two novel mutations D2437A and S2443T identified, which are located at the P6 and P1’ positions, respectively, of the NS5A-B cleavage site were investigated. Various mutations that apparently enhanced the JFH1 production ability, have already be described into the NS5A-B cleavage site, as the reported T2438I (Han et al., 2009) and V2440L (Kaul et al., 2007) mutations. Similarly, we determined that these mutations enhanced infectious particles production by trans-encapsidation, but surprisingly no similar effects were obtained when introduced into the JFH1 genome. These results suggest that the two mutations are specifically implicated into trans- but not cis-encapsidation mechanism.

In summary, we have established a trans-encapsidation system that can be easily adapted to produce high titres of single-round infectious particles. This achievement greatly broadens the scope of previous systems and opens up new perspectives for studying HCV assembly and developing modified, live-attenuated vaccines.

**METHODS**

**Cells and viruses.** Huh-7 human hepatoma and 293A human embryo kidney cells were grown in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% FBS. Recombinant adenoviruses were generated by using the ViraPower
(a) NS2 NS3 4A NS4B NS5A NS5B

W864R  Q1012R  S1215T  T1399S  S2047T  D2254G  D2292E  I2370T  S2341P  C2432R  D2437A

814 1030 1662 1716 1977 2443 3033

(b) S2047T  D2254G  I2370T  D2292E  S2341P  K2350E  C2432R  D2437A

1977 2003 2189 2226 2314 2328 2442

(c) HCV IU per µg total RNA

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Adenoviral Expression System (Invitrogen), according to the manufacturer’s recommendations.

Plasmid constructions. Plasmids encoding JFH1-Luc and JFH1-LucΔE1E2 were derived from pFL-J6/JFH-5-C19Rluc2AUbi (kindly provided by C. M. Rice, The Rockefeller University, New York, USA) and have been described previously (Helle et al., 2010). pJFH1-GND was kindly provided by T. Wakita, National Institute of Infectious Diseases, Tokyo, Japan (Wakita et al., 2005). Plasmids encoding JFH1-Puro and JFH1-PuroΔE1E2 were built after replacement of the luciferase gene by the puromycin resistance gene and the full-length 5′E1E2 open reading frame.

**Fig. 5.** Conserved, adaptive mutations in non-structural genes were selected during successive trans-encapsidation cycles. (a) A schematic drawing of HCV non-structural proteins. Adaptive mutations conserved in the replicon JFH1-PuroΔE1E2 in two independent experiments are given above the drawing. Numbers refer to the amino acid position in the JFH1 polyprotein. Mutations observed in series 1 and 2 are indicated in italic letters and normal letters, respectively. (b) Schematic representation of the mutations located in the NS5A domains. (c) For each of the cell populations, 2.4×10⁴ cells per well were plated into 12-well plates. Twenty-four hours later, intracellular HCV RNA was titrated by qRT-PCR. Data represent the mean ± SD of three independent experiments.

**Fig. 6.** D2437A and S2443T mutations improved the production of trans-encapsidated virions. (a) Listing and positions of mutations described near the junction of NSA and NS5B. Amino acid numbers correspond to those of JFH1 HCV polyprotein. NS5A-B cleavage site is highlighted by a grey box. (b) Replication of mutants, JFH1-LucΔE1E2-WT or JFH1-Luc was measured by Renilla luciferase activity at 24, 48, 72 h post-electroporation. Values are expressed relative to the quantity of luciferase activity measured at 4 h post-electroporation. (c, d) Stable cell lines replicating mutants, JFH1-LucΔE1E2-WT or JFH1-PuroΔE1E2-WT were transduced with Ad-E1E2-2a (m.o.i. = 10). Supernatants were harvested 72 h later and HCV core protein was quantified. Supernatants were placed in contact with naïve Huh-7 cells for 4 h. Luciferase (c) or f.f.u. (d) assays were performed 72 h post-infection. Data represent the mean ± SD of three independent experiments.
the puromycin acetyltransferase gene in pJFH1-Luc and pJFH1-LucΔE1E2, respectively. D2437A and S2443T mutations which are located at the P6 and P1′ positions, respectively, of the NS5A-B cleavage site were introduced into the plasmids by using the QuikChange II XL site-directed mutagenesis kit, according to the manufacturer’s recommendations (Agilent Technologies).

Sequences encoding the 21 carboxy-terminal residues of core with EI-E2 (aa 171–750), 5′ UTR-E2 or 3′ UTR-NS2 from the genotype 2a JFH1 strain (kindly provided by T. Wakita) were amplified by PCR, cloned into the pENTR Directional TOPO vector and transferred into pAdCMV/v5-DEST by gateway recombination. The same strategy was used to construct pAdCMV/v5-DEST encoding E1E2 from genotype 1a, 1b, 3a and 4 (UKN1A2-1, UKN1B2-16, UKN3A-1.28 and UKN4-21.16 strains, respectively, kindly provided by J. K. Ball, University of Nottingham, Nottingham, UK) or core to NS2 from genotype 1b (Lex strain, GenBank accession no. JN120912). The primer sequences are available on request. These constructs were used to generate adenovirus according to the manufacturer’s recommendations (Invitrogen).

In vitro transcription, electroporation and establishment of stable cell lines. HCV RNA were produced by in vitro transcription of JFH1-Puro-WT, JFH1-PuroAE1E2, JFH1-Luc-WT and JFH1-LucΔE1E2 genomes and delivered into HuH-7 cells by electroporation, as described previously (Helle et al., 2010). Electroporated cells were seeded at a density of 10⁵ cells per 25 cm² flask. For the establishment of JFH1-PuroAE1E2 cell populations, puromycin (Invitrogen) was added to DMEM supplemented with 10 % FBS to give a final concentration of 5 μg ml⁻¹.

Trans-encapsidation experiments. One million cells containing JFH1-PuroAE1E2 or JFH1-LucΔE1E2 replicon were seeded into a 25 cm² flask. On the following day, cells were exposed to the adenovirus (m.o.i. of 10) and incubated overnight. Supernatants were recovered 72 h later, centrifuged (1000 g for 5 min) and filtered through a 0.45 μm syringe filter.

Indirect immunofluorescence. Cells were fixed with paraformaldehyde for 10 min at room temperature, permeibilized with 0.5 % Triton X-100 and washed three times with PBS. Next, the cells were incubated with primary antibodies [rat anti-E2 mAb 3/11, kindly provided by J. A. McKeating, or anti-NS3 (Virogen)] for 1 h at room temperature, washed with PBS and then incubated with secondary antibodies [rhodamine red-X-conjugated affinityPure donkey anti-rat IgG (Jackson ImmunoResearch) or FITC-conjugated anti-mouse IgG (Rockland)] for 30–45 min at room temperature.

Western blot analysis. Cells were lysed with PBS and 1 % Triton X-100. Protein content of pre-cleared cell lysates was determined by the BCA method as recommended by the manufacturer (Sigma), using BSA as a standard. Total proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond-ECL; Amersham) and revealed with a specific mAb followed by anti-rat IgG conjugated to peroxidase (Jackson ImmunoResearch). The immune complexes were visualized by enhanced chemiluminescence detection (ECL; Amersham) as recommended by the manufacturer.

HCV core protein quantification. HCV core antigen secreted into the supernatant was quantified by a fully automated chemiluminescent microparticle immunoassay (Architect HCVAg; Abbott), according to the manufacturer’s instructions.

HCV RNA quantification. HuH-7 cells (1.2 × 10⁵) per well in a 12-well plate were inoculated for 4 h with 1 ml of trans-encapsidation supernatant. Cells were incubated overnight and washed with DMEM. Seventy-two hours later, total RNA in cell lysates was extracted using the Qiagen RNeasy kit. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and titrated in a quantitative, real-time RT-PCR assay (qRT-PCR) on an ABI Prism 7900 (Applied Biosystems) using primers within the NS3 region (sequences available on request). Values were normalized against the β-actin-specific signal. For replication analysis, stable cell lines P0, P1, P2, P3, P4 were plated in 12-well plates, 72 h post-plating total RNA was extracted and titrated as previously described.

Measurement of luciferase activity. Cells were inoculated as described for HCV RNA quantification. After 72 h, cell monolayers were lysed by the addition of 200 μl Renilla Luciferase Assay Lysis Buffer (Promega). Luminescence was measured according to the manufacturer’s instruction on a Centro XS LB960 luminometer (Berthold Technologies). For replication analysis, cells were electroporated with various mutants or wild-type RNAs, then were plated in 24-well plates and lysed with 100 μl 1 × Renilla lysis buffer (Promega) at 4, 24, 48 and 72 h post-electroporation. Fifty microliters of cell lysate was used to determine the luciferase activity. Values were normalized relative to those at 4 h.

HCV infectivity quantification. Naive HuH-7 cells were inoculated with 300 μl trans-encapsidation supernatant. Forty-eight hours after inoculation, the infected cells were fixed with paraformaldehyde, permeabilized with 0.5 % Triton X-100, immunostained with anti-NS3 antibody (Virogen) and counted for f.f.u.

Neutralization assay. Neutralization assays were performed by pre-incubating trans-encapsidation supernatants with the 3/11 anti-E2 mAb for 2 h at 37 °C (Helle et al., 2010). The infection efficiency was measured 72 h post-infection, as described above.

Sequencing of adaptive mutations. We determined the sequence of JFH1-PuroΔE1E2 replicons in the different HuH-7 cell populations (P0, P1, P2, P3 and P4) by directly sequencing overlapping PCR fragments spanning the entire ORF. The purified PCR products were directly sequenced with the BigDye Terminator v1.1 kit (Applied Biosystems). Sequences were read on an ABI Prism 310 genetic analyser (Applied Biosystems) and its related Sequence Navigator software. The sequences of primers used for the RT-PCR, PCR and sequencing reactions are available on request.

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REFERENCES


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