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Expression of hepatitis C virus (HCV) structural proteins *in trans* facilitates encapsidation and transmission of HCV subgenomic RNA

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A characteristic of many positive-strand RNA viruses is that, whilst replication of the viral genome is dependent on the expression of the majority of non-structural proteins *in cis*, virus particle formation can occur when most or all of the structural proteins are co-expressed *in trans*. Making use of a recently identified hepatitis C virus (HCV) isolate (JFH1) that can be propagated in tissue culture, this study sought to establish whether this is also the case for hepaciviruses. Stable cell lines containing one of two bicistronic replicons derived from the JFH1 isolate were generated that expressed non-structural proteins NS3–5B or NS2–5B. Release and transmission of these replicons to naïve Huh7 cells could then be demonstrated when baculovirus transduction was used to express the HCV proteins absent from the subgenomic replicons. Transmission could be blocked by a neutralizing antibody targeted at the E2 envelope protein, consistent with this phenomenon occurring via *trans*-encapsidation of replicon RNA into virus-like particles. Transmission was also dependent on expression of NS2, which was most effective at promoting virus particle formation when expressed *in cis* on the replicon RNA compared with *in trans* via baculovirus delivery. Density gradient analysis of the particles revealed the presence of a broad infectious peak between 1.06 and 1.11 g ml^{-1}, comparable to that seen when propagating full-length virus in tissue culture. In summary, the *trans*-encapsidation system described offers a complementary and safer approach to study HCV particle formation and transmission in tissue culture.

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INTRODUCTION

Current estimates are that 170 million individuals are persistently infected with hepatitis C virus (HCV) throughout the world. Although initially many HCV-positive individuals will exhibit few clinical signs of infection, a significant health burden is associated with long-term chronic virus infection, with recent estimates suggesting that there is a three-fold increased risk of death above background levels, primarily due to liver disease, suicide and possibly cardiovascular complications (Guiltinan *et al.*, 2008). Therapeutic intervention is available, but therapies are poorly tolerated and only partially effective (Deutsch & Hadziyannis, 2008). Furthermore, a vaccine for HCV is not available, and whilst it may be possible to develop one, our current understanding of how the immune system eliminates this virus suggests that such a vaccine would need to stimulate a cell-mediated immune response against a broad range of major histocompatibility complex class I and II epitopes (Day *et al.*, 2003; Lauer *et al.*, 2004). Therefore, there is a need for further research into various aspects of the HCV life cycle, both in terms of identification of novel targets for therapeutic intervention and to establish how the virus is able to circumvent both adaptive and innate immunity.

HCV is a positive-strand, enveloped RNA virus belonging to the family *Flaviviridae* and possesses a 9.6 kb genome containing a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). Translation of this ORF is directed by an internal ribosome entry site (IRES) found within the 5′ UTR, resulting in the production of a single polypeptide that is cleaved by host and viral proteases into structural and non-structural proteins, respectively (reviewed by Suzuki *et al.*, 2007). Historically, HCV research has been restricted by the inability to cultivate the virus effectively *in vitro*. However, two major advances have helped to alleviate this problem. The first of these was the development of the HCV replicon, a bicistronic HCV subgenomic RNA construct

lacking structural proteins, but expressing neomycin phosphotransferase to allow selection of cells capable of maintaining the replicon in culture (Lohmann *et al.*, 1999). This enabled identification of mutations in the HCV genome that promoted replication of HCV transcripts in tissue culture cells (Blight *et al.*, 2000; Krieger *et al.*, 2001; Lohmann *et al.*, 2001). However, these same mutations did not facilitate production of infectious particles (Pietschmann *et al.*, 2002) and were shown to attenuate virus infectivity *in vivo* (Bukh *et al.*, 2002). More recently, an HCV genotype 2a isolate (JFH1) has been identified, which is infectious *in vivo* but is also capable of replicating and producing virus particles in cell culture (HCVcc) without the need for culture adaptation (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). Importantly, replicons derived from JFH1 demonstrate robust replication in tissue culture without the requirement for culture-adaptive mutations (Date *et al.*, 2004; Kato *et al.*, 2003). We speculated that it should be possible to develop a *trans*-encapsidation system for HCV using a replicon-based system, given that it has been successfully reported for other members of the family *Flaviviridae* (Gehrke *et al.*, 2003; Jones *et al.*, 2005; Khromykh *et al.*, 1998), but that it would require the use of JFH1 constructs to avoid utilization of culture-adapted mutations. Our findings are consistent with this hypothesis and demonstrate that *trans*-encapsidation of the HCV genome can be achieved under laboratory conditions. Viral *trans*-encapsidation systems can be useful vaccine-delivery systems (Anraku *et al.*, 2002; Pushko *et al.*, 1997; Zhou *et al.*, 1994) and, with further development, HCV *trans*-encapsidation systems may provide novel ways of generating protective immune responses against infectious virus.

METHODS

Cells and viruses. Huh7 cells (a gift from Professor R. Bartenschlager, Department of Molecular Virology, University of Heidelberg, Germany) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine, 1 × non-essential amino acids, 25 mM HEPES, 50 U penicillin (Invitrogen) and 50 μg streptomycin ml^{-1} (Invitrogen). Sf9 cells were maintained in TC100 with 10 % FCS, 50 U penicillin and 50 μg streptomycin ml^{-1} and used to isolate, amplify and titrate baculovirus clones by using standard procedures. Recombinant baculovirus was generated by using the Bac-to-Bac system (Invitrogen) according to the manufacturer's recommendations. Concentrated baculovirus stocks were obtained by clarification of the virus supernatant using a 0.45 μm filter and centrifugation (26 000 r.p.m. in a Beckman SS28 rotor for 1 h at 4 °C), and were resuspended in PBS. For transduction experiments, cells containing replicons were seeded 20–24 h in advance at a cell density of 2.0×10^4 cells cm^{-2}. Unless otherwise stated, cells were then incubated with 4×10^7 p.f.u. baculovirus ml^{-1} for 4 h and allowed to recover for between 24 and 72 h before recovering the supernatant, which was filtered through a 0.45 μm syringe filter and assessed for the presence of *trans*-encapsidated replicon.

DNA constructs. pSGR-JFH1(GND), pSGR-JFH1 [referred to as pSGR-JFH1(NS3–5B) for the purposes of this study] and pJFH1 were gifts from Professor T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). To generate pSGR-JFH1(NS2–5B), the *Kpn*I–*Nsi*I fragment from pSGR-JFH1(NS3–5B) was first excised and replaced with the *Kpn*I–*Nsi*I fragment from pJFH1 to produce a construct lacking a portion of both the encephalomyocarditis virus (EMCV) IRES and NS2. The resulting plasmid was then linearized with *Kpn*I to allow insertion of the remaining EMCV IRES/NS2 sequence, the DNA for which was generated by a two-step PCR. The first round of this PCR utilized primer pairs EMCV(fwd) (5′-GCGTATTCAACAAGGGGCTGAAG-3′) and JFH_EMCV(revfus) (5′-GTGCGTCATACATGGTATTATCGTGTTTTTCAAAGG-3′), and JFH_NS2(fwdfus) (5′-GATAATACCATGTATGACGCACCTGTGCACGG-3′) and JFH_NS2(rev) (5′-CCTGCATGGGTGGTACCCACTC-3′), respectively, and the second round used primers EMCV(fwd) and JFH_NS2(rev). In order to generate the baculovirus constructs expressing JFH1 structural proteins, it was first necessary to create a Bac-to-Bac transfer vector for expression of genes under the control of a hybrid constitutive immediate-early human cytomegalovirus enhancer/chicken β-actin (CA) promoter. To do this, the *Sph*I (polished with *Pfu*I)–*Hin*dIII fragment from pBACMAM-2 (Novagen) was transferred to the pFB(*Xba*I–*Hin*dIII) vector (McCormick *et al.*, 2006) cut with *Xba*I (polished with *Pfu*I) and *Hin*dIII, generating pFBM. DNAs encoding the regions core–P7 and core–NS2 from pJFH1 were amplified by PCR using primer pairs CORE(JFH_RI) (5′-GAGAGAGAATTCGCCACCATGAGCACAAATCCTAAACC-3′) and P7(JFH_RI) (5′-GAGAGAGAATTCTTAGGCATAAGCCTGCCGGGGCAG-3′), and CORE(JFH_RI) and NS2(JFHRIrev) (5′-GAGAGAGAATTCTTAAAGGAGCTTCCACCCCTTGGAG-3′), respectively, and cloned into pFBM by using *Eco*RI to generate pFBM(JFH1)C–P7 and pFBM(JFH1)C–NS2. To produce pFBM-derived structural protein expression constructs containing the F172C and P173S mutations at the COOH end of the core, DNAs encoding the core region and the E1–P7 region were amplified by using primer pairs CORE(JFH_RI) and CORESPMOD (5′-CAGGGCCAGCAAGAAGATAGAAAAGCTGCAGCCGGGTAGGTTCCCTGTTGC-3′), and SPE1JFH(fwd) (5′-GAGAGAGAATTCGCCACCATGGGCTGCAGCTTTTCTATCTTCTTGCTGG-3′) and P7(JFH_RI). The PCR product encoding the E1–P7 region was cloned into pFBM by using *Eco*RI, generating pFBM(JFH1)E1–P7*, or both PCR products were digested with *Pst*I + *Eco*RI and cloned into *Eco*RI-cut pFBM as a three-way ligation, generating pFBM(JFH1)C–P7*. All FBM baculovirus constructs were derived from the respective pFBM vectors described above. The sequences of vectors are available on request.

Colony-forming assays. To produce RNA for transfection into cells, 5 μg each replicon-containing DNA construct was linearized with *Xba*I, polished with mung bean nuclease (NEB) and used as a template in a 50 μl T7 RNA polymerase reaction following the manufacturer's recommendations (NEB), but performed at 30 °C for 2 h using 2 mM each rNTP. RQ1 DNase (Promega) was then used to remove the DNA template, and the transcripts were purified by using an RNA Clean-up kit 25 (Zymo Research). The integrity of the transcripts was confirmed by MOPS/formaldehyde gel electrophoresis before electroporating 1 μg each transcript into Huh7 cells and performing a colony-forming assay. Details of this are essentially as described previously (McCormick *et al.*, 2004) except that, during the 2-week selection period, the concentration of G418 (Melford Laboratories) was reduced from 750 μg ml^{-1} (first feed) to 500 μg ml^{-1} (second feed) and finally to 250 μg ml^{-1} (third and fourth feeds).

To detect *trans*-encapsidated replicons, naïve Huh7 cells were seeded at 2×10^4 cells cm^{-2} in six-well dishes and allowed to recover overnight. The next day, the medium was replaced with 2 ml neat supernatant from *trans*-encapsidation experiments or gradient fractions (0.5 ml diluted with 1.5 ml medium) and the cells were left for a further 24 h before initiating selection with G418. The concentration and timing of the G418 selection schedule was the same

as that described above. Antibodies used in neutralization experiments were the anti-E2 monoclonal antibody (mAb) AP33 (Clayton *et al.*, 2002) and the anti-herpes simplex virus VP5 mAb DM165 (a gift from Dr F. Rixon, MRC Virology Unit, Glasgow, UK).

Equilibrium gradients. The low concentration of infectious particles containing replicon RNA in the supernatant necessitated the adoption of a modified protocol to assess particle density, similar to that described by others (Lindenbach *et al.*, 2006; Stone, 1974). Briefly, a 6.3 ml solution of a 2 : 1 mix of iodixanol : *trans*-encapsidated supernatant was placed in a 14 × 95 mm Beckman ultracentrifuge tube and neat *trans*-encapsidated supernatant was layered on top such that all air was excluded from the tube before sealing it with Parafilm. The tube was then left in a horizontal position at 4 °C for 24 h before being returned to a vertical position. The first 500 µl of the gradient was removed and discarded, and the remaining contents were centrifuged at 90 000 ***g*** for 24 h at 4 °C in a Beckman SW40 rotor. Fractions (1 ml) were harvested manually from the top of the gradient and the density of each was assessed by weighing. Colony-forming assays were performed by using 500 µl of each fraction and the RNA from a further 200 µl was harvested by using Total RNA Isolation reagent (ABgene) for analysis by quantitative RT-PCR (qRT-PCR).

Western blot analysis. Cells were lysed in RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 % (v/v) NP-40, 0.5 % (w/v) sodium deoxycholate, 0.1 % SDS] supplemented with 2 × Complete protease inhibitor cocktail (Roche), and the protein concentration of samples was determined by using BCA reagent (Pierce). Samples standardized by protein content (typically 5–10 µg per well) were separated by SDS-PAGE and transferred to PVDF membrane (Millipore). Membranes were blocked with 5 % (w/v) low-fat dried milk, 0.1 % Tween 20 (Merck) in Tris-buffered saline and incubated with sheep anti-NS3 sera or anti-NS5A (a gift from Dr M. Harris, Institute of Molecular and Cellular Biology, University of Leeds, UK), murine anti-E2 mAb ALP98 (Clayton *et al.*, 2002) or murine anti-core mAb 0126 (Biogenesis). Bound antibody was detected with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma) in conjunction with ECL reagent (Amersham Pharmacia Biotech), and light emissions were captured by film or using a Versadoc MP4000 imaging system (Bio-Rad).

qRT-PCR. For real-time analysis (qRT-PCR) of RNA after density-gradient ultracentrifugation, an absolute quantification reaction was performed. First, a cDNA standard was generated by reverse transcription of quantified JFH-1 RNA, made previously by *in vitro* transcription. Reverse transcription of standard RNA and unknown sample RNAs was performed by using TaqMan Reverse Transcription reagents (Applied Biosystems) with random primers. Serially diluted standard cDNAs and unknown sample cDNAs were analysed in triplicate by real-time PCR using TaqMan Fast Universal PCR Master Mix and No ampErase UNG (Applied Biosystems), with final probe and primer concentrations of 250 and 900 nM, respectively. The probe sequence was 5′-6-FAM-AAAGGCCTTGTGGTACTG-MGB-3′ (synthesized by Applied Biosystems), and primer sequences were 5′-TCTGCGGAACCGGTGAGTAC-3′ (forward) and 5′-GCACTC-GCAAGCACCCTATC-3′ (reverse) (Sigma). Real-time reactions were amplified under Fast Universal conditions on a 7500 Fast Real-Time PCR machine and data were analysed by using 7500 Fast System Software (SDS v. 1.3.1) (Applied Biosystems).

RESULTS

Establishment of donor cell replicon cell lines

Expression of NS2 is known to be essential for effective virus particle formation (Jones *et al.*, 2007; Pietschmann *et al.*, 2006; Yi *et al.*, 2007). Whilst there is no absolute requirement for NS2 to be expressed *in cis* with either p7 or NS3 (Jones *et al.*, 2007), it remained possible that the context within which it was expressed could still influence virus particle formation. For this reason, two separate JFH1-based subgenomic replicon constructs were used. The first was SGR-JFH1(NS3–5B), a bicistronic construct originally described by Wakita and colleagues (Kato *et al.*, 2003) that utilizes the HCV IRES and EMCV IRES to enable expression of neomycin phosphotransferase and the proteins NS3–NS5B, respectively (Fig. 1a). The second construct, SGR-JFH1(NS2–5B), was essentially identical to this construct except that NS2–NS5B was expressed from the latter cistron. To determine the effectiveness of both constructs at establishing stable replicon cell lines, T7 polymerase-derived RNA transcripts were transfected into Huh7 cells and a colony assay was performed. The results were consistent with the ability of both replicons to effectively establish replicon-containing cell lines, as their transfection led to the formation of G418-resistant colonies not observed in cells transfected with the polymerase knockout control replicon transcript, although approximately two-fold more colonies were seen in cells transfected with SGR-JFH1(NS2–5B) compared with those transfected with SGR-JFH1(NS3–5B) (Fig. 1b). The resulting polyclonal cell lines derived from these transfections were also subjected to Western blot analysis for the presence of NS3 and NS5A, which confirmed the presence of the replicons (Fig. 1c).

Provision of structural proteins *in trans* by using baculovirus

Having established JFH1-based subgenomic replicon cell lines, it was necessary to generate an efficient delivery system for expression of the remaining HCV proteins. Previous work has demonstrated the effectiveness of a baculovirus delivery system using a mammalian promoter for expression of HCV proteins in hepatocyte-derived cell lines (Fipaldini *et al.*, 1999; McCormick *et al.*, 2002). For this reason, cDNAs representing part of the JFH1 ORF encoding the core protein through to p7 and the core through to NS2 were cloned into a baculovirus transfer vector that was subsequently used to generate baculovirus constructs FBM(JFH1)C–P7 and FBM(JFH1)C–NS2 (Fig. 2a). Both baculoviruses promoted effective expression of core and E2 following their transduction into naïve Huh7 cells, although there were routinely higher levels of structural protein expression in cells transduced with FBM(JFH1)C–P7 (Fig. 2b). Similar results were obtained in cells harbouring the JFH1-based replicons. Furthermore, immunoprecipitation of E2 from cell lysates of Huh7 cells transduced with FBM(JFH1)C–P7 and FBM(JFH1)C–NS2 was found to co-precipitate a comparable amount of E1, consistent with appropriate E1–E2 heterodimer formation occurring when using either of these two constructs (data not shown).

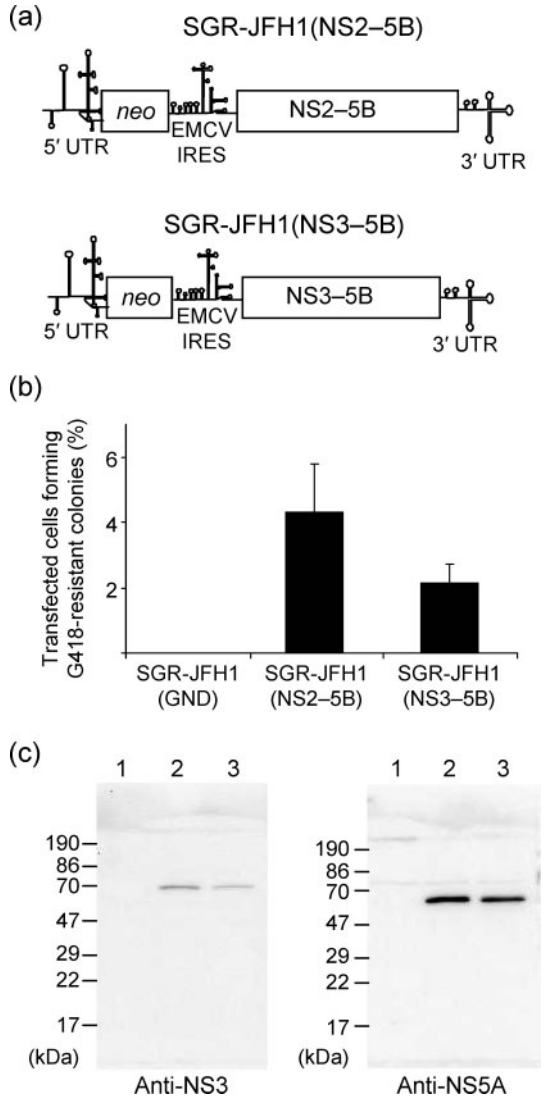

Fig. 1. Establishment of subgenomic JFH1-based replicon cell lines. (a) A schematic diagram of the two separate bicistronic replicon constructs used to generate the replicon-containing cell lines SGR-JFH1(NS2–5B) and SGR-JFH1(NS3–5B). The neomycin phosphotransferase gene is indicated by *neo*. (b) Results of three separate colony-forming assays performed after transfection of the replicon transcripts into Huh7 cells (mean ± SEM). (c) Western blot analysis was used to confirm expression of NS3 and NS5A in replicon polyclonal cell lines. Lanes: 1, naïve Huh7; 2, SGR-JFH1(NS2–5B); 3, SGR-JFH1(NS3–5B).

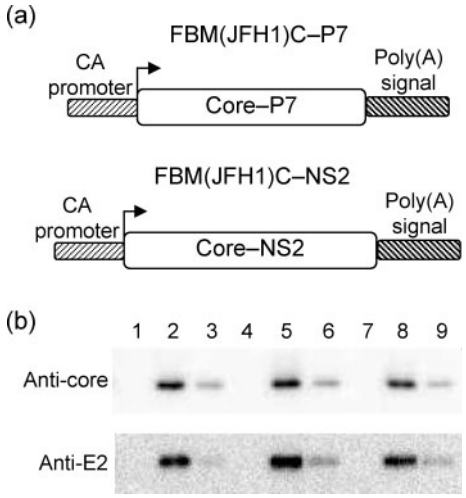

Fig. 2. Characterization of baculovirus constructs for expression of JFH1 structural proteins. (a) Schematic diagram of the baculovirus constructs FBM(JFH1)C–P7 and FBM(JFH1)C–NS2. (b) Western blot analysis was used to examine core and E2 expression in cell lines that were either mock-transduced (lanes 1, 4 and 7), transduced with FBM(JFH1)C–P7 (lanes 2, 5 and 8) or transduced with FBM(JFH1)C–NS2 (lanes 3, 6 and 9), using naïve Huh7 cells (lanes 1–3), SGR-JFH1(NS3–5B) (lanes 4–6) and SGR-JFH1(NS2–5B) replicon cell lines.

Trans-encapsidation and transmission of replicons to naïve Huh7 cells

To test whether *trans*-encapsidation of subgenomic replicons and subsequent particle assembly and secretion can occur when the virus structural proteins are supplied *in trans*, cell lines SGR-JFH1(NS2–5B) and SGRJFH1(NS3–5B) were transduced with the recombinant baculoviruses. Following incubation for 24 h, the medium from these cells was assessed for the presence of particles carrying *trans*-encapsidated replicon RNA by using a colony-formation assay (Fig. 3a). Consistent with the need for structural protein expression for virus particle formation, the supernatant from mock-transduced SGR-JFH1(NS2–5B) and SGR-JFH1(NS3–5B) cell lines did not facilitate the transmission of G418 resistance to naïve Huh7 recipient cells. Similarly, culture medium from naïve Huh7 donor cells transduced with FBM(JFH1)C–P7 and FBM(JFH1)C–NS2 did not confer G418 resistance to the recipient cells, ruling out any possibility that baculovirus transduction itself might modulate resistance to the antibiotic. However, when baculovirus transduction was used to promote HCV structural protein expression in SGR-JFH1(NS2–5B) and SGR-JFH1(NS3–5B) cell lines, a small but reproducible number of G418 colonies was observed. More importantly, there were significant differences in the number of colonies seen between these different experimental groups. The virus-like particles formed from FBM(JFH1)C–P7- or FBM(JFH1)C–NS2-transduced SGR-JFH1(NS2–5B) cells demonstrated the most efficient transfer to recipient Huh7 cells, with the former showing a slightly higher titre, a finding that may well relate to the overall level of structural protein expression from these two baculovirus constructs (Fig. 3a). In contrast, the number of virus-like particles in the medium of the transduced SGR-JFH1(NS3–5B) replicon cell line was significantly lower. Moreover, in the experimental group where the donor SGR-JFH1(NS3–5B) was transduced with FBM(JFH1)C–P7, only a single G418-resistant colony was observed from four separate

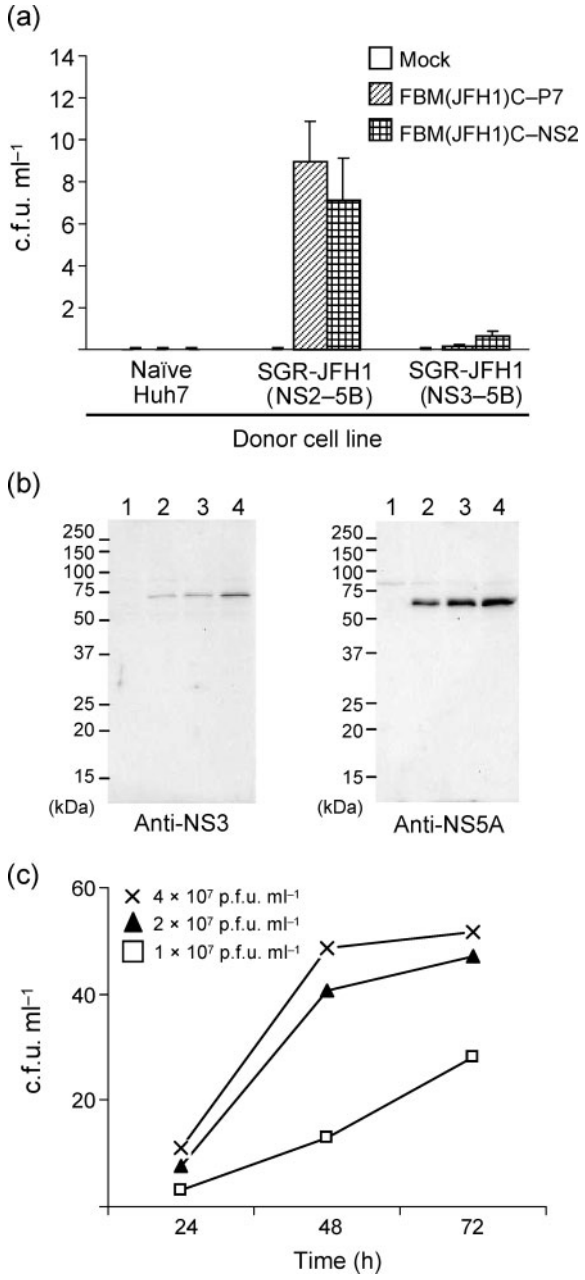

Fig. 3. Transmission of replicons from donor cells to recipient cells by expression of structural proteins. (a) Naïve Huh7 cells, SGR-JFH1(NS2–5B) and SGR-JFH1(NS3–5B) cell lines were either mock-transduced or transduced with 4×10^7 p.f.u. FBM(JFH1)C–P7 or FBM(JFH1)C–NS2 ml^{-1}. The supernatant was harvested 24 h later, placed on naïve recipient Huh7 cells and a colony-forming assay was performed. Data represent the mean ± SEM of four separate experiments. (b) G418-resistant polyclonal cells derived from these transmission experiments were analysed by Western blotting using anti-NS3 and NS5A antisera. Experimental groups included cells derived from SGR-JFH1(NS3–5B) transduced with FBM(JFH1)C–NS2 (lane 2), SGR-JFH1(NS2–5B) transduced with FBM(JFH1)C-P7 (lane 3) and SGR-JFH1(NS2–5B) transduced with FBM(JFH1)C–NS2 (lane 4). A cell lysate from mock-transduced cells was used as a negative control (lane 1). (c) Use of a colony-formation assay to assess transmission of SGR-JFH1(NS2–5B) to naïve Huh7 cells after transduction with 1, 2 or 4×10^7 p.f.u. FBM(JFH1)C–P7 ml^{-1} and harvesting the supernatant at a 24, 48 or 72 h time point. Results represent single data points from one of three comparable experiments.

experiments. This represents a level of transmission approximately 70-fold lower than that observed when the same baculovirus construct was used to transduce the SGR-JFH1(NS2–5B) donor cell line, supporting the proposition that replicon transmission is occurring through authentic virus particle formation, as NS2 is known to be required for efficient virus production and was absent in the former experimental group. Furthermore, this difference cannot be accounted for purely by variation in replicative capacities of the two replicons, because when SGR-JFH1(NS3–5B) was instead transduced with FBM(JFH1)C–NS2, a five-fold greater replicon transmission level was observed, despite the fact that this construct is less effective at facilitating expression of structural proteins.

In order to rule out the possibility that any mechanism other than transfer of replicon to recipient cells was occurring in these experiments, polyclonal cell lines derived from G418-resistant colonies were also examined for non-structural protein expression. Western blot analysis demonstrated the presence of both NS3 and NS5A, confirming that *trans*-encapsidation and transfer of the HCV replicon to these recipient cells had occurred (Fig. 3b).

Optimizing replicon transmission

The above experiments demonstrated that transduction of the SGR-JFH1(NS2–5B) cell line with FBM(JFH1)C–P7 facilitated the greatest number of replicon transmission events. However the overall numbers of colonies observed were low. For this reason, the SGR-JFH1(NS2–5B) cell line was transduced with different titres of FBM(JFH1)C–P7 and allowed to recover for varying periods of time between 24 and 72 h before the presence of encapsidated replicons was assessed. Colony-formation results indicated clearly that the highest titre of baculovirus used, which was the same as that used in the earlier experiments (4×10^7 p.f.u. ml^{-1}), facilitated the greatest levels of replicon transmission to naïve cells, although the next lowest titre examined (2×10^7 p.f.u. ml^{-1}) was nearly as effective (Fig. 3c). Lower levels of replicon transmission were observed when transducing with a titre of 1×10^7 p.f.u. FBM(JFH1)C–P7 ml^{-1}, despite the fact that structural protein expression could still clearly be detected in the donor cell line by Western blotting (data not shown). The release of the *trans*-encapsidated replicon also varied significantly over time. Low levels of replicon transmission were detected if the supernatant from the transduced SGR-JFH1(NS2–5B) cells was harvested 24 h post-transduction, but this increased significantly at 48 and 72 h, such that a five- to

ten-fold increase was observed. Interestingly, this time delay in release of *trans*-encapsidated replicons has also been reported to be a feature of JFH1 virus particles generated by using the HCVcc system (Pietschmann *et al.*, 2006). Although not necessarily linked, there was also an association between what appeared to be structural protein-induced cytopathic effect and release of *trans*-encapsidated replicons from the donor SGR-JFH1(NS2–5B) cell line, with extensive cytopathic effect being observed using 2×10^7 and 4×10^7 p.f.u. FBM(JFH1)C–P7 ml^{-1} after a 48 h period (data not shown).

Characterization of virus particles

It was clear that expression of structural proteins in the replicon-containing cells facilitated *trans*-encapsidation of the replicon, and that the characteristics of this phenomenon paralleled those reported for HCVcc. However, in order to establish further that *trans*-encaspidated HCV replicons represented an appropriate system to study virus transmission, it was necessary to demonstrate that these putative virus particles displayed antigenic and physical properties consistent with those observed for HCVcc.

To confirm that the infection of naïve cells by the replicon-containing particles was occurring via receptor-mediated cell entry, the ability of a broadly neutralizing mouse mAb, AP33 (Owsianka *et al.*, 2005), to block transmission was assessed. Medium collected from the SGR-JFH1(NS2–5B) cell line transduced with FBM-JFH1(C–P7) was pre-incubated for 1 h with AP33 or with DM165 (an irrelevant IgG1 subtype control) prior to performing a colony-forming assay. As shown in Table 1, only AP33 was found to reduce transmission of SGR-JFH1(NS2–5B), thus confirming that cell entry of replicon-containing particles was occurring via the interaction between HCV E2 and its receptor(s), analogous to that observed for HCVcc as well as retrovirus particles pseudotyped with HCV glycoproteins (Owsianka *et al.*, 2005; Tarr *et al.*, 2006).

We next determined the biophysical properties of the particles present in the medium collected from the SGR-JFH1(NS2–5B) cell line 72 h post-transduction by isopycnic centrifugation. Fractions collected from the gradient were tested for replicon RNA by qRT-PCR and assessed for the presence of infectious particles by a colony-forming assay (Fig. 4). Replicon RNA showed marked differences in distribution, although two distinct peaks of $\leqslant$1.03 and 1.13 g ml^{-1} were observed. Interestingly, the most infectious peak did not correlate with the peaks in HCV RNA level, but was much broader and distributed between 1.06 and 1.11 g ml^{-1}, similar to the density of infectious HCVcc (Lindenbach *et al.*, 2005; Yi *et al.*, 2007; Zhong *et al.*, 2005). One striking feature of these results was the high ratio of replicon transcripts to infectious particles in the supernatant; the sum of the data for the gradient analysis indicated that there were $4.9\pm1.1\times10^5$ genome equivalents per c.f.u. in the unfractioned supernatant (mean $\pm$ SEM).

Table 1. Inhibition of *trans*-encapsidated replicon infection of Huh7 cells by mAbs

Medium from the SGR-JFH1(NS2–5B) cell line transduced with FBM(JFH1)C–P7 was pre-incubated for 1 h with AP33 (a blocking anti-E2 mAb) or DM165 (an irrelevant IgG1 isotype control) and a colony-forming assay was performed. Values are shown as c.f.u. ml^{-1} and the percentage of the control (in parentheses), and represent the mean $\pm$ SD from triplicate readings generated from two separate experiments.

[mAb]	No antibody	AP33	DM165
2 μg ml^{-1}	112 $\pm$ 8	44 $\pm$ 3 (39 $\pm$ 3 %)	110 $\pm$ 8 (98 $\pm$ 7 %)
4 μg ml^{-1}	121 $\pm$ 13	21 $\pm$ 4 (17 $\pm$ 3 %)	121 $\pm$ 5 (100 $\pm$ 4 %)

A targeted mutation at the end of the core protein increases replicon transmission

It has been proposed that inefficient particle assembly and release, characteristic of the JFH1 strain of HCV, are, in part, related to two amino acids (Phe-172 and Pro-173) near the C terminus of the core protein and within the E1 signal peptide region (Delgrange *et al.*, 2007). These residues differ substantially from Cys and Ser as seen in the core consensus sequence. To establish whether these amino acids might also limit *trans*-encapsidation efficiency, a cDNA representing part of the JFH1 ORF encoding the core through to p7, but containing the mutations F172C and P173S, was used to generate the baculovirus construct FBM(JFH1)C–P7*. A control construct, FBM(JFH1)E1–P7*, expressing E1–P7 and which included the E1 signal peptide within the core sequence, was also generated. Western blot analysis of the donor SGR-JFH1(NS2–5B) cell line transduced with FBM(JFH1)C–P7, FBM(JFH1)C–P7* or FBM(JFH1)E1–P7* demonstrated that comparable levels of E2 expression were achieved by using all three constructs and confirmed that core expression was restricted to the first two of these constructs (Fig. 5a). Transmission of the SGR-JFH1(NS2–5B) replicon was only observed in experimental groups where baculovirus constructs expressing all of the structural proteins were used to transduce the donor cell line, confirming further that the transmission observed was due to genuine virus particle formation (Fig. 5b). More importantly, approximately fourfold more G418-resistant colonies were observed after Huh7 cells were incubated with supernatant from the FBM(JFH1)C–P7*-transduced donor cell line compared with the FBM(JFH1)C–P7-transduced donor cell line. Therefore, the original core-encoding region of JFH1 is not only suboptimal for full-length virus production, but also appears to limit replicon *trans*-encapsidation.

DISCUSSION

Identification of the JFH1 isolate and its use in the development of a cell-culture system have greatly facilitated

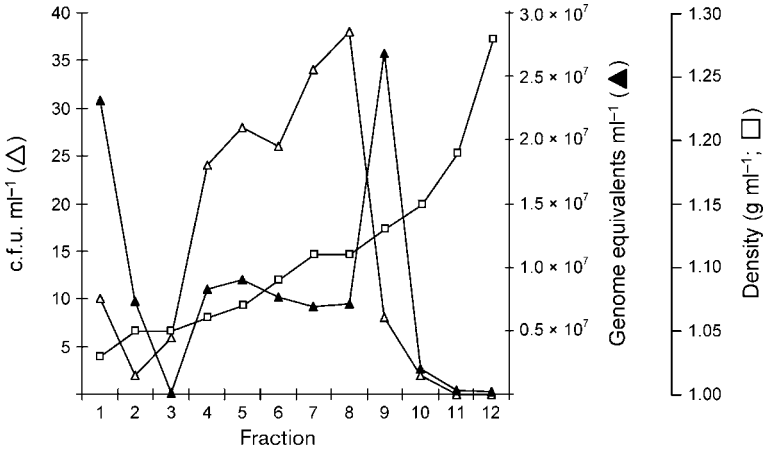

Fig. 4. Equilibrium centrifugation of the *trans*-encapsidated replicon using an iodixanol gradient. Cell supernatant from SGR-JFH1(NS2–5B) cells transduced with 4×10^7 p.f.u. FBM(JFH1)C–P7 ml^{-1} was harvested 72 h post-transduction and subjected to equilibrium centrifugation. Fractions from the gradient were assessed for density (□), replicon content (by qRT-PCR) (▲) and infectivity by G418 colony assay (△).

the study of virus particle morphogenesis and cell entry. We now report a further adaptation of the HCVcc system, namely the packaging of subgenomic replicons into infectious virus-like particles through provision of structural proteins *in trans*. During the preparation of this manuscript, two other groups also published evidence that subgenomic replicons could be packaged when structural proteins were expressed *in trans* (Ishii *et al.*, 2008; Steinmann *et al.*, 2008). Our work provides further support for this finding. The availability of a *trans*-encapsidation system for HCV is likely to provide a useful complement to other systems for studying virus particle formation and entry.

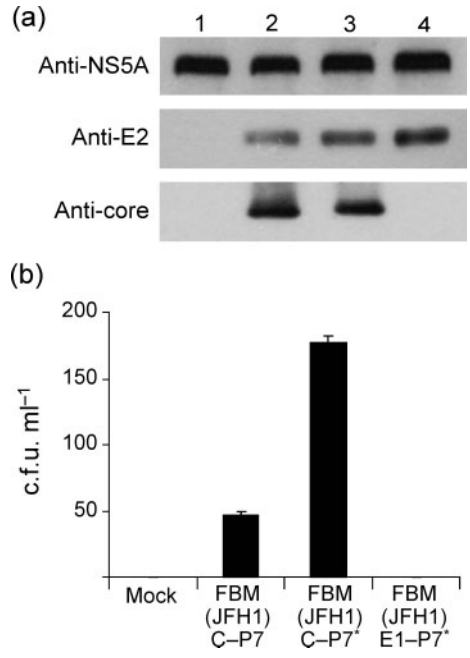

Fig. 5. Improved replicon transmission to donor cells using a modified JFH1 core sequence. (a) Western blotting was used to assess expression of HCV viral proteins in the SGR-JFH1(NS2–5B) donor cell line 20 h after mock transduction (lane 1) or transduction with FBM(JFH1)C–P7 (lane 2), FBM(JFH1)C–P7* (lane 3) or FBM(JFH1)E1–P7* (lane 4). (b) Supernatants from SGR-JFH1(NS2–5B) donor cells transduced with the same constructs were harvested 48 h post-transduction and assessed for infectivity by a G418 colony assay. Results represent mean ± SD from one of two separate experiments.

For *trans*-encapsidation to be an effective system for studying HCV biology, transmission of the *trans*-encapsidated replicon must occur through a process that mimics that seen for HCVcc. In this study, we found that (i) *trans*-encapsidation of the replicon showed a dependence on the presence of NS2, (ii) *trans*-expression of the envelope proteins in the absence of capsid failed to produce transmissible particles, (iii) the release of virus particles was maximal approximately 48 h after baculovirus transduction, (iv) an anti-E2 antibody neutralized the *trans*-encapsidated particle and (v) these infectious particles had a peak buoyant density of between 1.06 and 1.11 g ml^{-1}. All of these features are similar to those reported for replication of JFH1, the full-length construct from which all constructs used in this study were derived. Many of these observations also closely parallel those reported in the two recent HCV *trans*-encapsidation studies. However, one novel finding was that expression of NS2 *in cis* with the replicon-containing construct greatly enhanced replicon transmission to naïve recipient cells, compared with its provision *in trans*. The lack of a suitable antibody has prevented us from assessing NS2 expression directly in replicon-containing and baculovirus-transduced cells. However, it seems unlikely that NS2 expression levels are the limiting factor for *trans*-encapsidation of the replicon. Instead, as *trans*-encapsidation of SGR-JFH1(NS2–5B) correlated with the amount of FBM(JFH1)C–P7 used to transduce the cell line, it seems more likely that the structural proteins are the limiting factor for replicon packaging. Indeed, transduction of SGR-JFH1(NS2–5B) with FBM(JFH1)C–NS2 resulted in reduced levels of *trans*-encapsidation compared with transduction with FBM(JFH1)C–P7, despite the fact that levels of NS2

expression would have been greater. Therefore, two alternative explanations remain that may account for this apparent preference for expression of NS2 *in cis* in order to achieve *trans*-encapsidation. As SGR-JFH1(NS2–5B) replicates slightly more effectively than SGR-JFH1(NS3–5B), it is possible that these differences in the *trans*-encapsidation assay simply reflect the ability of the replicons to establish themselves once they have infected naïve Huh7 cells. Indeed, Steinmann *et al.* (2008) also observed that *trans*-encapsidation efficiency correlated at least partly with replication efficiency of the HCV transcript being packaged. However, a possible problem with this explanation is that there is only a two-fold difference in replicative capacity between SGR-JFH1(NS3–5B) and SGR-JFH1(NS2–5B) when electroporated into Huh7 cells, but there is a 10-fold difference in transmission efficiencies of these two replicons when structural proteins are provided *in trans* by FBM(JFH1)C–NS2. The other explanation is that NS2 expression *in cis* with other NS proteins in a replicating HCV transcript positions NS2 within a particular subcellular compartment to allow it to interact more effectively with host and viral components necessary for virus particle formation. This has been shown for NS5A, which has to be expressed *in cis* with other NS proteins in order to undergo appropriate post-translational modification (Koch & Bartenschlager, 1999; Neddermann *et al.*, 1999) and gain access to the replication complex (Appel *et al.*, 2005). Interactions between NS5A and the core protein are then thought to facilitate interactions between the replication complex and lipid droplets where particle assembly occurs (Appel *et al.*, 2008; Masaki *et al.*, 2008; Miyanari *et al.*, 2007). However, unlike the situation for NS5A, if this latter model is correct, then there is no obligatory requirement for NS2 to be expressed *in cis*, simply a preference for it. Further work is needed to establish exactly how *trans*-encapsidation might be affected by the context in which NS2 is expressed.

Trans-encapsidation in this study was achieved via transduction with baculoviruses expressing the HCV structural proteins, rather than through the generation of a packaging cell line or co-transfection with a helper virus as performed by others (Ishii *et al.*, 2008; Steinmann *et al.*, 2008). Unlike the use of helper virus, the baculovirus system also allows production of *trans*-encapsidated material that is free from other HCV replication-competent RNAs, thus simplifying data interpretation. This, coupled with the fact that baculovirus constructs expressing HCV structural proteins can be generated much more rapidly than stable cell lines, means that, for locations where an appropriate biocontainment facility for HCVcc work is unavailable, our system is likely to be more tractable for use in reverse-genetics studies of virus particle formation and entry. Indeed, we now have preliminary data showing that *trans*-encapsidation of transient luciferase-expressing replicons occurs after transduction with baculovirus expressing structural proteins (data not shown). Thus, the baculovirus-based system can achieve *trans*-encapsidation of subgenomic replicons without the use of helper virus or the need to establish either a stable packaging cell line or a replicon cell line. Furthermore, baculoviruses expressing structural proteins derived from diverse HCV genotypes and subtypes would allow generation of pseudotyped VLPs, thus facilitating studies on the effects of genetic variation on virus particle assembly and entry. We have not tested whether expression of structural proteins by transient transfection of plasmid might also allow *trans*-encapsidation of replicons. However, given the typically low efficiency with which plasmid transfection occurs into Huh7 cells, coupled with our observation that high levels of structural protein expression are necessary to achieve efficient packaging, it is likely that levels of *trans*-encapsidation would be much lower and possibly below a detectable threshold.

The maximum level of replicon *trans*-encapsidation that we observed when using the original JFH1 structural coding region was approximately 10^2 c.f.u. ml^{-1}, although there was some variability between experiments. This maximum level is comparable with the levels of *trans*-encapsidation observed by Ishii *et al.* (2008), who also used JFH1-based constructs, but is in marked contrast to the very high level of replicon transcripts released into the supernatant, suggesting that most of these transcripts are released either as extracellular membrane-associated RNAs or as defective virus particles, a feature of HCVcc and even more so of the HCV *trans*-encapsidation systems. Steinmann *et al.* (2008) found that the use of structural proteins from the J6 genotype 2b infectious clones, combined with the use of a chimaeric NS2 sequence, improved the production of infectious particles by several log orders of magnitude. We have also found that titres of infectious particles can be increased by changing the structural ORF, although in our study this was achieved by targeted mutations within the core-encoding region rather than by use of a chimaeric construct. We anticipate that further modifications, perhaps comparable to those used by Steinmann *et al.* (2008), could be used to improve baculovirus-based *trans*-encapsidation further.

In conclusion, we have developed a robust system that permits experimental separation of HCV replication and packaging functions, and which can be used without the need for stringent biocontainment. This system complements the existing HCVcc and retrovirus-based surrogate HCV pseudoparticle systems as a valuable tool for the study of HCV virus particle assembly and entry.

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