A hepatitis C virus (HCV) NS3/4A protease-dependent strategy for the identification and purification of HCV-infected cells

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As a tool for the identification and/or purification of hepatitis C virus (HCV)-infected cells, a chimeric form of the Gal4VP16 transcription factor was engineered to be activated only in the presence of the HCV NS3/4A protease and to induce different reporter genes [choramphenical acetyltransferase (CAT), green fluorescent protein (GFP) and the cell-surface marker H-2Kk] through the (Gal4)5-E1b promoter. For this, the NS5A/5B trans-cleavage motif of HCV of genotype 1a was inserted between Gal4VP16 and the N terminus of the endoplasmic reticulum (ER)-resident protein PERK, and it was demonstrated that it could be cleaved specifically by NS3/4A.

Accordingly, transient transfection in tetracycline-inducible UHCV-11 cells expressing the HCV polyprotein of genotype 1a revealed the migration of the Gal4VP16 moiety of the chimera from the ER to the nucleus upon HCV expression. Activation of the chimera provoked specific gene induction, as shown by CAT assay, first in UHCV-11 cells and then in Huh-7 cells expressing an HCV replicon of genotype 1b (Huh-7 Rep). In addition, the GFP reporter gene allowed rapid fluorescence monitoring of HCV expression in the Huh-7 Rep cells. Finally, the chimera was introduced into Huh-7.5 cells infected with cell culture-generated HCV JFH1 (genotype 2a), allowing the purification of the HCV-infected cells by immunomagnetic cell sorting using H-2Kk as gene reporter. In conclusion, the Gal4VP16 chimera activation system can be used for the rapid identification and purification of HCV-infected cells.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus that infects 3% of the world’s population, with development of chronic hepatitis in 60–90% of infected individuals, occurrence of cirrhosis in 0–5–30% of cases and hepatocellular carcinoma at a rate of 1–3% per year (Zoulim et al., 2003). The HCV genome (approx. 9.6 kb) encodes a single polyprotein precursor that undergoes a complex proteolytic cleavage to generate its structural (C, E1, E2) and non-structural (NS2–NS3–NS4A–NS4B–NS5A–NS5B) proteins (reviewed by Penin, 2003).

The functional organization of the HCV genome, as well as insights on its interaction with its cellular host, were determined progressively through cloning of virus strains with known infectious capacity (Kolykhalov et al., 1997; Yanagi et al., 1997), establishment of cell lines or transgenic mouse models expressing some or all of the HCV proteins (Lerat et al., 2002; Moradpour et al., 1998), three-dimensional protein analysis (Bressanelli et al., 1999; Yan et al., 1998), replicative viral models expressing part or all of...
the HCV genome (Bartosch et al., 2003; Blanchard et al., 2002; Buonocore et al., 2002; Matsuura et al., 2001) and construction of viral replicons of genotype 1b (Blight et al., 2000; Guo et al., 2001; Ikeda et al., 2002; Lohmann et al., 1999; Pietschmann et al., 2002), 1a (Bligh et al., 2003; Yi & Lemon, 2004) and 2a (Kato et al., 2003). A major breakthrough arose recently with the production of infectious particles of HCV genotype 2a from cell cultures (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

In vivo, HCV replicates well in the liver, its cellular host, as shown by high viral-load titres in sera from infected patients (10^8 RNA equivalents ml^-1; Terrault et al., 1997). In contrast, it infects primary cultures of human hepatocytes poorly, with limited virus propagation (Fournier et al., 1998; Rumin et al., 1999).

With the aim to identify and purify HCV-infected cells, including HCV-infected human hepatocytes, we developed a strategy based on the ability of the viral NS3 protease and its cofactor NS4A to cleave the majority of the HCV nonstructural proteins (NS4A–NS4B–NS5A–NS5B) in trans, including HCV-infected human hepatocytes, we developed a strategy based on the ability of the viral NS3 protease and its cofactor NS4A to cleave the majority of the HCV nonstructural proteins (NS4A–NS4B–NS5A–NS5B) in trans, in addition to cleavage activity in cis (NS3–NS4A). The HCV motifs cleaved in trans contain an acidic residue at P6, a Cys residue at position P1 and a small residue, such as Ser, Ala, Gly or Asn, at position P1' (Beyer et al., 2001). The presence of NS4A increased the stability of the substrate-acceptor site downstream of the cleavage site (Landro et al., 1997; Yan et al., 1998).

We used the NS3/4A trans-cleavage activity on the NS5A/NS5B domain of genotype 1a to engineer a transcription factor that will be released only upon HCV expression and we monitored its activity through the specific induction of different reporter genes. While our study was in progress (Meurs et al., 2003), another group reported a similar approach in which the NS3/4A-cleaved product was designed to locate at the cell surface (Paci et al., 2004). Our approach allows an amplification of the signal through the released transcription factor and enhanced detection of HCV-infected cells, as well as the possibility to vary the nature of the reporter genes.

We show here that the NS3/4A-mediated trans-activation assay can be used to monitor HCV expression in different cell lines. Importantly, with the use of a cell-surface marker as a reporter gene, we demonstrate the possibility to purify HCV-infected cells. This procedure opens the possibility to study the interaction of HCV with its natural cell host, the hepatocyte, as well as with other cells permissive for HCV infection.

**METHODS**

**Plasmids.** The plasmid pG5CAT contains five consensus Gal4-binding sites (Gal4[12mers x 5]) and an adenovirus E1b minimal promoter upstream of the choramphenical acetyltransferase (CAT) gene. The plasmid pM3VP16 expresses a fusion of the DNA-binding domain of the yeast Gal4 gene to the activation domain of the herpesvirus transcription factor VP16. Both were purchased from Clontech (Matchmaker system). The plasmid pMACS K^+ I contains the gene encoding a truncated, trypsin-resistant mouse major histocompatibility complex class I molecule, H-2K^k, and was purchased from Miltenyi Biotec. The defective lentiviral vectors HR'TRIPAU3 CMV GFP and H'RTRIPAU3 EF1αGFP were as described previously (Zennou et al., 2000).

All oligonucleotides used for the construction of plasmids described below are listed in Supplementary Table S1, available in JGV Online.

**pcDNA3/AMP chimera S or R.** (i) The 1733 bp N terminus of human PERK was copied by PCR from pcDNA3/PERK (a gift from R. Wek, Indianapolis, IN, USA) in the presence of 5% DMSO with the primers S1 and AS1. After subcloning into cCR2.1 TOPo, it was inserted into pcDNA3/AMP (Invitrogen). (ii) For the chimera S and R constructs, the 739 bp Gal4VP16 sequence was copied by PCR from pM3VP16 (Clontech) using primers S2 and AS2,3 (chimera S) or S3 and AS2,3 (chimera R), subcloned and transferred to pcDNA3/AMP(Nter–PERK) by using EcoRV. The plasmids pcDNA3/AMP(chimera S or R) could only be recovered from bacteria growing at 30°C.

**pcDNA3.1(T7-NS3), pcDNA3.1(T7-NS3/4A) and pcDNA3.1(T7-NS3/4AS139A).** The NS3 and NS3A4A fragments were removed from HR'TRIPAU3-NS3 and H'RTRIPAU3(CMV)-NS3/4A (see below), respectively, and inserted into pcDNA3.1/Hygro(+)T7 (a gift from D. Poncet, CNRS-INRA, Gil-sur-Yvette, France) after digestion by KpnI/BamHI. The S139A mutation was introduced by directed mutagenesis (QuikChange kit; Stratagene) by using oligonucleotides described by Foy et al. (2005).

**HR'TRIPAU3(Gal4)_2-E1b-H-2K^k.** The 159 bp promoter Gal4[S6mers x 5]E1b, or (Gal4)_2-E1b for short, was copied by PCR from pG5CAT with primers S4 and AS4, subcloned into pCR2.1 TOPo, cut with Nhel/AgeI and transferred to pMACS K^+ II deleted of its H-2K^k promoter with Nhel/AgeI. The 1246 bp (Gal4)_2-E1b-H-2K^k fragment was then excised by digestion with Smal/KpnI. HR'TRIPAU3 EF1αGFP was cut with MluI/KpnI to remove the EF1α promoter and green fluorescent protein (GFP). This vector was then ligated with the adapter O1/O2 and referred to as HR'TRIPAU3 ‘Adapter’. It was cut with Smal/KpnI to insert (Gal4)_2-E1b-H-2K^k.

**H'RTRIPAU3(CMV) adapter.** This plasmid was obtained by removing the GFP sequence from H'RTRIPAU3(CMV)GFP with BamHI/Xhol and inserting the adapter O3/O4.

**H'RTRIPAU3(CMV)-chimera S or R.** The entire sequence for chimera S or R was copied by PCR from pcDNA3/AMP (chimera) with the primers S5 and AS5 as described for PERK. After subcloning, the 2.5 kb cDNAs were cut with BglII and inserted into the compatible BamHI site of H'RTRIPAU3(CMV).

**H'RTRIPAU3(CMV)-SMSYGal4VP16.** This plasmid was designed to encode the Gal4VP16 moiety of the chimera plus the SMSY residues at its N terminus, in order to mimic exactly the sequence of this transcription factor when it is cleaved from the chimera by NS3/4A. The Gal4VP16 sequence was copied by PCR from pM3VP16 using the primers S6 and AS6. After subcloning, the SMSYGal4VP16 fragment was cut with BglII/Sall and inserted in H'RTRIPAU3(CMV) cut with BamHI/Xhol.

**H'RTRIPAU3(CMV)-PERKVCC.** This plasmid was designed to encode the PERK moiety of the chimera plus the VVCC residues at its C terminus, in order to mimic exactly the sequence of the
endoplasmic reticulum (ER)-retained part of the chimera after cleavage by NS3/4A. The PERK sequence was copied by PCR from pcDNA3/AMP chimera (chimera) using the primers S7 and AS7. After subcloning, the fragment was cut with BglII/Sall and inserted into HR'TRIP(3CMV) cut with BamHI/XhoI.

**HR'TRIPAU3(CMV)-NS3 and HR'TRIPAU3(CMV)-NS3/4A.** The sequence for NS3 and NS4A was amplified with primers S8 and AS8 or S9 and AS9 from a vaccinia virus vector expressing HCV 1a NS52–5B (C. Wychowski, unpublished data). After subcloning, NS3 was cut with BamHI/KpnI and inserted into HR'TRIPAU3(CMV). NS4A was cut with Smal/KpnI and inserted into HR'TRIPAU3(CMV-NS3).

**HR'TRIPAU3(CMV-H-2k**). The H-2k coding sequence was excised from pMACS K2.11 after Agel linearization, Klenow filling and KpnI digestion. After purification, it was inserted into HR'TRIPAU3(CMV) previously digested by Smal/KpnI.

**HR'TRIPAU3(Gal4-E1b-GFP).** The 159 bp (Gal4)-E1b promoter was amplified from pG5CAT with the primers S10 and AS10, subcloned, cut with MluI/BamHI and inserted into pTRIP-EF1z-GFP cut with the same enzymes to replace the EF1z promoter.

**Cell cultures.** The tetracycline (Tet)-regulated UHCV-11 cell line was cultured as described previously (François et al., 2000; Moradpour et al., 1998). Human embryonic kidney (HEK) 293T cells, the Huh-7 cell line, the Huh-7.5 cell line (Bligh et al., 2002; Lindenbach et al., 2005) and Huh-7 Rep cells (Breiman et al., 2005) were cultured as described previously (Breiman et al., 2005).

**Virus.** To prepare the JFH1 virus, Huh-7.5 cells were electroporated with an RNA transcribed from the plasmid JFH1 encoding HCV genotype 2a (Wakita et al., 2005; Zhong et al., 2005), grown for two successive passages (approx. 20 days) and their supernatants were applied to naïve Huh-7.5 cells. The infected cells were cultivated for two passages, their supernatant was applied to other naïve Huh-7.5 cells and the virus supernatant was collected after two passages. Two different virus stocks were prepared, which titrated 2.3 ± 10^6 and 3.1 ± 10^6 genome equivalents ml^-1, as measured by quantitative RT-PCR (qRT-PCR).

**Production of lentiviral vectors.** Lentiviral vectors were produced by a three-plasmid cotransfection of HEK 293T cells (140 cm^2 plates; 10 ± 10^6 cells plates) as described previously (Naldini et al., 1996; Zennou et al., 2000), using 10 μg of the envelope-encoding plasmid pSVG, 20 μg of the packaging plasmid pCMVΔR8.91 (obtained from D. Trono, University of Geneva, Switzerland) and 20 μg of the HR'TRIPAU3-based vectors containing the different transgenes. After 48 h, supernatants were applied to 5 ml 20% sucrose/TSE buffer [10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0-1 mM PMSF], concentrated by ultracentrifugation (25,000 r.p.m. for 90 min), resuspended in TSE, aliquotted and stored at −80° C. The titres were determined with an HIV-1 p24 ELISA kit (Perkin Elmer).

**Immunoblot analysis.** Transfection and preparation of cytoplasmic extracts of HEK 293T cells were done as described previously (Breiman et al., 2005). Preparation of nuclear and cytoplasmic extracts was as described by Kanazawa et al. (2004). Identical amounts of protein from each sample were loaded onto 12.5% SDS/acrylamide gels and analysed by immunoblotting (Breiman et al., 2005).

**Transfection and CAT assay.** UHCV-11 cells were transfected by using the calcium phosphate precipitation/glycerol shock technique as described previously (Bonnet et al., 2000). Huh-7 Rep cells and Huh-7.5 cells were transfected preferentially with Lipofectamine 2000 (Invitrogen) as described previously (Breiman et al., 2005). Twenty-four hours after transfection, the cells were washed three times in cold PBS, lysed in 250 μl lysis buffer (CAT ELISA; Roche Molecular Biochemicals), centrifuged (12,000 g; 2 min) and processed for CAT assay according to the manufacturer's instructions.

**Immunomagnetic separation.** Cells expressing the H-2k reporter gene were isolated by trypsin treatment, resuspended in phosphate buffer/2 mM EDTA (PBE) containing 10% heat-inactivated fetal bovine serum, washed once and resuspended in 2 ml of this buffer. They were then incubated with gentle agitation for 40 min at 4 °C with 50 μl anti-H-2k magnetic beads (MACSelect H-2k, Miltenyi Biotec). After centrifugation for 7 min at 1200 r.p.m., the cells/beads mix was washed once in 2 ml PBE, resuspended in this buffer, the cell number was counted and the H-2k-positive cells were selected by the POSell program on an AUTOMACS apparatus (Miltenyi Biotec). The percentage of H-2k-positive cells was calculated as the ratio of the number of cells present in the positive fraction to the total number of cells.

**Real-time RT-PCR analysis.** Total cellular RNA was extracted by acid/guanidinium thiocyanate/phenol/chloroform using RNAlab (Eurobio) according to the manufacturer's instructions. The sets of HCV primers were synthesized as described previously (Castet et al., 2002). For normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers were designed by using the LightCycler Probe Design software (Roche) by choosing primers on each side of an intron (human GAPDH, GenBanck accession no. J04038; primers S12 and AS12; Supplementary Table S1, available in JGV Online). The CDNA-synthesis step was performed on 1 μg total RNA as described previously (Castet et al., 2002), using rTh polymerase (Applied Biosystems) and one of the antisense primers (AS11 or HCV 38–56). For each cDNA synthesis, GAPDH reverse transcription was performed in the same tube. The cDNA was then purified with a High Pure PCR Product purification kit (Roche Applied Science) in a final volume of 50 μl. PCR amplification was performed at an annealing temperature of 65 °C with 2–5 μl purified cDNA in a 10 μl reaction mixture containing 1 μl LightCycler FastStart DNA Master SYBR green kit (Roche), 0.5 μM each primer and 4 mM MgCl2. qPCR was performed on a LightCycler apparatus using SYBR Green primers KY 78 and KY 80, as described previously (Castet et al., 2002). Standard curves were established by using tenfold serial dilutions of the GAPDH plasmid or of HCV synthetic minus-strand RNAs. The measured amounts of each mRNA were normalized to the amounts of GAPDH mRNA. To demonstrate the specificity of the detection of HCV negative-strand RNA, serial dilutions of positive- and negative-strand HCV RNA transcripts corresponding to the 5' non-coding region of HCV were reverse-transcribed in the presence of the HCV 38–56 sense primer (Fournier et al., 1998) and qRT-PCR was performed as described above.

**RESULTS**

**Engineering of an NS3/4A-sensitive transcription factor**

The Gal4VP16 transfection factor was engineered to be retained at the ER through the sequence GADTE^DVVCC^S^MY, which represents the NS3/4A trans-cleavage motif between NS5A and NS5B of HCV of genotype 1a (Yanagi et al., 1997) and is equally sensitive to NS3/4A of genotype 1a, 1b (Beyer et al., 2001). It is also sensitive to NS3/4A of genotype 2a (see below). The ER-anchoring site was provided by the N terminus and the
membrane-spanning domain of PERK, a naturally ER-localized protein (Harding et al., 1999; Shi et al., 1998). Gal4VP16 is therefore located close to NS3/4A, itself anchored to the ER through NS4A (De Francesco et al., 2003). Two chimeras have been obtained, one sensitive to NS3/4A (chimera S) and one insensitive (chimera R), where Cys at P1 and Ser at P1′ were substituted for Ala and Arg (Fig. 1a). As Gal4VP16-inducible reporter genes, we chose CAT, GFP or the cell-surface protein H-2Kk and placed them under the control of the (Gal4)5-E1b promoter (Fig. 1b).

Specific cleavage of the chimera by NS3/4A and migration of Gal4VP16 to the nucleus

HEK 293T cells were transfected with the chimera constructs in the absence or presence of NS3/4A, either wild type (wt) or catalytically inactive (NS3/4A SA) (Foy et al., 2005), and analysed for association of the Gal4VP16 transcription factor with the nuclear fractions. The results show that Gal4VP16 was specifically expressed above background levels in the extract expressing chimera S in the presence of NS3/4A wt, thus indicating specific cleavage of the chimera. However, efficiency of cleavage was low compared with expression of Gal4VP16 alone (Fig. 2a).

Expression of chimera S or R was then assayed by immunofluorescence after transfection into the Tet-inducible UHCV-11 cell line (Moradpour et al., 1998). As controls, cells were cotransfected with plasmids expressing each half of the chimera (N-terPERKVVCC and SMSYGal4VP16) to generate conditions where both parts of the chimera are expressed fully. The results (Fig. 2b) show a nuclear localization of the Gal4VP16 transcription factor whether the cells were induced (Tet−) or not (Tet+), as expected, because its expression is independent of HCV. In the absence of HCV expression (Tet+), both chimeras S and R are expressed in the cytoplasm at the ER level. Induction of the HCV proteins did not affect chimera R localization, but provoked a more disorganized distribution of chimera S in the cytoplasm, with some nuclear localization. These results show that the chimera constructs are correct in terms of cellular localization and that some of the Gal4VP16 moiety of chimera S can migrate to the nucleus in response to HCV expression.

Specific induction of reporter genes by the NS3/4A-sensitive chimera

We then analysed whether the amount of Gal4VP16 released from chimera S was sufficient for gene trans-activation. HEK 293T cells were cotransfected with plasmids expressing

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**Fig. 1.** Schematic representation of the chimera and of its activation by HCV NS3/4A. (a) The Gal4VP16 transfection factor is fused to the ER-resident protein PERK through the central part of the NS5A/5B motif sensitive to the protease action of NS3/4A (chimera S) or to the same motif in which the P1 Cys and P1′ Ser residues have been substituted by Ala and Arg (chimera R). S, Signal peptide; TM, transmembrane domain. (b) Different reporter genes (CAT, GFP or a trypsin-resistant version of the murine H-2Kk gene) have been placed under the control of the Gal4VP16-inducible promoter (Gal4)5-E1b. Upon expression of NS3/4A, the Gal4VP16 moiety of the chimera is cleaved and migrates to the nucleus, where it can induce the expression of reporter genes. Expression of CAT is determined by ELISA and that of GFP by fluorescence and immunoblot. Expression of H-2Kk at the cell surface was used for immunomagnetic cell sorting of HCV-expressing cells.
the Gal4VP16-inducible GFP reporter gene with either Gal4VP16, chimera S or chimera R and NS3/4A or NS3. The results show GFP induction only upon chimera S and NS3/4A coexpression (Fig. 3a). NS3 alone was inactive or barely active, confirming that its full protease activity requires association with NS4A (Bartenschlager, 1999). Thus, Gal4VP16 can be cleaved specifically from the chimera by NS3/4A to promote gene expression. The GFP induction increased with NS3/4A concentration to reach expression levels similar to those induced by free Gal4VP16. Therefore, despite a limited cleavage efficiency, chimera S can release enough Gal4VP16 to provoke full gene trans-activation.

Fig. 2. Specific cleavage of Gal4VP16 from the chimera by NS3/4A, expressed alone or in the context of the HCV polyprotein of genotype 1a. (a) HEK 293T cells were transfected with pM3VP16 (1-5 μg) or vectors expressing chimera S (0.4 μg) or chimera R (0.4 μg) in the absence or presence of vectors expressing either NS3/4A wt (4 μg) or NS3/4A S139A mutant (SA; 4 μg). Three hours after the beginning of transfection, 5 μM lactacystine was added to prevent protein degradation. After 24 h, nuclear extracts were prepared for analysis of Gal4VP16, either as such or after cleavage from the chimera, whilst cytoplasmic extracts were used for the analysis of NS3/4A and actin. An immunoblot was performed using polyclonal antibodies directed against VP16 (1/1000; Clontech) for the detection of Gal4VP16, against T7 (1/5000; Novagen) for the detection of the T7-tagged NS3/4A constructs and a mAb against actin (1/1500; Sigma) for loading control. (b) UHCV-11 cells were seeded in eight-chamber slides (Lab-tek) at a density of 20 000 per well in the presence of 1 μg Tet ml⁻¹. After 24 h, the cells were washed four times with PBS to remove Tet and then incubated in the presence (Tet⁺) or absence (Tet⁻) of Tet for another 24 h. Three series of transfection were performed: in the first series, the cells were cotransfected with 1 μg HR'TRIPΔU3(CMV)-SMSYGal4VP16 and 1 μg HR'TRIPΔU3(CMV)-PERKVVCC in order to mimic conditions when both parts of the chimera are expressed after cleavage (top); in the second and third series, cells were transfected either with 1 μg pcDNA3/AMP/chimera R (middle) or with 1 μg pcDNA3/AMP/chimera S (bottom). After 24 h, the cells were processed for immunostaining with mAb anti-Gal4 (1/200; GV-3-As; Euromedex) (Texas red) and with polyclonal anti-NS3 antibodies (1/100; Roche) (fluorescein isothiocyanate). Analysis was performed by confocal microscopy with × 40 magnification (Leica).
We noticed that codetection of NS3/4A and the chimera was localization at the ER, as in the Tet and S revealed either homogeneous or disorganized localization at the ER, as in the (Tet and S) chimera constructs. Next, we assayed chimera activity in cells expressing a full-length HCV expression (Fig. 3b). Induction increased as a function of the concentration of chimera S, with a maximum observed at 60% of the control. This demonstrates that, when expressed in the presence of all of the HCV proteins, NS3/4A cleaves sufficient Gal4VP16 from the chimera to generate important signal amplification.

Specific activation of the NS3/4A-sensitive chimera in a cell line expressing a full-length HCV replicon

The activity of the chimera was then assayed in the presence of all of the HCV proteins, using the Tet-inducible UHCV-11 cell system and CAT reporter gene. The results showed specific induction of CAT by chimera S in cells induced for HCV expression (Fig. 3b). Induction increased as a function of the concentration of chimera S, with a maximum corresponding to 60% of the control. This demonstrates that, when expressed in the presence of the other HCV proteins, NS3/4A cleaves sufficient Gal4VP16 from the chimera to generate important signal amplification.

Specific activation of the NS3/4A-sensitive chimera in a cell line expressing a full-length HCV replicon

Next, we assayed chimera activity in cells expressing a full-length HCV replicon of genotype 1b (Breiman et al., 2005; Pietschmann et al., 2002). Ectopic expression of chimeras R and S revealed either homogeneous or disorganized localization at the ER, as in the (Tet−) UHCV-11 cells. We noticed that codetection of NS3/4A and the chimera was observed rarely, possibly due to a very high level of expression and largely cytoplasmic distribution of the chimera (data not shown).

In Huh-7 Rep cells, the chimera-mediated CAT reporter assay proved specific, but indicated a lower efficiency (20%) than that observed in the UHCV-11 cells (60%) (Fig. 4a). This may be related to a heterogeneity in the expression levels of the viral proteins in these cells (Breiman et al., 2005). As a second read-out, we used GFP as reporter gene and monitored its induction by fluorescence. These results showed specific GFP induction in the presence of chimera S, but not in the presence of chimera R, and confirmed the specific activation of the chimera in Huh-7 Rep cells (Fig. 4b).

![Figure 3](image_url)
Purification of HCV-infected cells with the NS3/4A-dependent chimera, using the JFH1 virus and an in vitro culture model of HCV infection

It is now possible to generate HCV particles in cell culture, or HCVcc, from Huh-7 cells expressing HCV of genotype 2a (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We used in vitro RNAs transcribed from the JFH1 plasmid to prepare virus stocks, using the Huh-7.5 cell-culture system (Wakita et al., 2005; Zhong et al., 2005), to determine the ability of such viruses to activate the chimera system. Huh-7.5 cells were transfected with the inducible CAT-expressing vector in the presence of vectors expressing chimera S, chimera R or Gal4VP16. The cells were then either not infected or infected with different concentrations of the JFH1 virus. In the absence of infection, CAT expression was highly induced in the presence of Gal4VP16, barely induced in the presence of chimera S and not induced in the presence of chimera R (Fig. 5a, lanes 1–4). Infection with JFH1 provoked a specific induction of CAT expression in the presence of chimera S, which represents 20–28 % of the CAT expression induced with Gal4VP16. In contrast, CAT expression remained low (5 % of CAT expression with Gal4VP16) in the presence of chimera R (Fig. 5a, lanes 5–9). Note that, although the chimera S-mediated CAT in the induction increased with JFH1 concentration, the difference of induction was not very pronounced (between 20 and 28 %), whereas there is a 2-log difference in the JFH1 concentration. The reason for this is not known, but may be related to a decrease in the efficiency of expression of the viral proteins, and hence NS3/4A protease, as the concentration of HCV-infected cells increases.

In contrast to the possibility to perform efficient infection of cells in culture with HCVcc, infection of primary cultures of human hepatocytes with HCV-containing sera is poorly efficient (Castet et al., 2002) and may reflect more natural conditions of infection with HCV. The possibility to purify HCV-infected hepatocytes would therefore allow characterization of the components of the host required for permissivity. With this in mind, we infected Huh-7.5 cell cultures with JFH1 at a low m.o.i. to validate the use of the NS3/4A-dependent chimera system for the purification of HCV-infected cells. In order to do this, the chimera/gene reporter constructs were transduced into cells after their transfer to HR'TRIP lentiviral vectors and generation of the corresponding pseudovirus. The transduction procedure is very efficient (transfer of material into >80 % of cells) and was necessary here to increase the probability of coexpression of the constructs with HCV. In addition, this procedure would be essential to transfer the chimera and the gene reporters to primary cultures of human hepatocytes, as these cells do not divide in culture (Ferrini et al., 1997; Giannini et al., 2003). Huh-7.5 cells were infected with JFH1 (2 × 10^5 genome equivalents per 8 × 10^5 cells) at a virus:cell ratio of 1:4 (2 × 10^5 genome equivalents per 8 × 10^5 cells), which corresponds to a situation intermediate to the ratio used in Fig. 5(a) in order to have an efficient activation of the chimera, as well as an efficient HCV infection. The cells were then transduced after 24 h with pseudovirus expressing CMV-H-2K^k or expressing (Gal4)_5-E1b-H-2K^k alone, or cotransduced with the (Gal4)_5-E1b-H-2K^k pseudovirus in the presence of pseudovirus expressing Gal4VP16, chimera S or chimera R. Four days after transduction, cells were processed for immunomagnetic cell sorting by

Fig. 4. Specific activation of the NS3/4A-sensitive chimera in Huh-7 cells expressing a full-length HCV replicon of genotype 1b. (a) Huh-7 Rep cells (100,000 per well; 24-well plates) were transfected with 0.5 μg of the reporter plasmid pG5CAT alone (first lane), in the presence of 1 μg pM3VP16 (second lane) or in the presence of 0.5–3 μg plasmid expressing chimera S or chimera R. After 24 h, the cells were washed twice with PBS and CAT expression was measured by ELISA (Roche) and expressed as the mean of duplicate wells and the figure is a representative of different, independent experiments. (b) Huh-7 Rep cells were transfected with 500 ng reporter plasmid expressing (Gal4)_5-E1b-GFP, either alone (Control) or in the presence of 500 ng pM3VP16 (Gal4VP16), pcdNA3/AMP chimera S (Chimera S) and pcdNA3/AMP chimera R (Chimera R). Three hours after the beginning of transfection, the medium was removed and replaced with fresh medium. Specific increase of fluorescent cells over background levels was observed after 24 h for the chimera S-expressing cells. Photographs were taken 3 days after transfection, using Metaview Software coupled to a Leica microscope with ×10 magnification.

Purification of HCV-infected cells with the NS3/4A-dependent chimera, using the JFH1 virus and an in vitro culture model of HCV infection

It is now possible to generate HCV particles in cell culture, or HCVcc, from Huh-7 cells expressing HCV of genotype 2a (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We used in vitro RNAs transcribed from the JFH1 plasmid to prepare virus stocks, using the Huh-7.5 cell-culture system (Wakita et al., 2005; Zhong et al., 2005), to determine the ability of such viruses to activate the chimera system. Huh-7.5 cells were transfected with the inducible CAT-expressing vector in the presence of vectors expressing chimera S, chimera R or Gal4VP16. The cells were then either not infected or infected with different concentrations of the JFH1 virus. In the absence of infection, CAT expression was highly induced in the presence of Gal4VP16, barely induced in the presence of chimera S and not induced in the presence of chimera R (Fig. 5a, lanes 1–4). Infection with JFH1 provoked a specific induction of CAT expression in the presence of chimera S, which represents 20–28 % of the CAT expression induced with Gal4VP16. In contrast, CAT expression remained low (5 % of CAT expression with Gal4VP16) in the presence of chimera R (Fig. 5a, lanes 5–9). Note that, although the chimera S-mediated CAT in the induction increased with JFH1 concentration, the difference of induction was not very pronounced (between 20 and 28 %), whereas there is a 2-log difference in the JFH1 concentration. The reason for this is not known, but may be related to a decrease in the efficiency of expression of the viral proteins, and hence NS3/4A protease, as the concentration of HCV-infected cells increases.

In contrast to the possibility to perform efficient infection of cells in culture with HCVcc, infection of primary cultures of human hepatocytes with HCV-containing sera is poorly efficient (Castet et al., 2002) and may reflect more natural conditions of infection with HCV. The possibility to purify HCV-infected hepatocytes would therefore allow characterization of the components of the host required for permissivity. With this in mind, we infected Huh-7.5 cell cultures with JFH1 at a low m.o.i. to validate the use of the NS3/4A-dependent chimera system for the purification of HCV-infected cells. In order to do this, the chimera/gene reporter constructs were transduced into cells after their transfer to HR'TRIP lentiviral vectors and generation of the corresponding pseudovirus. The transduction procedure is very efficient (transfer of material into >80 % of cells) and was necessary here to increase the probability of coexpression of the constructs with HCV. In addition, this procedure would be essential to transfer the chimera and the gene reporters to primary cultures of human hepatocytes, as these cells do not divide in culture (Ferrini et al., 1997; Giannini et al., 2003). Huh-7.5 cells were infected with JFH1 (2 × 10^5 genome equivalents per 8 × 10^5 cells) at a virus:cell ratio of 1:4 (2 × 10^5 genome equivalents per 8 × 10^5 cells), which corresponds to a situation intermediate to the ratio used in Fig. 5(a) in order to have an efficient activation of the chimera, as well as an efficient HCV infection. The cells were then transduced after 24 h with pseudovirus expressing CMV-H-2K^k or expressing (Gal4)_5-E1b-H-2K^k alone, or cotransduced with the (Gal4)_5-E1b-H-2K^k pseudovirus in the presence of pseudovirus expressing Gal4VP16, chimera S or chimera R. Four days after transduction, cells were processed for immunomagnetic cell sorting by
Fig. 5. Purification of HCV-infected cells with the NS3/4A-dependent chimera, using the JFH1 infection in vitro culture model. (a) Huh-7.5 cells, plated at 80,000 cells in 24-well plates, were transfected with 0.5 µg of the reporter plasmid pG5CAT alone (lanes 1 and 5), in the presence of 5 µg pM3VP16 (lane 2) or in the presence of 5 µg of plasmids expressing chimera S (lanes 3 and 6–8) or chimera R (lanes 4 and 9). Three hours after transfection, they were either incubated as such (lanes 1–4) or in the presence of different concentrations of the JFH1 virus stock (3 × 10⁵ genome equivalents ml⁻¹), such as 5 × 10⁶ (lanes 6 and 9), 5 × 10⁵ (lane 7) and 5 × 10⁴ (lane 8) genome equivalents ml⁻¹. After 18 h, the cells were washed three times and further incubated for 24 h in fresh medium. After another 24 h, cells were washed twice with PBS, and CAT expression was measured as described in the legend to Fig. 3. CAT expression levels were 0.004 and 0.007 pg CAT (µg total protein)⁻¹ without addition of the activator (lanes 1 and 5) and increased to 1.55 pg CAT (µg total protein)⁻¹ in the presence of Gal4VP16 (lane 2). The results are expressed as the percentage of the highest CAT expression (here, in lane 2), as described in the legend to Fig. 3. The presence of the virus in Huh-7.5 cells infected with the three different dilutions was determined by immunofluorescence in separate wells using a 1:250 dilution of rat anti-HCV E2 3/11 mAbs (Rouillé et al., 2006) and Cy3-coupled anti-rat antibodies (Jackson Laboratories). Nuclei were stained with DAPI. Images were obtained by using a Zeiss microscope (× 40 magnification; apotome diffraction) and acquired by using the Axiosvision 4.4 software. (b) Huh-7.5 cells were plated at 800,000 cells per 60 mm dish in the presence of either 70 µl of the JFH1 virus stock titrating 2.3 × 10⁶ genome equivalents ml⁻¹ (in one experiment) or 7 µl of the stock titrating 3 × 10⁷ genome equivalents ml⁻¹ (in a separate experiment, performed in duplicate). After 24 h, the medium was replaced by fresh medium. The cells were transduced with the different VSV-pseudotyped lentiviral vectors, as follows: 500 ng for CMV-H-2Kk, Gal4VP16, chimera S or chimera R and 200 ng for (Gal4)_5-E1b-H-2Kk. After 4 days, the cells were trypsinized and processed for immunomagnetic cell sorting as described in Methods. The results are expressed as a percentage of the H-2Kk-positive cells and represent the mean of the three assays performed in the two different experiments. RNA was extracted from the AUTOMACS-sorted cells and assayed for the presence of HCV negative-strand RNA by qRT-PCR as described in Methods. The results are expressed as the fold increase of HCV(−) RNA in all samples compared with its expression in the RNA extracted from the (Gal4)_5-E1b-H-2Kk-expressing cells. The specificity of the assay was determined by qPCR on tenfold serial dilutions of positive- and negative-strand HCV RNA after reverse transcription with the negative strand-specific sense primer (graph on the right). The slope of the linear plots is indicated for each (+) and (−) RNA.
The percentage of positive cells expressing H-2K\textsuperscript{k} either from its constitutive promoter CMV or from its inducible promoter in the presence of Gal4VP16, was 32 and 50%, respectively, thus showing the efficacy of the technique. A low percentage of positive cells was obtained from cells transduced with the H-2K\textsuperscript{k} reporter gene only (6.7%) or in the presence of chimera R (5.7%), which probably reflects some leakage from the promoter. In contrast to this, 21.7% of H-2K\textsuperscript{k}-positive cells were collected from cells transduced with the H-2K\textsuperscript{k} reporter gene in the presence of chimera S, thus demonstrating a specific gene induction through the activation of the chimera during HCV infection (Fig. 5b, top). Specific purification of the HCV-infected cells in the chimera S-expressing cells was further confirmed by qRT-PCR on the HCV negative replicative strand, or HCV(−), using the strand-specific rTth technique described previously (Castet et al., 2002) (Fig. 5b, insert). We observed a net increase in HCV(−) expression in these cells compared with the other H-2K\textsuperscript{k}-positive fractions (Fig. 5b, bottom).

Altogether, these results confirm the data observed successively with UHCV-11 cells and Huh-7 Rep cells, and demonstrate firmly that the NS3/4A-dependent chimera can be used to monitor HCV infection and to perform the purification of HCV-infected cells.

**DISCUSSION**

Here, we have exploited the NS3/4A protease activity of HCV to develop a strategy for the identification and selection of HCV-infected cells. We set up a two-component procedure in which the Gal4VP16 transcription factor was modified to become a target of NS3/4A. Under these conditions, liberation of Gal4VP16 allows signal amplification through induction of a specific promoter. The Gal4VP16 chimera construction was designed to contain the NS3/4A trans-cleavage motif located between NS5A and NS5B, which is present in different genotypes, such as 1a, 1b and 3a (Beyer et al., 2001). To prevent its non-specific migration to the nucleus in the absence of NS3/4A, Gal4VP16 was linked to the N terminus of the PERK protein, an ER-resident protein. As ER is the cellular site for the biosynthesis of HCV proteins, NS3/4A is therefore expressed close enough to the chimera to provoke its cleavage. PERK is an eIF2\textgreek{a} kinase (Harding et al., 1999; Shi et al., 1999) that is activated by dimerization through its N terminus in response to stress situations (i.e. protein overload or misfolding) (Kauffman, 1999). PERK has a dual effect, as it can either inhibit protein synthesis (in the majority of cases) or increase the translation of specialized mRNAs presenting internal ribosome entry site structures (Subkhankulova et al., 2001), including HCV RNAs (Fernandez et al., 2002). The chimera construct does not contain the catalytic domain of PERK and therefore cannot interfere with the general protein synthesis per se. Indeed, a qRT-PCR analysis revealed no change in the expression levels of HCV RNA in the presence of the chimera (data not shown). The HCV E2 protein has previously been reported to affect PERK activity through interaction with the PERK catalytic domain (Pavio et al., 2003). As the chimera lacks this domain, it cannot be affected by the presence of E2. The hypothesis that the PERK luminal domain of the chimera interfered with NS3/4A expression because of a possible homodimerization with endogenous PERK (Ma et al., 2002) was also ruled out by constructing a chimera with a large deletion (Δ112–500) in the PERK luminal homodimerization domain. This construct proved as efficient as the full-length chimera in the CAT assay (not shown).

Other groups have previously exploited the trans-cleavage activity of the NS3 protease to activate the expression of transgenes. In a pioneer work, Shimotoho and colleagues generated a chimera in which the human T-lymphotropic virus Tax transactivating factor was fused to an ER-retained HCV NS2 protein through a 33 aa NS5A/NS5B junction sequence and used a reporter vector in which CAT was under the control of a Tax-responsive element (Hirowatari et al., 1995). Another group generated a susbtrate vector in which enhanced GFP (EGFP) was fused to secreted alkaline phosphatase (SEAP) through the decapeptide NS4A/NS4B junction sequence. The latter system allowed specific release and secretion of SEAP in the presence of the NS3/4A protease, expressed either ectopically or from an HCV subgenomic replicon (Lee et al., 2003). These two systems were designed for the screening or evaluation of protease inhibitors. More recently, a system allowing the sorting of cells expressing a subgenomic HCV replicon, with a fourfold enrichment, was reported by Migliaccio and colleagues. In this system, the CD8 cell-surface marker was designed to be retained at the plasma membrane, after expression in the ER lumen upon NS3/4A cleavage, and is therefore directly dependent upon NS3/4A cleavage for its expression. This involves a complex, multicomponent chimeric protein anchored at the ER membrane through transmembrane (TM) domains crossing the membrane three times and presenting a NS3/4A trans-cleavage site in the cytosolic domain immediately preceding the last TM domain linked to the transgene (Pacini et al., 2004).

In line with these precedent studies, the PERK-NS5A/5B-Gal4VP16 chimera, constructed here, was also cleaved specifically by NS3/4A. The efficiency of cleavage was limited, but sufficient to trigger a strong signal through gene amplification by the Gal4VP16 transcription factor. By using CAT, GFP or H-2K\textsuperscript{k} as reporter genes, we demonstrated that the chimera could operate in different cellular systems expressing NS3/4A, either alone (HEK 293T cells) or in the context of HCV expression. In this respect, the chimera was activated by NS3/4A of different HCV genotypes, such as 1a (UHCV-11 cells), 1b (Huh-7 Rep cells) and 2a (Huh-7 cells infected with HCV of genotype 2a produced in cell culture).

We observed that the chimera-mediated induction of the reporter varied depending on the HCV-expressing system. In addition to the deletion of the luminal PERK domain, we
tried other modifications in the chimera sequence to improve its efficacy, such as insertion of 10–20 aa between the TM and trans-cleavage domains for better accessibility of NS3/4A, or introduction of a longer NS3/4A cleavage sequence, such as the one described by Pacini et al. (2004). Only the latter resulted in a higher efficacy of cleavage by NS3/4A, but did not yield better induction of the reporter gene (not shown). It is therefore probable that the efficacy of the chimera-mediated trans-activation assay is more dependent on the NS3/4A expression level than on the structure of the chimera. Indeed, we found that the best activation of the chimera occurred in the UHCV-11 cell line and in JFH1-infected Huh-7 cells, which showed high expression of the viral proteins, whereas chimera activation was less important in Huh-7 Rep cells, where we consistently observed that no more than 30–40% of cells expressed detectable amounts of viral proteins (Breiman et al., 2005).

At present, the only efficient in vitro cell-culture system for HCV is infection of Huh-7,5 cells by the JFH1 genome of genotype 2a or by the chimeric J6/JFH1, containing the structural genes from an infectious clone of genotype 2a (HC-16) and the non-structural genes of JFH1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The infectivity of these HCVcc could be demonstrated in vivo (Lindenbach et al., 2006; Wakita et al., 2005) and, interestingly, comparison of HCVcc grown only in cell culture or passaged in vivo revealed some differences in their buoyant density and infectivity (Bukh & Purcell, 2006; Lindenbach et al., 2006). At present, only HCV of genotype 2a allows comparison between in vitro and in vivo growth. Therefore, there is a need for a cellular system allowing screening of different HCV genotypes for their ability to grow in culture and to determine the influence of different factors on their infectivity, such as human lipoproteins and apolipoproteins (Bukh & Purcell, 2006; Maillard et al., 2006). The NS3/4A-dependent chimera system, described here, can now be used for these purposes. In particular, it can be used for the purification of HCV-infected human hepatocytes, either upon infection with JFH1 virus or upon infection with HCV from the sera of infected patients. In the latter case, concentration of HCV from the sera will be necessary in order to increase the efficiency of infection, as we observed that the H-2Kk cell-sorting procedure requires that at least 10% of cells are infected.

The H-2Kk immunomagnetic cell-sorting procedure cannot allow discrimination between cells expressing only a few H-2Kk molecules at their surface, resulting from some leakage of transcription in the absence of the inducer, and cells expressing several molecules of H-2Kk at their surface as a result of authentic HCV infection. Based on this information and taking advantage of the flexibility of our system, by changing the reporter gene to the luciferase gene, we are now adapting the chimera system for the detection of HCV-infected cells by bioluminescence analysis with a wide-field microscope. Only authentically infected cells will be revealed by their intense luminescence. This procedure will give information both on the percentage of infected cells and on the quality of the infection, as well as leading to the analysis of the efficacy of different HCV inhibitors during an ongoing infection. As our strategy of detection is at the cellular level, it will conveniently allow us to detect infection by different HCV genotypes, including HCV from a natural source (serum from patients), without the requirement to concentrate the sera first.

In conclusion, the NS3/4A-dependent chimera system described in this study can be delivered to a number of different cells and can be activated in response to different HCV genotypes, such as 1a, 1b and 2a, and has been designed to be flexible in order to accommodate different reporter genes. This system can now be used to explore the ability of different HCV genotypes to infect cells and to determine the factors involved in the permissivity of the cells to infection.

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