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A reporter cell line for rapid and sensitive evaluation of hepatitis C virus infectivity and replication

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ABSTRACT

The human pathogen hepatitis C virus (HCV) is associated with chronic liver disease. The recent development of the cell culture infectious HCV (HCVcc) system has opened up avenues for detailed studies on the life cycle of the virus and its interaction with the host cell. Current methods to quantitate virus infectivity in cell culture are time-consuming and labor-intensive. This study describes the generation of a cell-based secreted alkaline phosphatase (SEAP) reporter assay to facilitate in vitro studies of HCV infection and replication. This assay is based on a novel reporter cell line stably expressing the enhanced green fluorescent protein (EGFP) fused in-frame to the secreted alkaline phosphatase via a recognition sequence of the viral NS3/4A serine protease. The SEAP reporter from a similar construct has previously been shown to be released from the fusion protein and be secreted into the extracellular culture medium following cleavage by the viral NS3/4A protease. The reporter cell line enabled rapid and sensitive quantification of HCV infection and viral replication in cell culture. The utility of this system for investigating virus entry, and for high throughput screening of entry inhibitors and other antiviral compounds was demonstrated using several inter- and intra-genotypic chimeras of HCV.

1. Introduction

It is estimated that about 170 million people world-wide are currently infected with hepatitis C virus (HCV), a major cause of chronic liver disease (Liang et al., 2000; Poynard et al., 2003). Current treatment regimens fail to produce a sustained response in a significant proportion of these patients, many of whom progress to chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). Therefore there is an urgent need for the development of new antiviral agents.

HCV is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family Flaviviridae. The viral genome encodes a single polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases to yield the mature structural proteins, composed of core and envelope glycoproteins E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The structural proteins and ion channel protein p7 are processed by endoplasmic reticulum (ER) signal peptidases, whereas the non-structural proteins are processed by the viral autoprotease complex NS2/3 and the NS3/4A serine protease (Moradpour et al., 2007). The NS3/4A protease recognizes the conserved NS4A/4B sequence **DEMEEC-S**XXX and **DEMEEC-A**XXX (Fig. 1A) as well as the conserved motif **D**XXXX**C-S**XXX and **E**XXXX**T-A**XXX, which is found at the NS3/4A, NS4B/5A, and NS5A/5B junctions (Grakoui et al., 1993; Kim et al., 1996; Kou et al., 2007). Cleavage by NS3/4A is essential for the maturation of the NS3/4A serine protease itself, the NS3 RNA helicase, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase, which are required for viral replication (Moradpour et al., 2007).

There are six distinct HCV genotypes and multiple subtypes; the viral genotypes vary by at least 30% at the nucleotide level (Bourlière et al., 2002; Pawlotsky, 2003; Simmonds, 1995). The recent advent of cell culture infectious HCV (HCVcc) has allowed in vitro studies on various aspects of the virus life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Prior to this, most research on HCV replication has been carried out in cell lines harbouring autonomously replicating subgenomic and genome-length viral RNA (Moradpour et al., 2007). The HCVcc system is limited

Abbreviations: FCS, fetal calf serum; FFU, focus forming units; HCV, hepatitis C virus; HCVcc, cell-cultured HCV; IFN-α, interferon-alpha; IgG, immunoglobulin G; IRES, internal ribosome entry site; IU, international unit; MAb, monoclonal antibody; MLV, murine leukemia virus; NS, non-structural; PBS, phosphate-buffered saline; RLU, relative light units; RT-qPCR, real-time-quantitative polymerase chain reaction; SEAP, secreted alkaline phosphatase; $CCID_{50}$, 50% cell culture infective dose; WT, wild type; Pur^R, puromycin resistance gene.

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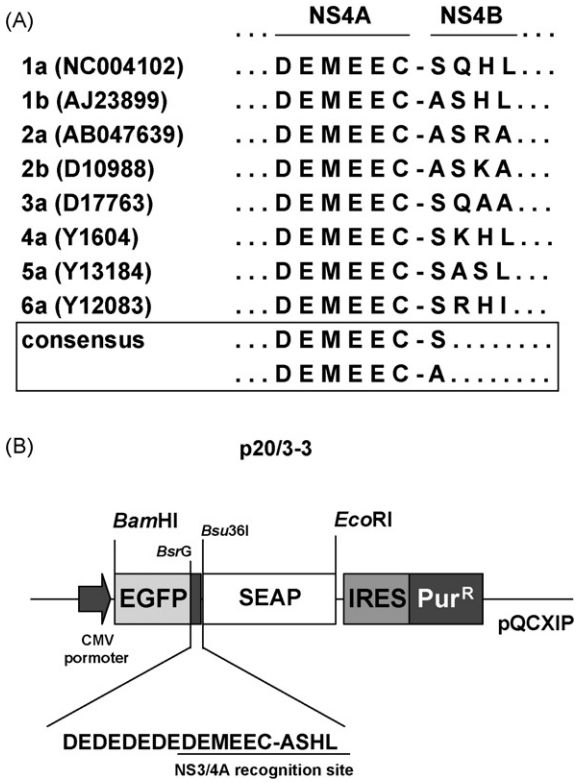

Fig. 1. Schematic diagram of fusion protein construct. (A) Alignment of the conserved amino acid residues representing the cleavage site of NS3/4A of diverse HCV genotypes. The accession numbers of the sequences of the HCV genotypes are shown in brackets. (B) The construct p20/3-3 contains the sequence encoding EGFP and SEAP linked by the octapeptide DEDEDEDE and the HCV genotype 1b NS4A/4B substrate sequence DEMEEC-ASHL (representing the recognition site for the viral NS3/4A protease) cloned in the pQCXIP vector upstream of the internal ribosome entry site (IRES) and the puromycin resistance gene (Pur^R). The recognition site of the NS3/4A protease is underlined and '-' denotes the cleavage site.

to a genotype 2a strain JFH1, although chimeric viruses expressing the structural and some of the non-structural proteins of other genotypes can be generated in the JFH1 background, which partly overcomes this limitation (Gottwein et al., 2009; Pietschmann et al., 2006).

Several methods are currently used to quantify HCV infection and replication in culture. These include assays to determine focus forming units (FFU), 50% cell culture infective dose ($CCID_{50}$) (Lindenbach et al., 2005), and to quantify virus RNA levels by real-time PCR. Recombinant viruses carrying reporter genes in their genomes, typically luciferase, or green fluorescent protein (GFP), are also used for the measurement of viral RNA replication. A chimeric bicistronic JFH1 (genotype 2a) virus that carries the luciferase reporter gene in the viral cDNA sequence, was developed to characterize the early steps of HCV entry (Koutsoudakis et al., 2006). An analogous chimeric monocistronic reporter virus system was used to demonstrate time- and temperature-dependent activation of HCV for low-pH-triggered entry (Tscherne et al., 2006). However, chimeric reporter viruses generally tend to have attenuated replication levels and are somewhat impaired in producing infectious progeny compared to their parent genomes. More importantly, each virus under study needs to be genetically modified to express the reporter protein.

The aim of this study was to develop a cell line that, upon infection with any HCVcc isolate, would secrete an easily measurable reporter protein whose activity would correlate with virus infection, translation and replication. It had previously been shown, using transient transfection, that a fusion protein composed of the enhanced green fluorescence protein (EGFP) and secreted alkaline phosphatase (SEAP) linked by an octapeptide spacer and the HCV NS4A/4B cleavage site (Fig. 1B), could act as a substrate for the viral serine protease NS3/4A (Chou et al., 2007; Lee et al., 2003). The role of EGFP is to retain the entire fusion protein within the cell. The HCV NS3/4A protease, when supplied in trans, releases SEAP from the fusion protein, thus enabling its N-terminal signal peptide to direct its secretion into the extracellular culture medium. This study describes the generation and characterization of a human hepatoma reporter cell line, Huh7-J20, that stably expresses an almost identical fusion construct. Huh7-J20 has several distinct advantages: (i) it offers a rapid and convenient infectivity assay in which SEAP activity is directly measured in a sample of the culture medium without compromising cell integrity; (ii) it provides a tool to indirectly measure infectivity, translation and viral RNA replication; (iii) it can be used in virus neutralization assays and for high throughput screening of viral entry inhibitors and other antiviral compounds; and (iv) it can be used with any inter- or intra-genotypic derivative of HCV JFH1, and possibly other cell culture-infectious viral isolates that may be isolated in future.

2. Materials and methods

2.1. Cell culture

Human hepatoma Huh7 cells (Nakabayashi et al., 1982) and human epithelial kidney (HEK)-293T cells (ATCC CRL-1573) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 M nonessential amino acids. The Huh7-J20 cells were propagated in the same medium supplemented with 2 μg/ml puromycin (see next section).

2.2. Plasmid construction and establishment of the reporter cell line, Huh7-J20

The plasmid p20/3-3 encoding EGFP fused in-frame with the octapeptide DEDEDEDE followed by the NS4A/4B protease recognition sequence DEMEEC-ASHL and SEAP (EGFP-oct-Δ4A/4B-SEAP) was assembled by PCR amplification and cloned into the bicistronic murine leukaemia virus (MLV) transfer-vector pQCXIP (Clontech), which also expresses the puromycin resistance gene (see Fig. 1B). To generate retroviral pseudoparticles, HEK-293T cells were co-transfected with plasmids expressing the vesicular stomatitis virus G protein, the MLV Gag-Pol packaging vector and p20/3-3 as described previously (Bartosch et al., 2003). Following incubation at 37 °C for 24 h, the medium containing the released retroviral pseudoparticles was collected, filtered through 0.45 μm pore-sized membrane and used to transduce Huh7 cells. At 3 days post-transduction, the cells were cultured in the presence of 2 μg/ml puromycin and the surviving reporter-expressing cells, designated Huh7-J20, were pooled and used in experiments. Flow cytometry analysis confirmed that all cells expressed EGFP (data not shown).

2.3. Generation of strain JFH1 HCVcc and its inter- and intra-genotypic derivatives

The plasmid pJFH1 containing the full-length genomic cDNA sequence of the HCV genotype 2a strain JFH1 was a kind gift from Takaji Wakita (Wakita et al., 2005). The intra-genotypic chimera J6/JFH1 was constructed by replacing cDNA encoding amino acids 1–864 (i.e. core to the loop region between the transmembrane domains 1 and 2 of NS2) of strain JFH1 with those from a chimpanzee-infectious genotype 2a strain J6CF (Yanagi et al., 1999) in pJFH1. The HCV genotype 2b intra-genotypic chimera 2B1.1/JFH1 has been described previously (Owsianka et al., 2008).

The HQL virus genome was constructed by introducing three adaptive mutations into the intergenotypic chimera expressing HCV genotype 1a strain H77c (Yanagi et al., 1997) core to NS2 in the JFH1 background described previously (Pietschmann et al., 2006). These adaptive mutations in HQL, Y835H in NS2, K1402Q in NS3 (unpublished data) and V2440L in NS5A (Kaul et al., 2007), increased the titers of the intergenotypic H77c-JFH1 chimera from 1×10^3 to 1×10^6 $CCID_{50}$/ml (data not shown).

HCVcc were generated essentially as described previously (Wakita et al., 2005). Briefly, linearized plasmids carrying the full-length HCV sequence were used as a template to generate viral genomic RNA by in vitro transcription. Ten microgram of this RNA was electroporated into Huh7 or Huh7-J20 cells.

2.4. Flow cytometry analysis and counting of cells

Cells were trypsinized and fixed with 1% paraformaldehyde and permeabilized with 0.1% saponin. They were then stained using anti-NS5A mouse monoclonal antibody (MAb) 9E10 (Lindenbach et al., 2005) followed by an anti-mouse phycoerythrin (PE) conjugated secondary antibody for analysis by flow cytometry on a BD FACSCalibur using CellQuest Pro software (BD Biosciences). The absolute number of cells was determined using Centrifuge Tubes for Biomass Determination (Sartorius Stedim Biotech).

2.5. Virus yield

Culture medium of infected cells was harvested 4 days post-transfection, filtered through a 0.45 μm-pore membrane, and used as virus stock for infection assays. Serially diluted virus (replicates of six) was titrated by infecting Huh7 cells, followed by fixation in methanol and probing with the anti-HCV NS5A mouse MAb 9E10 and a FITC-conjugated anti-mouse IgG antibody. The cells were examined under a UV microscope and the virus titer determined as $CCID_{50}$, as described by Lindenbach et al. (2005).

2.6. Indirect immunofluorescence

Huh7 cells grown on glass coverslips were infected with the virus and incubated for 2 days at 37 °C. The cells were washed twice with phosphate-buffered saline (PBS), fixed for 20 min in methanol at −20 °C, and blocked with PBS containing 2% FCS for 10 min at room temperature. The cells were probed for 1 h with the anti-E2 MAb AP33 (Clayton et al., 2002; Owsianka et al., 2001) or the sheep polyclonal anti-NS5A antiserum (Macdonald et al., 2003). Subsequently, the cells were washed extensively with PBS containing 0.05% Tween 20 and incubated for 1 h at room temperature with an anti-species IgG conjugated with either FITC or TRITC (Invitrogen). The nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, 1 mg/ml, diluted 1:1000, for 1 min). After washing with PBS, the coverslips were mounted on a glass slide and examined with a Zeiss Laser Scanning LSM510 META inverted confocal microscope (Carl Zeiss Ltd., UK).

2.7. SEAP assay

Huh7-J20 cells were seeded at 1×10^4 cells/well in a 48-well tissue culture dish and incubated at 37 °C. Next day, cells were infected with 100 μl of virus stock for 3 h after which the inoculum was replaced with 400 μl of fresh medium and the cells incubated at 37 °C for various time intervals. To measure the extracellular SEAP activity, 90 μl of culture medium was collected and mixed with 10 μl of 10-times concentrated lysis buffer to a final concentration of 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl and 0.5% Triton X-100 to inactivate the virus. The SEAP assay was performed using the Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech) by mixing 12.5 μl of the cell medium with 37.5 μl of assay buffer and 50 μl of the SEAP substrate solution in a black 96-well microplate. The SEAP activity was measured using a Hidex Chameleon plate-reader and expressed as relative light units (RLU).

2.8. RNA extraction and relative quantification of virus RNA by real-time-quantitative polymerase chain reaction (RT-qPCR)

Huh7-J20 cells were seeded in 48-well tissue culture dishes and infected with HCVcc as described above. At appropriate times post-infection, the cells were washed with PBS and total RNA was isolated using the RNAeasy Kit (Qiagen). To quantitate HCV RNA, a relative quantification reaction was performed, where each sample was normalized to an endogenous control gene (GAPDH). cDNA was generated by reverse transcription of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems), with random primers. Each sample was analyzed in triplicate in singleplex reactions to detect either HCV positive-strand target cDNA with a FAM labelled probe (250 nM) and HCV specific primers (900 nM each), or GAPDH with a pre-validated endogenous control VIC-probe/primer mix (Applied Biosystems). The HCV probe sequence was 6-FAM-AAA GGC CTT GTG GTA CTG-MGB (Applied Biosystems), and primer sequences were: 5′-TCT GCG GAA CCG GTG AGT AC-3′, forward, and 5′-GCA CTC GCA AGC ACC CTA TC-3′, reverse (Sigma-Genosys). Real-time reactions were run using TaqMan Fast Universal PCR Master Mix, no ampErase UNG (Applied Biosystems), under Fast Universal conditions on a 7500 Fast Real-Time PCR machine and data analyzed using 7500 Fast System Software (Applied Biosystems).

2.9. Antibody-mediated neutralization of virus infection

Medium containing HCVcc was mixed with serial dilutions of the anti-HCV E2 MAb AP33 and incubated for 1 h at 37 °C. The mixture was then used to infect Huh7-J20 reporter cells seeded the previous day in a 48-well tissue culture dish and incubated at 37 °C for 3 h. The inoculum was replaced with 400 μl of fresh medium and the cells incubated at 37 °C for 3–4 days. For the anti-CD81 blocking experiments, Huh7-J20 cells were pre-incubated with serially

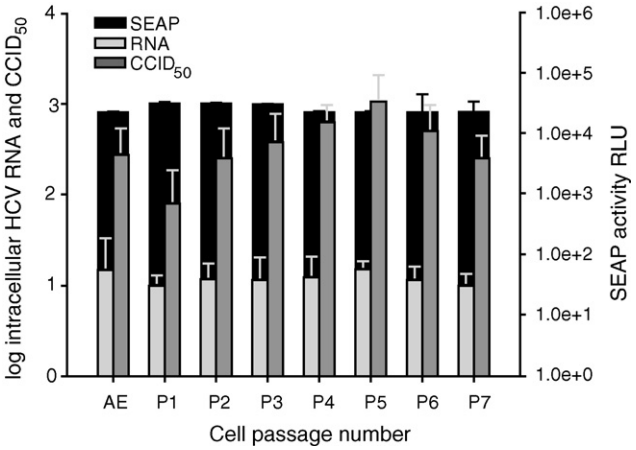

Fig. 2. Correlation between extracellular SEAP activity, intracellular HCV RNA and virus yield during serial passage of transfected cells. Huh7-J20 cells were electroporated with in vitro transcribed WT JFH1 RNA and incubated for 4 days (AE). Electroporated cells were serially passaged (1:10) when subconfluent up to seven times (P1–P7). At each stage, the SEAP activity (black bars) in the medium was measured and compared with the level of intracellular viral RNA (light grey bars) and the infectious virus yields (dark grey bars) as determined by RT-qPCR and $CCID_{50}$ assays, respectively. The values for SEAP activity, intracellular viral RNA levels and virus yield were normalized with respect to the number of infected cells, as determined using flow cytometry. Each point represents the average of three independent experiments with error bars indicating the standard deviation.

diluted anti-CD81 MAb (BD Pharmingen) for 1 h at 37 °C. HCVcc was added directly to the antibody-containing medium and incubated with cells at 37 °C for a further 3 h. The inoculum was then replaced with 400 μl of fresh medium and the cells incubated as above. The SEAP activity in the infected cell medium was analyzed as described in Section 2.7.

2.10. Inhibition of HCVcc replication by interferon (IFN)-α and other antiviral compounds

Huh7-J20 cells seeded in 48-well tissue culture dish were pre-treated or not for 18 h with increasing concentration of IFN-α (Sigma). Subsequently, cells were infected with the WT JFH1 virus for 3 h after which the inoculum was replaced with fresh medium and the cells were incubated at 37 °C for various time intervals. Moreover, naïve Huh7-J20 reporter cells were electroporated with WT JFH1 RNA and plated in 12-well tissue culture dish. Next day, the culture medium was replaced with fresh medium containing different concentrations of IFN-α. At 72 h post-infection, the medium was analyzed for SEAP activity.

The HCV NS3 protease inhibitor, VX-950 (Reesink et al., 2006), and the viral polymerase inhibitor, 2′-C-methyladenosine (Carroll et al., 2003; Paeshuyse et al., 2008), were a kind gift from Johan Neyts (Rega Institute for Medical Research) and Craig Gibbs (Gilead Sciences), respectively. Huh7-J20 cells were infected with WT JFH1 virus for 3 h after which the inoculum was replaced with fresh medium containing different amounts of VX-950 or 2′-C-methyladenosine and the cells were incubated at 37 °C for 72 h. The levels of virus infectivity were determined by measurement of SEAP activity in the medium.

3. Results

3.1. Characterisation of the SEAP reporter cell line Huh7-J20

Huh7-J20 cells were transfected with WT JFH1 RNA by electroporation and passaged seven times over a total period of 34 days. At each passage the accumulated SEAP in the culture medium was quantified. In parallel, the total intracellular RNA and the virus yield were also determined at each passage. Furthermore, cells were counted and analyzed by flow cytometry to quantify the absolute number of infected cells. When normalized with respect to the number of HCV replicating cells, the SEAP activity correlated with virus RNA levels during serial passaging (Fig. 2). However, the virus yield was seen to fluctuate, a feature observed previously in persistently infected cells (Zhong et al., 2006). Separately, Huh7-J20 cells were compared with the parent Huh7 cells for permissivity for virus entry and replication. Immunofluorescence analysis 3 days

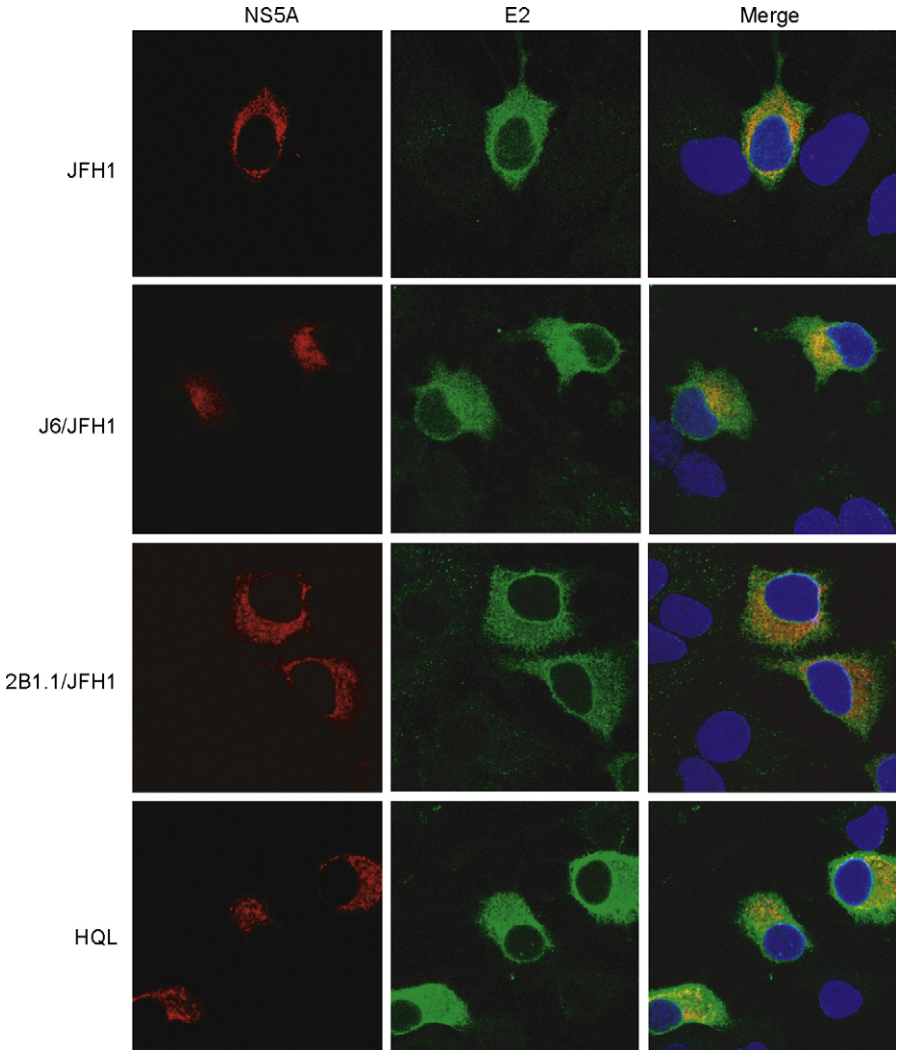

Fig. 3. Detection of HCV proteins E2 and NS5A by immunofluorescence. Huh7 cells were infected with WT JFH1, J6/JFH1, 2B1.1/JFH1 and HQL. After incubation for 2 days at 37 °C the cells were fixed and probed with E2- and NS5A-specific antibodies. Nuclei were visualized by DAPI staining.

post-infection showed fewer cells per foci in the Huh7-J20 cells compared to those in Huh7 cells (data not shown), indicating that virus spread was reduced. In accordance with this observation the Huh7-J20 cells had a ~3-fold reduction in the number of infected cells and a ~5-fold reduction in viral RNA levels as measured by flow cytometry and RT-qPCR, respectively. Despite this reduction the Huh7-J20 cells were still suitable for the accurate and sensitive detection of HCV infection (see below).

3.2. Correlation between SEAP activity and HCV replication

The SEAP reporter cell line Huh7-J20 was characterized in more detail using WT JFH1 and three inter- or intra-genotypic chimeric derivatives, J6/JFH1, 2B1.1/JFH1, and HQL expressing the structural proteins from viral genotypes 2a, 2b and 1a, respectively. All chimeras showed comparable viral NS5A and E2 expression in infected cells as detected by immunofluorescence (Fig. 3). To demonstrate that the SEAP activity secreted by infected cells correlates with virus RNA replication levels, Huh7-J20 cells were infected with WT JFH1, or the chimeric viruses and the SEAP activity released into the cell medium was measured at different time points post-infection. In parallel, the amount of intracellular viral RNA was determined by RT-qPCR at each time point. As shown in Fig. 4, a time-dependent increase in SEAP activity was observed in the medium of cells infected with all HCVcc viruses. As expected, no SEAP was released into the medium of mock-infected cells (data not shown). The level of SEAP activity differed between chimeras, but correlated with their respective replication levels up to 96 h post-infection, at which time cells reached confluency (Fig. 4). These data clearly demonstrate the potential of Huh7-J20 as a reporter cell line in HCV infection assays. At 120 h post-infection, there was a general reduction in viral RNA levels, whereas the SEAP activity continued to increase. This effect was observed in cells infected with all viruses. Together, these data show a strong correlation between the SEAP activity and virus RNA replication in actively dividing cells. It is unclear why HCV replication is limited in confluent cells, but similar observations have been made previously (Koutsoudakis et al., 2006). Possible explanations could be cell death due to over-confluency, depletion of the nucleoside triphosphate pools in resting cells (Stuyver et al., 2003), and/or cell cycle-dependent effects on virus RNA replication and translation (Honda et al., 2000).

3.3. Determination of the sensitivity of the SEAP reporter system

One of the most important characteristics of any reporter system is its sensitivity. Therefore, the SEAP assay was compared to the RT-qPCR method for detecting virus infection, the most sensitive method to detect HCV replication currently available. Huh7-J20 cells in 6-well dishes were infected with WT JFH1 at different dilutions. The infected cells were incubated in 2 ml medium/well and the extracellular SEAP activity was measured 3 days post-infection essentially as described in Section 2. In parallel, the number of infected cells in each sample was determined by flow cytometry.

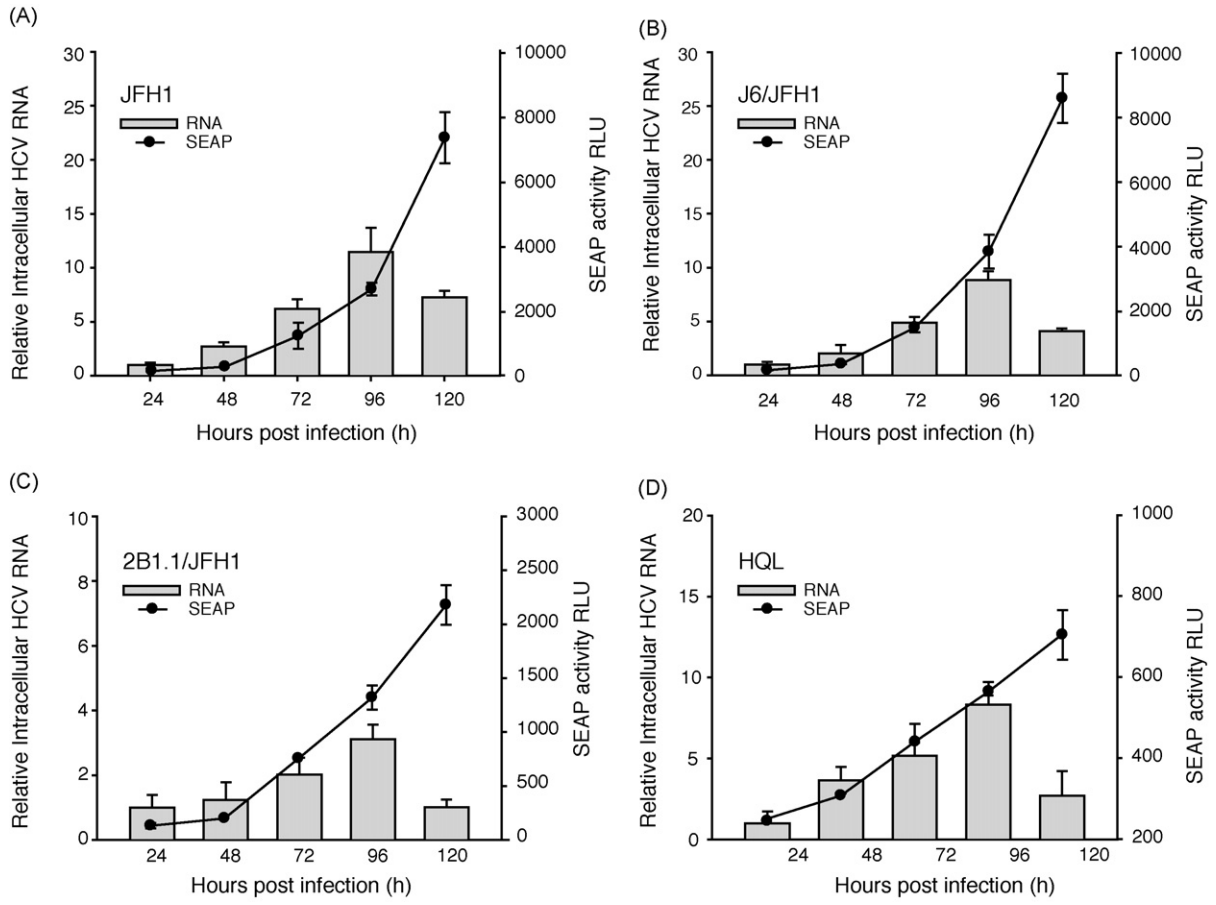

Fig. 4. Correlation between extracellular SEAP activity and HCV-RNA replication levels. Huh7-J20 cells were infected with (A) WT JFH1, (B) J6/JFH1, (C) 2B1.1/JFH1 at an MOI of 0.1 $CCID_{50}$/cell and (D) HQL at an MOI of 0.005 $TCID_{50}$/cell. The extracellular SEAP activity (●) was analyzed at various time points post-infection as shown. At each time point total RNA was isolated and analyzed by RT-qPCR. The grey bars represent the relative quantification of intracellular HCV RNA. The relative RNA values for each genotype were calibrated against their respective 24 h time point value. Each point represents the average of three independent experiments with error bars indicating the standard deviation.

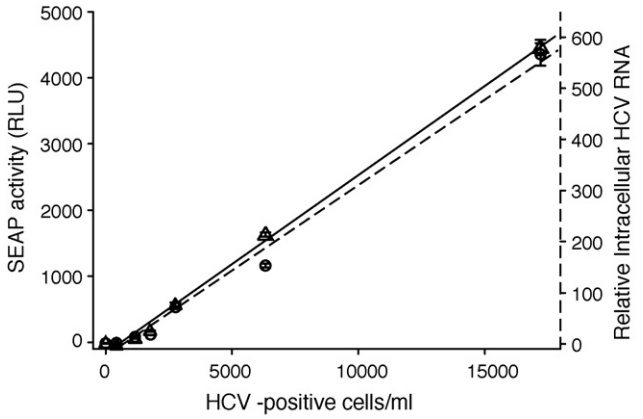

Fig. 5. Sensitivity of the SEAP reporter system. Correlation between the number of infected cells, extracellular SEAP activity and intracellular viral RNA levels. Huh7-J20 cells in 6-well dish were infected in triplicate with different dilutions of WT JFH1 HCVcc as described in the text. At 3 days post-infection, the extracellular SEAP activity (solid line, △) was determined. Furthermore, total cellular RNA from each sample was isolated and the viral RNA levels (dashed line, ○) determined by RT-qPCR. The relative RNA values were calibrated against the lowest value. The experiment showed a significant correlation between HCV-positive cells and both SEAP-activity and RNA levels (r^2 = 0.996 and 0.990, respectively).

Furthermore, total RNA from each sample was isolated and analyzed by RT-qPCR. As shown in Fig. 5, the levels of both SEAP activity and viral RNA correlated significantly (r^2 = 0.996 and 0.990, respectively) with the number of infected cells. Thus, the sensitivity of the SEAP assay in detecting and quantifying virus infection was comparable to the RT-qPCR method.

3.4. Determination of antibody-mediated virus neutralization by SEAP assay

The utility of the Huh7-J20 cell line for virus neutralization assays was examined using two well-characterized neutralizing MAbs; AP33 targeting a conserved epitope in the viral E2 glycoprotein (Clayton et al., 2002; Owsianka et al., 2005; Tarr et al., 2007), and a MAb recognizing the cell surface protein CD81, a co-receptor required for virus entry into target cells (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). WT and chimeric viruses were pre-incubated with MAb AP33 over a range of concentrations before infecting Huh7-J20 cells. The SEAP activity in infected cell medium was determined after 3 days. A clear antibody dose-dependent effect on SEAP activity was observed, with the 50% inhibitory concentration (IC_{50}) of MAb AP33 ranging from 0.6 μg/ml for HQL to 11 μg/ml for J6/JFH1 virus (Fig. 6A). Similarly, an antibody concentration-dependent decrease in SEAP activity was seen in the medium of Huh7-J20 cells that had been pre-incubated with anti-CD81 prior to infection with different viruses (Fig. 6B). Consistent with previous reports (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005), almost complete neutralization was achieved with the anti-CD81 MAb at a concentration of 5–10 μg/ml.

3.5. Inhibition of virus replication by IFN-α and other antiviral compounds

The ability of IFN-α to inhibit virus replication was examined using the SEAP reporter cell line. As shown in Fig. 7A, virus replication was inhibited in a dose-dependent manner in response to pre-treatment of Huh7-J20 cells with IFN-α. In the medium of cells pre-treated with 100 IU IFN-α/ml, the SEAP activity was reduced to 13.2% of the untreated control at 72 h post-infection. A similar effect was observed in WT JFH1 RNA-electroporated Huh7-J20 cells incubated in the presence of IFN-α, where the relative SEAP activity was reduced to 23.2% at an IFN-α concentration of 100 IU/ml (Fig. 7B).

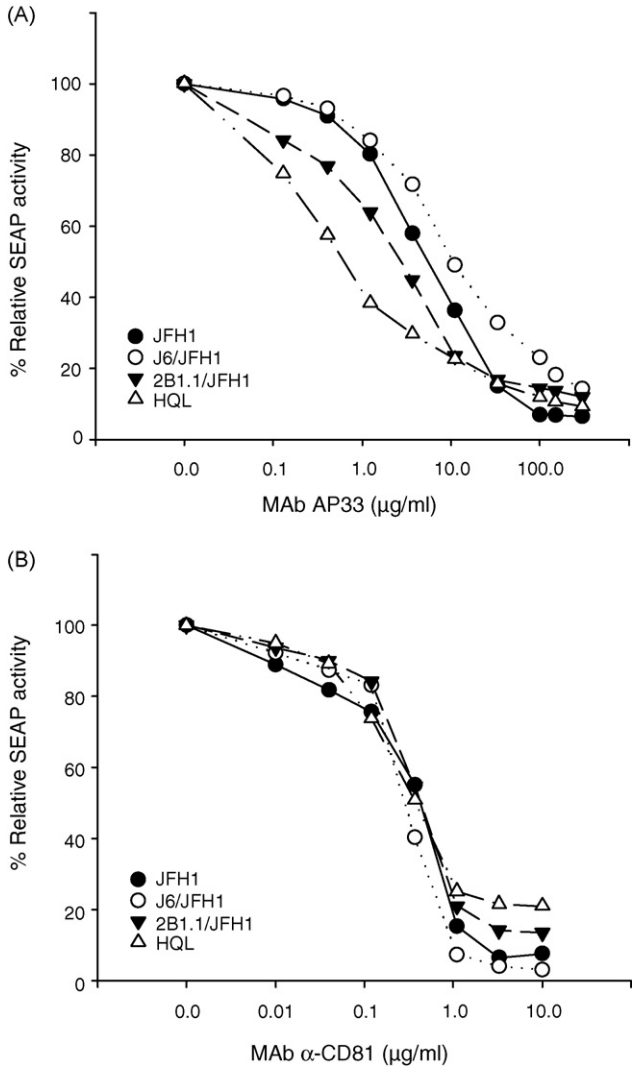

Fig. 6. Quantitation of HCVcc neutralization by anti-E2 or anti-CD81 antibody using the SEAP reporter readout. (A) WT JFH1 (●), J6/JFH1 (○), 2B1.1/JFH1 (▼) and HQL (△) viruses were pre-incubated with different amounts of anti-E2 MAb AP33 for 1 h at 37 °C prior to infection of Huh7-J20 cells. (B) Huh7-J20 cells were pre-incubated for 1 h at 37 °C with different concentration of the anti-CD81 MAb prior to infection with different viruses described above. In both cases, the extracellular SEAP activity was measured 3–4 days post-infection. The neutralizing activity is expressed as percentage of inhibition, normalized to the no-antibody control and is shown as the average of three independent experiments.

The utility of the SEAP readout was further validated using two other inhibitors of virus replication, VX-950 and 2′-*C*-methyladenosine. VX-950 is a viral NS3 protease inhibitor which has shown promising efficacy in clinical trials (Forestier et al., 2007; Reesink et al., 2006). The nucleoside 2′-*C*-methyladenosine has been shown to inhibit the viral NS5B RNA-dependent RNA polymerase activity (Carroll et al., 2003). As shown in Fig. 7C and D, both VX-950 and 2′-*C*-methyladenosine inhibited HCVcc replication in Huh7-J20 cells in a concentration-dependent manner. The IC_{50} of VX-950 and 2′-*C*-methyladenosine derived from the SEAP readout was 0.08 μM and 2.2 μM, respectively, values that are comparable to those reported previously in cells carrying viral replicons (Paeshuyse et al., 2008). Collectively, these data demonstrate that the Huh7-J20 reporter cell line offers a useful tool for rapid and convenient screening of antiviral compounds.

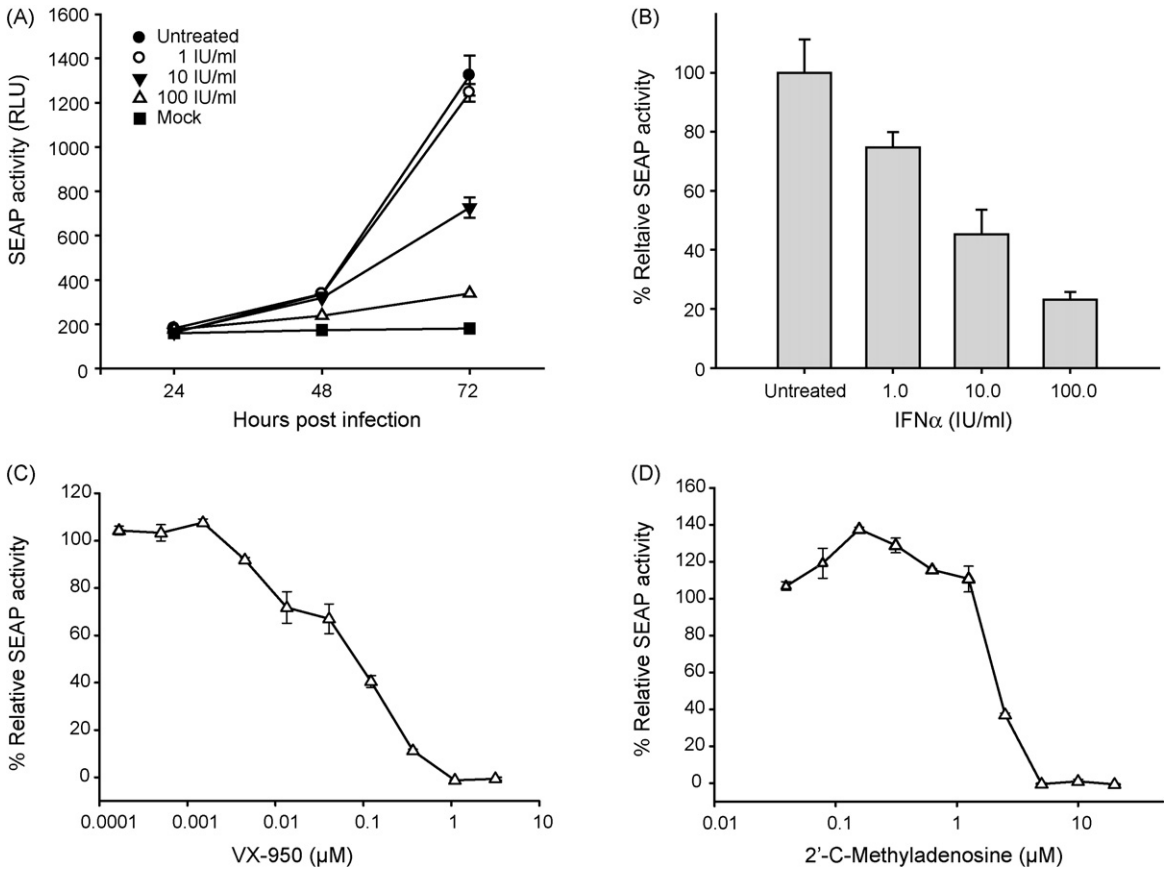

Fig. 7. Measurement of inhibition HCVcc replication in Huh7-J20 cells by antiviral compounds. (A) Huh7-J20 cells were either untreated (●) or pre-treated for 18 h with 1 IU/ml (○), 10 IU/ml (▼) or 100 IU/ml (△) of IFN-α prior to infection with WT JFH1 at an MOI of 0.1 $CCID_{50}$/cell. SEAP activity in the culture medium was detected at various time points post-infection. The medium of mock-infected cells was used as a negative control (■). (B) Huh7-J20 cells electroporated with WT JFH1 RNA were either untreated or treated with indicated concentrations of IFN-α. The SEAP activity in the culture medium was analyzed at 72 h post-incubation. The inhibition is expressed as percentage of relative SEAP activity, normalized to the untreated control. Each value represents the average of three independent experiments with error bars indicating the standard deviation. (C and D) Huh7-J20 cells were infected with WT JFH1 HCVcc in the presence of varying amounts of inhibitor of the viral (C) protease (VX-950) or (D) polymerase (2′-C-methyladenosine) as described in Section 2. The SEAP activity in the culture medium was analyzed at 72 h post-infection. The inhibition is expressed as described in (B).

4. Discussion

We have established a robust SEAP reporter cell line, Huh7-J20, which despite being slightly less permissive for virus infection in comparison to the parent line allows for a rapid and accurate quantitation of HCV replication in cell culture. This cell line stably expresses a fusion protein (EGFP-oct-Δ4A/4B-SEAP) from which the SEAP is cleaved by the HCV NS3/4A protease leading to its release into the culture medium following virus infection. It is noteworthy that no SEAP activity is detected in lysate of uninfected Huh7-J20 cells (data not shown) indicating that the uncleaved fusion protein is enzymatically inactive. During serial passaging of cells electroporated with viral RNA, the relative SEAP activity in infected cell medium correlated directly with viral RNA levels, but not with infectious virus yield. Fluctuation in the virus yield of transfected cells during passaging of persistently infected cells has been observed in previous studies and likely reflects co-evolutionary events that favor adaptation of the virus and its host cell (Zhong et al., 2006). The reasons for the reduction in virus spread in Huh7-J20 cells are not immediately apparent. It is unlikely to be due to alteration in virus receptor expression levels given that these are polyclonal cells derived directly from the parent Huh7 line. However, we observed that Huh7-J20 cells do tend to grow at a lower rate than Huh7, which may explain these observations. Another possibility may be that the recruitment of the NS3/4A serine protease for cleavage of the fusion protein has a negative impact on viral replication.

The experimental data demonstrate that SEAP activity in the medium of infected Huh7-J20 cells correlated precisely with virus infectivity and RNA replication levels. Furthermore, the SEAP readout was comparable to the RT-qPCR method. The utility of this system in investigating virus entry and antibody-mediated neutralization of infection was also demonstrated using chimeric HCVcc displaying glycoproteins of diverse genotypes. In addition, SEAP readout of Huh7-J20 cells infected in the presence of selected inhibitors (IFN-α or inhibitors of viral protease or polymerase) showed the potential of this cell line in rapid and convenient screening of compounds that inhibit virus replication.

Another major advantage of this reporter system is that it alleviates the need to generate individual recombinant viruses carrying a reporter gene, as the SEAP secretion from Huh7-J20 is equally responsive to infection by chimeric as well as WT JFH1 HCVcc. The results show that the genotype 2a JFH1 protease readily cleaves the genotype 1b-based NS4A/4B protease substrate sequence that links the EGFP–SEAP fusion protein in this cell line. Given the high conservation of the NS4A/4B recognition site of NS3/4A protease across different HCV genotypes (Grakoui et al., 1993) (Fig. 1B), it is possible that this reporter cell line may be useful in quantitating infection of any novel cell culture infectious HCV (HCVcc) of diverse genotype/subtypes that are isolated in future.

The advantages of SEAP as a reporter in biological systems are well known. Specifically, it dispenses with the need to wash and lyse cells as the enzyme is released into the medium, and its activity is easily detectable by chemiluminescence. These properties, in combination with a stable cell line, are particularly conducive for screening large numbers of patient sera, specific antibodies or compounds for inhibition of HCV infection and replication, if necessary in a high throughput format. Moreover, the Huh7-J20 cell line would be an effective tool in studies dissecting the mechanisms of virus entry.

In conclusion, the Huh7-J20 reporter cell line offers significant advantages for rapid quantification of virus infectivity and replication in terms of ease and expense over the more conventional methods such as the FFU, $CCID_{50}$ and RT-qPCR assays. Furthermore, it represents a valuable tool for studying the process of HCV infection, and for screening and developing novel antiviral compounds.

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