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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.

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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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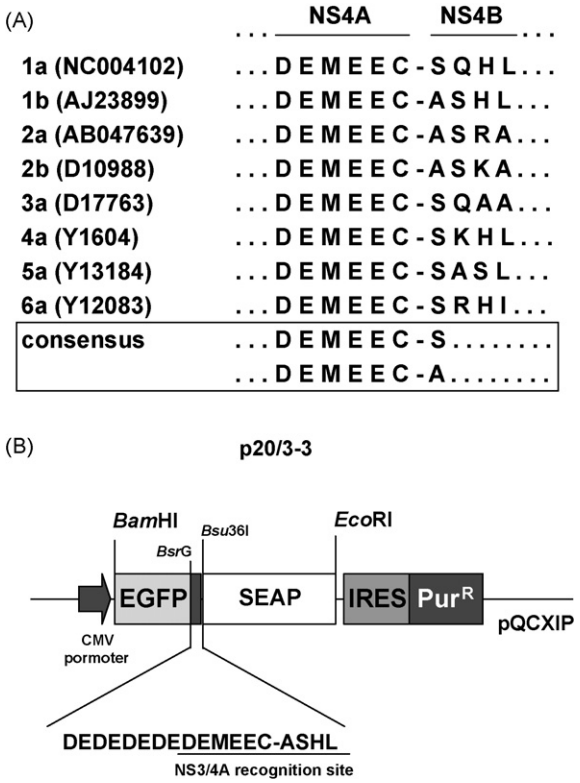

Fig. 1. Schematic diagram of fusion protein construct. (A) Alignment of the conserved amino acid residues representing the cleavage site of NS3/4A of diverse HCV genotypes. The accession numbers of the sequences of the HCV genotypes are shown in brackets. (B) The construct p20/3-3 contains the sequence 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).

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