Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 359 (2007) 57–62
BBRC
www.elsevier.com/locate/ybbrc

Development and characterization of a recombinant cDNA-based hepatitis C virus system

Jean Ndjomou [a], Ying Liu [a], John O'Malley [a], Maria Ericsson [b], Johnny J. He [a,c,d,*]

[a] Department of Microbiology and Immunology, Indiana University School of Medicine, R2 302, 950 W. Walnut Street, Indianapolis, IN 46202, USA
[b] Electron Microscopy Facility, Harvard Medical School, Boston, MA 02115, USA
[c] Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[d] Walther Cancer Institute, Indianapolis, IN 46206, USA

Received 4 May 2007
Available online 21 May 2007

Abstract

Invention of subgenomic HCV replicon a few years ago and recent success of *in vitro* production of infectious HCV have improved our knowledge of the HCV life cycle, replication, pathogenesis, and screening of anti-HCV therapeutics. However, the highly genotype-dependent nature of the *in vitro* HCV production system has limited its potential for HCV research. In this study, we constructed a recombinant DNA-based HCV system that contained EF-1α promoter-driven HCV genotype 1b with HCV E1/E2 deleted and replaced by GFP. We co-transfected this recombinant cDNA with HCV E1/E2 or VSV-G expression plasmid into 293T cells, and we showed HCV protein expression and processing and demonstrated production of HCV-like particles in culture supernatant of co-transfected cells. These results support potential use of this system for studies on expression and processing of the HCV polyprotein and assembly and release of HCV-like particles.

Keywords: HCV; Recombinant system; 293T cells; Virus-like particles

Hepatitis C virus (HCV) belongs to the *flaviviridae* family and the *hepacivirus* genus. HCV often induces lifelong persistent infection that frequently evolves to develop severe chronic liver diseases including cirrhosis and hepatocellular carcinoma [3]. There are more than 170 million people infected worldwide [23]. The virus is enveloped and has a 9.6-kb single-stranded genomic RNA of positive polarity. It contains a 5′ untranslated region (UTR) and a 3′UTR, a single open reading frame (ORF) encoding a polyprotein of approximately 3011 aa, which is processed co- and post-translationally by host and viral proteases into structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [20].

Most insights into the cellular and viral requirements for HCV replication are based on the *in vitro* genotype 1b subgenomic replicon [14]. Recently, new *in vitro* systems capable of producing infectious HCV particles have been described for genotype 2a [22,27] and 1a [25]. Undoubtedly, these systems will advance our understanding of HCV life cycle and facilitate development of anti-HCV therapeutics. Nevertheless, they also have limitations. Some evidences such as the poor responsiveness of some genotypes to IFN-α/ribavirin therapies [15] and the high frequency of association of genotype 1 with cirrhosis and liver cancer [26] point to biological differences among genotypes. Thus, alternative *in vitro* propagation systems are needed for HCV genotype 1b as well as other genotypes. In this study, we constructed a recombinant cDNA HCV system and took advantage of the highly transfectable nature of non-hepatocyte 293T cells to characterize expression of HCV proteins

* Corresponding author. Address: Department of Microbiology and Immunology, Indiana University School of Medicine, R2 302, 950 W. Walnut Street, Indianapolis, IN 46202, USA. Fax: +1 317 274 7592.
E-mail address: jjhe@iupui.edu (J.J. He).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.073

and RNA as well as HCV-like particle production in these cells.

Materials and methods

Plasmids. VSV-G expression plasmid was described elsewhere [8] and HCV Con1E1/E2 construct was provided by Dr. J. McKeating. pEF.HCV was constructed by cloning the full-length HCV 1b cDNA (Accession No. D89815) from plasmid pBR322T703X (gift from Dr. T. Miyamura) into the pEF6/V5-His-TOPO/LacZ-derived backbone (Invitrogen, Carlsbad, CA) using standard molecular cloning techniques. pEF.HCV.E-.GFP was made in the context of pEF.HCV plasmid, in which E1/E2 genes were replaced by GFP gene and thus encodes HCV core, GFP, p7, and all HCV nonstructural proteins. pEF.HCV.E-.GFP.5BΔ10 was derived from pEF.HCV.E-.GFP by deleting 10 amino acids that are required for HCV 5B polymerase activity using QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). pEF.Core.GFP was constructed in a similar fashion and only encodes core and GFP proteins. The GFP gene was derived from pEGFP-C1 (Clontech, Mountain View, CA). The HCV coding sequences in these constructs are under the control of the human translational elongation factor 1-α (EF-1α) promoter. The constructs were verified by DNA sequencing.

Cell cultures and transfection. 293T cells were obtained from ATCC and propagated in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 1% L-glutamine at 37 °C in 5% CO_2. The cells were seeded at a density of 2×10^6 cells per 10-cm plate, and the transfections were carried out by the calcium phosphate precipitation method.

Cell lysates, subcellular fractionation, and Western blotting. For HCV protein expression analysis, transfected cells were washed twice with cold PBS, harvested and suspended in RIPA buffer plus 1 mM PMSF and incubated on ice for 20 min. The lysates were obtained by centrifugation at 14,000 rpm for 15 min at 4 °C. For subcellular fractionation, transfected cells were washed as above, recovered by centrifugation at 500*g* for 5 min, suspended in 1 ml hypotonic buffer (10 mM Tris–HCl pH 7.8, 10 mM NaCl) plus 1 mM PMSF and 1% protease inhibitor cocktail (Roche, Indianapolis, IN), incubated on ice for 20 min, and homogenized by passing 20 times through a 27-gauge needle. The nuclei were recovered by centrifugation at 1500*g* for 10 min at 4 °C. The membranes were pelleted from the post-nuclear supernatant by ultracentrifugation through 6% (w/v) sucrose cushion in Beckman SW55Ti rotor at 150,000*g* for 1 h at 4 °C. The proteins in the cytosolic fraction (supernatant) were precipitated with 20% trichloroacetic acid and washed with 80% acetone. The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The proteins were resolved on 12% SDS–PAGE, transferred to membranes, and probed with 1:20 dilution of HCV mAbs for core, NS5A, and NS3 (ViroStat, Portland, ME), or with human HCV patient sera, followed by incubation with appropriate peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were revealed by ECL Plus kit (Amersham Biosciences).

RNase protection assay (RPA). To generate sense (+) and anti-sense (−) HCV-specific RNA probes, the HCV 5′UTR was amplified from pEF.HCV.E-.GFP by PCR with primers containing SmaI sites and cloned into SmaI digested-pGEM-3Z (+) vector (Promega, Madison, WI) to obtain the plasmid pGEM-3Z-5′UTR. The probes were synthesized with Maxiscript RNA transcription kit (Ambion, Austin, TX) using linearized pGEM-3Z-5′UTR as template and T7 RNA polymerase (for sense probe) and SP6 RNA polymerase (for anti-sense probe) following the manufacturer's instructions. Total RNA was extracted from the cells using TRIzol total RNA kit (Invitrogen), treated with DNase I at 37 °C for 45 min, and purified by phenol/chloroform extraction. The RPA was performed with BD RiboQuant™ RPA kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Briefly, 40 μg total RNA was hybridized with 8×10^4 cpm each probe. The RNA hybrids were treated with RNase A/T1 followed by proteinase K and purified as above. The protected probes were analyzed by electrophoresis on 6% polyacrylamide–7.5 M urea gel and visualized by autoradiography.

Virus preparation and viral protein and RNA detection. 293T cells were transfected with pEF.HCV.E-.GFP in combination with HCV E1/E2 or VSV-G expression plasmid. Forty-eight hours post-transfection, the supernatant was collected and clarified by centrifugation at 3000 rpm for 1 h at 4 °C. Virus was concentrated by addition of one-fourth volume of sterile 40% polyethylene glycol/0.4 M NaCl in TNE buffer and incubation at 4 °C overnight. The virus precipitates were collected by centrifugation at 20,000 rpm for 3 h at 4 °C in SW48 rotor (Beckman–Coulter, Fullerton, CA). The pellet was suspended in SDS sample buffer, resolved on 12% SDS–PAGE and analyzed for HCV core by Western blotting. Alternatively, RNA was extracted from the pellet with TRIzol kit (Invitrogen) and viral RNA was detected by RPA with HCV 5′UTR anti-sense probe. For sucrose density gradient analysis of HCV virus-like particles, supernatant from transfected cells was clarified and concentrated as above, the pellets were suspended in 2 ml TNE buffer plus 1 mM PMSF and overlaid onto 10–60% sucrose gradient in TNE buffer. The mixture was then ultracentrifuged at 178,000*g* for 20 h at 4 °C in SW41 rotor (Beckman–Coulter). Fractions of 1 ml were collected from top to bottom of the gradient and analyzed for VSV-G by Western blotting and for HCV RNA by RT-PCR.

Electron microscopic analysis of HCV-like particles. The cell culture supernatant from transfected 293T cells was clarified by centrifugation at 3000 rpm for 1 h at 4 °C. The cell-free preparation was fixed with 2.5% glutaraldehyde for 30 min on ice, loaded onto 30% sucrose cushion in TNC buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$) containing 1 mM PMSF, and ultracentrifuged at 178,000*g* for 20 h at 4 °C in a SW 41Ti rotor. The virus pellet was suspended in 100 μl TNC buffer and 5 μl of the sample was adsorbed to a carbon coated grid that had been made hydrophilic by a 30 s exposure to a glow discharge in an Edwards Auto 306 vacuum evaporator. Excess liquid was removed with filter paper and the samples were stained with 1% uranyl acetate for 1 min. The grids were examined using a JEOL 1200EX transmission electron microscope and digital Images were taken with DITABIS imaging plates.

Results and discussion

Construction of a recombinant DNA-based HCV clone

Recent success of HCV virus production in cell cultures offers the opportunity to look into the virus life cycle but has the limitation that it appears to be highly dependent on the JFH-1 genotype 2a strain. Thus, in this study we attempted to develop a surrogate model and constructed a recombinant HCV cDNA under the control of EF-1α promoter (Fig. 1). The cDNA derived from HCV genotype 1b isolate NIHJ1, which was shown to be infectious for humans and chimpanzees [1]. This system allows convenient delivery of HCV genome into the cells in the form of DNA by transfection. We removed the envelope genes to allow pseudotyping of progeny HCV-like virions with different viral envelopes by in *trans* co-expression. Besides the preferency of HCV to infect and replicate in liver cell lines, studies have demonstrated the replication in non-hepatocyte cell lines [2,12]. As 293T cells are highly susceptible to the calcium phosphate precipitation-mediated DNA transfection, we chose these cells as a platform to characterize this recombinant HCV cDNA.

HCV protein expression and localization in 293T cells

Next, we transfected the new recombinant HCV cDNA clone into 293T cells and determined the HCV protein expression. Western blot analysis showed that HCV core

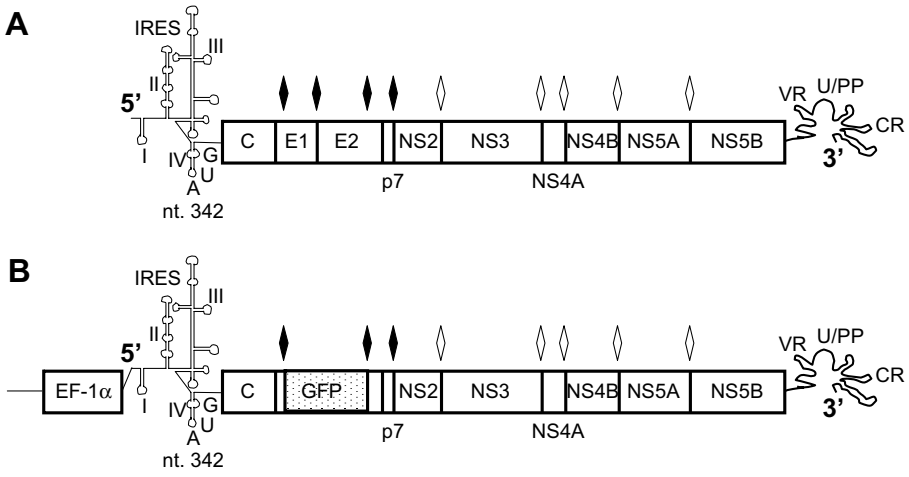

Fig. 1. HCV genomic organization and recombinant HCV construct. (A) HCV genome consists of a 5′UTR containing an IRES within the stem loops II, III, and IV, a single ORF encoding a polypeptide, which is processed into core (C), glycoproteins E1/E2, nonstructural proteins p7, NS2, NS3, NS4A/B, NS5A/B, and a 3′UTR organized into variable region (VR), polyuridine/polypyrimidine tract (U/PP), and conserved region (CR). (B) The recombinant HCV DNA construct pEF.HCV.E-.GFP developed in this study. GFP gene was inserted in place of E1/E2, and part of the N-term of E1 and C-term of E2 were retained to ensure proper processing of the polyprotein. Closed diamond: cleavage sites of cellular signal peptidase; open diamond: cleavage sites of HCV NS2-NS3/NS3 proteases.

and NS5A proteins were expressed at expected molecular weights (Fig. 2A). Previous study has identified two forms of core protein (p23 and p21): core precursor (191 aa) and matured core (174 aa) protein [24]. Likewise, we detected these two forms of core protein. In addition, we also detected two forms of NS5A using an anti-NS5A antibody, which probably represented the basal phosphorylated (p56) and the hyperphosphorylated (p58) forms of NS5A, as

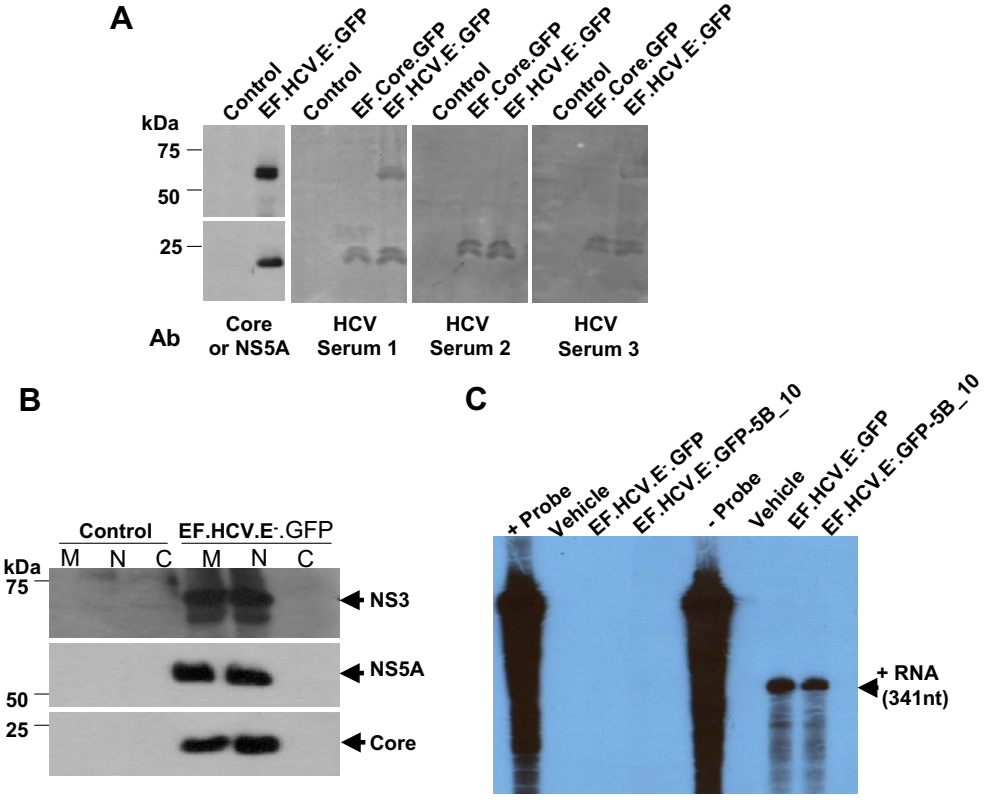

Fig. 2. Expression, subcellular localization of HCV proteins, and HCV RNA expression. (A) 293T cells were transfected with pEF.HCV.E-.GFP, pEF.Core.GFP, or pEF empty vector control. Transfected cells were lysed in RIPA buffer, and analyzed by Western blotting using anti-core and anti-NS5A mAbs, or HCV-positive patient sera. (B) Transfected cells were subjected to subcellular fractionation and proteins in the cytosolic (C), membrane (M), and nuclear fractions (N) were analyzed by Western blotting. (C) 293T cells were transfected with pEF.HCV.E-.GFP, pEF.HCV.E-.GFP-5BΔ10, or pEF empty vector control; total RNA was isolated and HCV negative and positive RNAs were determined by RPA using strand-specific probes.

expected [11]. These findings clearly show that HCV proteins in this newly engineered system were efficiently expressed and more importantly, correctly processed and post-translationally modified. Considering the requirement of viral proteases NS2-NS3/NS3 for processing of HCV polyprotein, expression of core and NS5A indicate that other HCV proteins encoded by the recombinant clone were expressed and biologically active.

We then determined subcellular localization of core, NS3, and NS5A proteins in 293T cells. Western blot analysis of nuclear, membrane, and cytosolic fractions showed that these proteins were all present in membrane and nuclear but not the cytosolic fractions (Fig. 2B). The association of these proteins with the intracellular membrane is consistent with other studies [5,7]. Their presence in the nuclear compartment may be attributable to an incomplete fractionation. However, there is a possibility that some of them are indeed present in the nucleus, as there is a nuclear localization signal in HCV core [6] and NS5A [9]. Besides, these proteins have been shown to be involved in transcription regulation [4,19]. Moreover, HCV NS3 was also found to associate with a nuclear protein [10]. Collectively, these results suggest that this recombinant cDNA clone was able to express HCV polyprotein in 293T cells and that these cells offered a cellular environment in which proper processing, intracellular targeting, and post-translational modifications of HCV proteins can take place.

Expression of HCV RNA transcripts in 293T cells

HCV replication involves expression of HCV proteins and synthesis of negative-strand HCV RNA intermediate from positive-strand HCV RNA genome [13]. Thus, we next determined expression of the positive and negative-strand HCV RNAs in 293T cells by RPA using strand-specific HCV RNA probes. Using *in vitro* transcribed sense and anti-sense non-labeled 5′UTR RNAs, we first confirmed that this assay was highly strand-specific with a detection limit of lower than 10 ng target RNA (data not shown). We then transfected 293T cells with this recombinant cDNA or a replication defective mutant (NS5BΔ10) and harvested cells for total RNA isolation. Using the RPA, we found that hybridization of isolated RNA with anti-sense 5′UTR probe gave rise to a protected RNA signal of expected size of 341 nt, indicating expression of the HCV positive-strand RNA in these cells (Fig. 2C). However, hybridization with sense 5′UTR probe failed to detect any RNA signal of negative polarity, suggesting that no expression of negative-stranded HCV RNA or no HCV replication took place in these cells, or HCV replication in these cells was beyond the detection limit of the assay.

Analysis of the production of HCV virus-like particles in cell culture supernatant

One of our goals was to produce and characterize HCV-like viruses in human non-hepatocyte cells *in vitro*. Successful expression of HCV genomic RNA as well as proteins from the recombinant cDNA clone raised the possibility of producing HCV virus-like particles in the culture supernatant. Thus, we next tested this possibility by co-expressing this cDNA clone with HCV E1/E2 or VSV-G envelope expression plasmid. The virus-like particles were purified from cell-free supernatant by sucrose centrifugation and analyzed for HCV core protein by Western blotting and HCV RNA by RPA. Core protein and VSV-G (Fig. 3A), HCV envelope protein (data not shown) as well as positive-strand HCV RNA (Fig. 3B) were detected in the virus pellets of co-transfected cells. To ensure production of virus-like particles, we further purified the viruses using the sucrose band ultracentrifugation and performed electron microscopic analysis. Both HCV E1/E2 and VSV-G co-transfected cells produced virus-like particles that were morphologically homogeneous with a diameter of approximately 30 nm (Fig. 4A). Although HCV or HCV-like particles produced in other *in vitro* systems were larger in size i.e., approximately 50 nm in diameter [16,22], HCV particles isolated from HCV-infected patients are quite heterogeneous in sizes ranging from 20 to 60 nm [17,21]. Thus, the discrepancy of the particle size may likely attribute to different sources of producing cells in which their membrane biochemical composition such as lipid and lipoproteins are different.

To further characterize the virus-like particles, we performed the equilibrium density ultracentrifugation of HCV/VSV-G like-viruses in 10–60% sucrose gradient

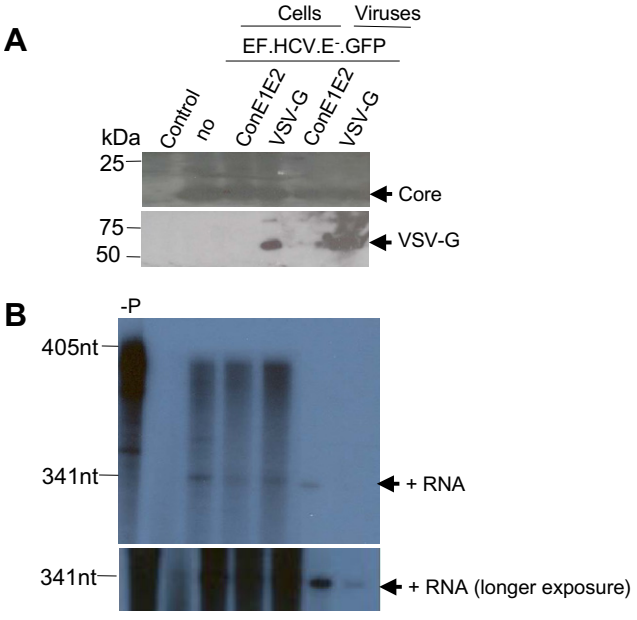

Fig. 3. Production of HCV viral-like particles. 293T cells were transfected with pEF empty vector, pEF.HCV.E-.GFP alone (no env), or pEF.HCV.E-.GFP with HCV ConE1/E2 (E1E2) or VSV-G plasmid. Supernatant was collected, cleared of cell debris, centrifuged to pellet the viruses, and viral protein lysates and RNA were prepared. Both cell and virus lysates were analyzed by Western blotting for VSV-G and HCV core proteins (A) or by RPA for HCV + RNA using anti-sense probe (B).

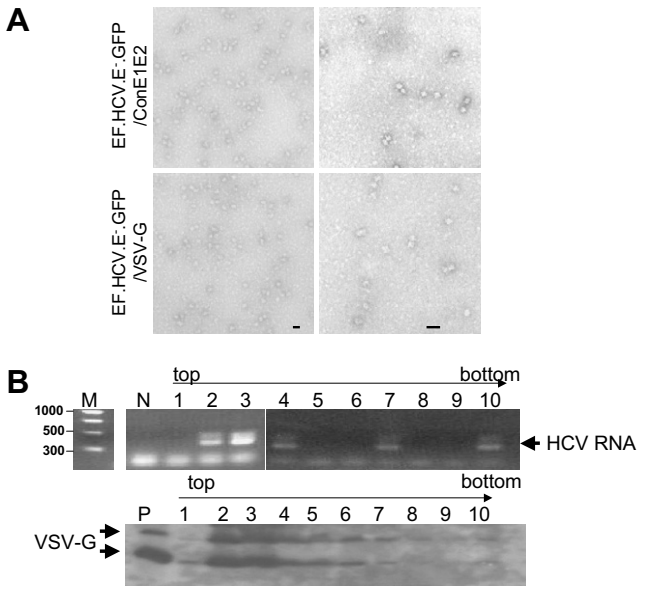

Fig. 4. Electron microscopy of virus-like particles and sucrose density analysis of virus preparation. The virus pellets were subjected to purification by passing through a 30% sucrose cushion at 178,000*g* for 20 h at 4 °C. Purified viruses were fixed in 2% formaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 and then visualized by negative staining electron microscopy (A). Scale bar: 50 nm. For virus-like particle fractionation, viral pellets were overlaid over 10–60% sucrose gradient and ultracentrifuged as above. Fractions of 1 ml were collected from top to bottom and analyzed for VSV-G by Western blotting and for HCV RNA by RT-PCR (B).

followed by fractionation and analysis for VSV-G protein and HCV RNA. HCV RNA was found to co-fractionate with VSV-G in most fractions with both higher abundance in the third fraction (Fig. 4B), suggesting a physical association of HCV RNA and VSV-G protein. Nevertheless, HCV RNA containing-fractions without VSV-G were also detected, which is consistent with the notion that different types of HCV RNA complexes were released from the transfected cells. In fact, HCV RNA which is not associated to viral glycoproteins has been described in hepatoma cell lines [18,25]. The "naked" RNA may represent either nonspecific release of HCV RNA or it may indeed be associated to membranous complexes or cellular components. The co-fractionation of viral RNA with VSV-G on sucrose gradient along with the electron microscopic analysis clearly demonstrated that viral-like particles were assembled and released into the culture medium. Similar results were obtained with HCV cognate envelope proteins (data not shown).

In summary, in this study we established and characterized a recombinant HCV cDNA that allowed HCV protein and RNA expression as well as HCV-like particles production in a non-hepatocyte human cell line. The finding that the viral-like particles produced in this system were smaller compared to those obtained in other *in vitro* systems suggests that virion morphogenesis and egress are different between liver and non-liver cells. Thus, this system may also offer an opportunity to study the processes of HCV assembly and release in non-hepatocyte cells.

Acknowledgments

We thank Dr. S.-L. Tan for HCV-positive human sera, scientific discussion, and critical reading of the manuscript, Dr. J. Mckeating for HCV ConE1/E2 expression plasmid, and Dr. T. Miyamura for the full-length HCV cDNA.

References

[1] H. Aizaki, Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, T. Miyamura, Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample, Hepatology 27 (1998) 621–627.

[2] S. Ali, C. Pellerin, D. Lamarre, G. Kukolj, Hepatitis C virus subgenomic replicons in the human embryonic kidney 293 cell line, J. Virol. 78 (2004) 491–501.

[3] H.J. Alter, L.B. Seeff, Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome, Semin. Liver Dis. 20 (2000) 17–35.

[4] A. Bergqvist, C.M. Rice, Transcriptional activation of the interleukin-2 promoter by hepatitis C virus core protein, J. Virol. 75 (2001) 772–781.

[5] V. Brass, E. Bieck, R. Montserret, B. Wolk, J.A. Hellings, H.E. Blum, F. Penin, D. Moradpour, An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A, J. Biol. Chem. 277 (2002) 8130–8139.

[6] S.C. Chang, J.H. Yen, H.Y. Kang, M.H. Jang, M.F. Chang, Nuclear localization signals in the core protein of hepatitis C virus, Biochem. Biophys. Res. Commun. 205 (1994) 1284–1290.

[7] N. El Hage, G. Luo, Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA, J. Gen. Virol. 84 (2003) 2761–2769.

[8] J. He, Y. Chen, M. Farzan, H. Choe, A. Ohagen, S. Gartner, J. Busciglio, X. Yang, W. Hofmann, W. Newman, C.R. Mackay, J. Sodroski, D. Gabuzda, CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia, Nature 385 (1997) 645–649.

[9] Y. Ide, L. Zhang, M. Chen, G. Inchauspe, C. Bahl, Y. Sasaguri, R. Padmanabhan, Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A, Gene 182 (1996) 203–211.

[10] A. Iwai, Y. Hasumura, T. Nojima, T. Takegami, Hepatitis C virus nonstructural protein NS3 binds to Sm-D1, a small nuclear ribonucleoprotein associated with autoimmune disease, Microbiol. Immunol. 47 (2003) 601–611.

[11] T. Kaneko, Y. Tanji, S. Satoh, M. Hijikata, S. Asabe, K. Kimura, K. Shimotohno, Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome, Biochem. Biophys. Res. Commun. 205 (1994) 320–326.

[12] T. Kato, T. Date, M. Miyamoto, Z. Zhao, M. Mizokami, T. Wakita, Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon, J. Virol. 79 (2005) 592–596.

[13] T. Laskus, M. Radkowski, A. Piasek, M. Nowicki, A. Horban, J. Cianciara, J. Rakela, Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes, J. Infect. Dis. 181 (2000) 442–448.

[14] V. Lohmann, F. Korner, J.O. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[15] M.P. Manns, J.G. McHutchison, S.C. Gordon, V.K. Rustgi, M. Shiffman, R. Reindollar, Z.D. Goodman, K. Koury, M.H. Ling, J.K. Albrecht, Peginterferon alfa-2b plus ribavirin compared with

interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial, Lancet 358 (2001) 958–965.
[16] E. Matsuo, H. Tani, C.K. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A.H. Patel, T. Miyamura, Y. Matsuura, Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus, Biochem. Biophys. Res. Commun. 340 (2006) 200–208.
[17] M.A. Petit, M. Lievre, S. Peyrol, S. De Sequeira, P. Berthillon, R.W.H. Ruigrok, C. Trepo, Enveloped particles in the serum of chronic hepatitis C patients, Virology 336 (2005) 144–153.
[18] T. Pietschmann, V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, R. Bartenschlager, Persistent and transient replication of full-length hepatitis C virus genomes in cell culture, J. Virol. 76 (2002) 4008–4021.
[19] S.J. Polyak, K.S.A. Khabar, D.M. Paschal, H.J. Ezelle, G. Duverlie, G.N. Barber, D.E. Levy, N. Mukaida, D.R. Gretch, Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response, J. Virol. 75 (2001) 6095–6106.
[20] K.E. Reed, C.M. Rice, Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties, Curr. Top. Microbiol. Immunol. 242 (2000) 55–84.
[21] A. Trestard, Y. Bacq, L. Buzelay, F. Dubois, F. Barin, A. Goudeau, P. Roingeard, Ultrastructural and physicochemical characterization of the hepatitis C virus recovered from the serum of an agammaglobulinemic patient, Arch. Virol. 143 (1998) 2241–2245.
[22] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.
[23] World Health Organization Hepatitis C, Wkly. Epidemiol. Rec. 72 (1997) 65–69.
[24] K. Yasui, T. Wakita, K. Tsukiyama-Kohara, S.I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J.R. Wands, M. Kohara, The native form and maturation process of hepatitis C virus core protein, J. Virol. 72 (1998) 6048–6055.
[25] M. Yi, R.A. Villanueva, D.L. Thomas, T. Wakita, S.M. Lemon, Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells, Proc. Natl. Acad. Sci. USA 103 (2006) 2310–2315.
[26] N.N. Zein, Clinical significance of hepatitis C virus genotypes, Clin. Microbiol. Rev. 13 (2000) 223–235.
[27] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. USA 102 (2005) 9294–9299.