# Promising Targets for Anti-Hepatitis C Virus Agents

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**Abstract:** Hepatitis C virus (HCV) infection is a serious global health problem, with 3-4 million new cases reported each year. Chronic HCV infection places 170 million people at risk of developing liver cirrhosis and hepatocellular carcinoma. However, difficulties in preparing HCV particles *in vitro* have delayed development of effective anti-HCV therapies. In 2005, Wakita *et al.* developed an *in vitro* method to prepare HCV particles, thereby enabling researchers to better understand the mechanism of HCV infection. Other recent advances include development of a virus-free system for evaluating HCV replication and the identification of HCV receptors, such as claudin-1 and occludin, that may serve as targets for anti-HCV drugs. In this review, we discuss recent findings in HCV infection research, including discovery of new potential targets for anti-HCV therapy.

**Keywords:** Hepatitis c virus, CD81, claudin-1, NS3 helicase, cyclophilin, miRNA122.

## INTRODUCTION

It is estimated that approximately 170 million people worldwide are infected with hepatitis C virus (HCV). Chronic HCV infection induces cirrhosis of the liver or hepatocellular carcinoma. Currently, no vaccines or inhibitors that block HCV entry into cells are approved for clinical use. Standard therapy for chronic HCV infection is the combination of pegylated interferon (IFN) and ribavirin (RBV); however, only 50% to 60% of infected patients get a sustained anti-viral response by this therapy. In addition, the severe side effects typical of IFN and RBV treatment often lead patients to stop treatment, and development of novel treatments with fewer serious side effects are therefore necessary.

Hepatitis C virus is a single-stranded RNA virus belonging in the family *Flaviviridae*. The viral genome is approximately 9,600 nucleotides, containing a 5'untranslated region (5' UTR), a region encoding a polyprotein of about 3,000 amino acids, and a 3' UTR. An internal ribosome-entry site (IRES) in the 5' UTR induces cap-independent translation. Once translated, the viral polyprotein is proteolytically processed by cellular signal peptidases and viral proteases into at least 10 mature viral proteins. Three of these proteins (Core, E1, and E2) are the structural proteins included in virions. It is unclear whether protein p7 is included in virions. Non-structural proteins (NS) include NS2, NS3, NS4A, NS4B, NS5A, and NS5B, and all except NS2 are necessary for formation of the complex associated with viral replication. In this review, we summarize recent developments in anti-HCV agents and discuss potent targets for anti-HCV agents.

## INHIBITORS OF HCV ENTRY INTO CELLS

### HCV Receptors

Hepatitis C virus contains two glycosylated envelope proteins, E1 and E2. While the role of E1 in infection is poorly understood, E2 is known to play a critical role through binding to the cell surface receptor and facilitating virus entry. Several receptors and co-receptors are involved in HCV infection, including CD81, scavenger receptor class B type I (SR-BI), low-density lipoprotein receptor (LDLR), claudin-1, and occludin [1-5]. Although it has been demonstrated that both CD81 and SR-BI directly bind to E2, there is no evidence that claudin-1 and occludin bind the HCV envelope, suggesting that claudin-1 and occludin may interact with other co-receptors to induce HCV entry.

Development of inhibitors that block envelope protein E2 from interacting with cellular receptors is an important area of anti-HCV research. One such class of inhibitors, (ssDNA) aptamers that recognize the HCV E2 protein, was isolated using a living cell surface technique (Systematic Evolution of Ligands by Expotential Enrichment). The ssDNA aptamer ZE2 binds to E2 with high affinity and inhibits its interaction with CD81, and was shown to block HCV infection *in vitro* [6].

Other inhibitors of HCV infection include proteins that bind to or modulate the activity of CD81 and prevent its interaction with CD81 with E2. Salicylate derivatives identified through virtual screening inhibit HCV infection by binding to the open conformation of the large extracellular loop (LEL) of CD81 and preventing its binding to E2. Benzyl salicylate inhibits the interaction of CD81-LEL with E2 by 25% at 50 μM [7]. Another modulator of CD81 activity is PSCK9, a regulator protein of membrane-bound receptors such as LDLR, ApoER2, and very low-density lipoprotein receptor. A recent study showed that PSCK9 deregulates the cell surface localization of CD81. Soluble PCSK9 inhibits HCV infection *in vitro* in a dose-dependent manner [8].

Claudin-1 has been identified as a co-receptor involved in HCV entry into cells, and its interaction with CD81 may help facilitate the early and late stages of HCV entry [4]. Claudin-1 is estimated to be a co-receptor that interacts with CD81. Recently, a claudin-1 antibody was developed that helped elucidate the role of claudin-1 in HCV infection. Anti-claudin-1 inhibited HCV infection at the same stage of HCV entry at which an anti-CD81 antibody did [9]. Since there is no evidence that claudin-1 binds directly to any HCV envelope proteins, it is believed that claudin-1 interacts with CD81 to form a complex that enables HCV cell entry, and may thus serve as a target for development of new HCV entry inhibitors.

Several HCV entry inhibitors that target neither the HCV envelope proteins nor cellular receptors have also been developed. One such inhibitor is C5A, an amphipathic α-helical peptide derived from the membrane anchor domain of HCV NS5A. C5A prevents initiation and spread of HCV infection by destabilizing virions, and has been shown to destroy the integrity of other viral particles, including other *Flaviviridae* (West Nile virus and dengue virus), some paramyxoviruses, and human immunodeficiency virus [10, 11]. C5A might recognize lipid composition of virus membranes, leading to the antiviral activity of C5A to the other viruses [11].

Arbidol is a broad-spectrum antiviral agent that inhibits virus-induced membrane fusion [12-14]. Arbidol is an effective inhibitor of both hepatitis B and C, as well as a wide range of other viruses, including influenza A and B, parainfluenza virus 3, respiratory syncytial virus, and rhinovirus 14. Other agents that block viral entry into host cells include Peptide 75, a peptide derived from the HCV E2 protein transmembrane domain [15], and the Lamiridosins, compounds extracted from *Lamium album* [16]. While the mechanisms through which these agents act to inhibit viral infectivity are poorly understood, continued research may lead to development of additional novel series of inhibitors.

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### Protein p7 Inhibitors

Viroporin protein p7 has two membrane-spanning helices and oligomerizes to form a cation-selective ion channel or pore [17-19]. However, the function of p7 and its importance in the HCV infection cycle is unclear. Recent studies revealed that p7 is critical for HCV entry as well as the release of infectious virions both *in vitro* and *in vivo* [20-23]. While p7 is not required for HCV RNA replication [24, 25], the protein is necessary for assembly of the viral particle [22], suggesting that p7 may be a virion component.

Amantadine is an inhibitor of the influenza A virus M2 protein, which is also a viriporin protein. Amantadine also blocks HCV p7 ion channel activity [26, 27]. Several clinical trials showed that amantadine treatment may be effective in patients with chronic hepatitis C infection [28-33]. Other inhibitors of protein p7 have been also identified, including iminosugar derivatives, hexamethylene amiloride, rimantadine, and GSK1-3 [20, 34-37]. More importantly, three-dimensional structure and functional amino acids of protein p7 have been determined [38, 39]. These findings will facilitate the development of new inhibitors against this important HCV protein.

## INHIBITORS OF REPLICATION-ASSOCIATED VIRAL PROTEINS AND THE VIRAL GENOME

### IRES Inhibitors

The internal ribosome-entry site (IRES) is a well-defined structure of about 340 nucleotides in the 5' UTR of the HCV genome [40]. The host 40S ribosomal subunit and eukaryotic initiation factor 3 (eIF3) recognize the HCV IRES and synthesize viral polyproteins in a cap-independent manner.

The IRES consisting of three domains (domain II-IV) is an attractive target for antiviral drugs because the sequences of subdomains IIIe and IIId are well conserved in all HCV sub-types. Oligodeoxynucleotides targeting IRES domain IIId had antiviral effects [41]. The structures of sub-domain IIa and IIIb are also highly conserved among many HCV sub-types [42, 43]. The L-shaped conformation of sub-domain IIa directs the apical hairpin loop of domain IIb towards the ribosomal E site in the proximity of the active site [44, 45]. The L-shaped architecture of domain II is essential for binding of the 40S ribosome to the IRES. Benzimidazole targets domain IIa and inhibits HCV replication by inducing a widening of the RNA interhelical angle in sub-domain IIa, thereby leading to inhibition of IRES-driven translation [46]. Sub-domain IIIb consists of a hairpin loop, an internal loop and two mismatched cytosine bases. The sequence is not well conserved, but the three-dimensional structure is well conserved. Recognition of the structure of sub-domain IIIb by eIF3 is essential for IRES-dependent translation, indicating that modulation of sub-domain IIIb conformation may inhibit the HCV replication [47]. IRES domain IV contains the HCV translation start codon. The GCAC sequence near the initiator AUG codon is also essential for ribosome assembly [48, 49]. Several inhibitors, including shRNA, siRNA, and hammerhead ribozyme, target the GCAC sequence and efficiently inhibit HCV replication by blocking the initiation of translation [50-52].

### NS3 Helicase Inhibitors

Helicase NS3 possesses multifunctional enzymatic activities and plays an essential role in HCV replication [53]. The N-terminus of NS3 is a serine protease involved in viral polyprotein processing, while the C-terminus is an RNA helicase/nucleotide triphosphatase [54]. A number of inhibitors of NS3 protease activity, such as boceprevir, telaprevir, SCH-900518 and VX-813, have been clinically used as inhibitors of HCV replication [55, 56]. NS3 helicase unwinds RNA in a 3' to 5' direction on a 3' overhang region, using any NTPs or dNTPs as an energy source [57-60]. There are a variety of known NS3 helicase inhibitors with diverse modes of action. Benzimidazole and benzotriazole derivatives, acridone-4-carboxylic acid derivatives, triphenylmethane derivatives, QU663, and NS3 peptide (p14) have all been identified as inhibitors of the NS3 helicase [55, 61-65]. Acridone-4-carboxylic acid derivatives intercalate into RNA and inhibit both NS3 helicase and NS5B polymerase activities, while triphenylmethane derivatives inhibit NS3 helicase by preventing NTPase hydrolysis and RNA substrate binding. QU663 is a nucleotide-mimicking compound that inhibits NS3 helicase activity by competing with the enzyme for nucleic acid substrates. Finally, peptide p14, a highly conserved arginine-rich sequence of NS3 helicase, inhibits the enzyme by binding to domain I.

### NS4A

NS4A forms a stable heterodimeric complex with NS3 and has serine protease activity. The NS3/4A heterodimer cleaves viral polyprotein into mature viral proteins. An important inhibitor of NS4A is ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)- 3-(pyridine-3-carbonyl)thiourea] (ACH-806). EC50 values for ACH-806 were 30 and 14 nM in genotype 1a and 1b replicon systems, respectively [66]. ACH-806 has synergistic activity with the NS3 protease inhibitor as well as the NS5B polymerase inhibitor, and has no cross-resistance to either inhibitor [67, 68]. A clinical study has revealed that ACH-806 is an effective antiviral agent against HCV genotype 1 [69].

### NS4B

NS4B is believed to induce the formation of intracellular membrane structures termed the membranous web [70]. HCV replication complex consisting of NS4A, 5B and other NS proteins is co-localized with HCV RNA in the membranous web. An amphipathic N-terminal helix in NS4B mediates membrane association and forms the replication complex [71]. An arginine-rich motif in the C-terminus of NS4B specifically binds the 3' terminus of the negative HCV RNA strand, which is essential for HCV replication. Clemizole hydrochloride inhibits binding of NS4B to the negative RNA strand and thereby disrupts HCV replication [72]. However, the underlying mechanism has not been currently understood.

### NS5A

NS5A is a 56- to 58-kDa membrane-associated phosphoprotein consisting of three domains (domains I, II, and III). In its basally phosphorylated form (p56), NS5A is active in viral replication, whereas the hyperphosphorylated form (p58) is active in viral packaging [73, 74]. Domain I, located in the N-terminus of NS5A, contains a membrane anchoring helix and zinc- and RNA-binding motifs. Domain I has multiple functions, including promoting membrane association of the replication complex, zinc-binding, RNA-binding, and dimerization of NS5A. NS5A is localized in the replication complex on endoplasmic reticulum *via* the domain I, and NS5A interacts with 3'-ends of HCV plus and minus RNA strands. A class of compounds with a thiazolidinone core structure (BMS-824, -858, and -665) inhibits HCV replication *in vitro* by interfering with one or more of the functions of NS5A domain I. These compounds target 76 N-terminal amino acids of NS5A, and they may interfere with RNA-binding or NS5A dimerization. The resultant inhibition of hyperphosphorylation of NS5A might inhibit HCV replication [75]. Another class of NS5A inhibitors, the piperazinyl-N-phenylbenzamides, prevents HCV replication by blocking dimerization of NS5A [76].

### Cyclophilin

The immunosuppressant compound cyclosporin A (CsA) is one of the most well known HCV inhibitors. Cyclosporin A acts by

targeting cellular proteins involved in HCV replication [77, 78]. Several sub-types of cyclophilin (CyP), CyPA and CyPB, have been reported to be CsA targets [79-81]. A recent study involving the knockdown of individual CyP sub-types revealed that CyP40 is a novel target of CsA [82]. CyPB facilitates HCV replication *via* the regulation of the RNA binding ability of NS5B [81]. CyPA and CyPB are likely to play different roles in HCV replication than does CyPA. Non-immunosuppressive CsA analogs, such as NIM811, Debio-025, and SCY635, inhibit both CyPA and CyPB [83-85], while CyPB and CyP40 facilitate HCV replication in CsA-resistant cells. There is thus considerable interest in development of CyPB or CyP40 inhibitors [82].

### Lupus Autoantigen (La)

Many cellular proteins that interact with IRES elements and stimulate IRES-driven translation have been reported, including the lupus autoantigen (La), polypyrimidine tract binding protein (PTB), poly rC binding protein 2 (PCBP2), C23 nucleolin, and NS1-associated protein 1 [86-94]. These transacting proteins are termed IRES-transacting factors (ITAFs). Granzyme H interferes with La-mediated HCV-IRES translational activity by cleaving the La protein [95]. A synthetic peptide (named LAP) that corresponds to the 18 N-terminal amino acids of La efficiently blocks HCV replication [96]. It is believed that LAP competitively blocks La from interacting with the ITAFs PTB and PCBP2, suggesting the possibility that ITAFs would be suitable targets for inhibition of HCV replication [97].

### microRNAs

A few microRNAs (miRNAs) associated with HCV replication have been reported. A liver-specific miRNA, miR-122, facilitates HCV RNA replication by binding the 5' UTR of the viral genome [98]. It has been suggested that down-regulation of miR122 is involved in the anti-HCV activity of IFN [99]. Recently, development of a novel therapeutic agent targeting miR122 was reported. Silencing miR-122 with a locked nucleic acid (LAN)-modified phosphorothioate oligonucleotide (SPC3649) efficiently blocked HCV RNA replication in chronically infected chimpanzees [100].

Another miRNA, miR-199a, has anti-HCV activity that is independent of the IFN pathway. The target sequence of miR-199a is a

**Table 1. Targets for HCV Entry**

| Targets | Anti-HCV agents | Mechanisms |
|---|---|---|
| E2 | ssDNA aptamers (ZE2) | Inhibit the interaction of E2 with CD81 by binding to E2 |
| CD81 | Salicylate derivatives | Inhibit the interaction of E2 with CD81 by binding to LEL of CD81 |
| | Soluble PSCK9 | Deregulate the cell surface localization of CD81 |
| Claudin-1 | Claudin-1 antibody | Inhibit cell entry of HCV by blocking cladin-1 |
| Viral membrane | C5A | Destabilize virions |
| Cellular membrane | Arbidol | Inhibit virus-induced membrane fusion |
| Unknown | Peptide 75 | Not understood |
| | Lamiridosins | Not understood |
| p7 | Amantadine | Inhibit p7 ion channel activity |
| | Amino sugar derivatives | Inhibit p7 ion channel activity |

**Table 2. Targets for HCV Replication**

| Targets | Anti-HCV agents | Mechanisms |
|---|---|---|
| IRES | Benzimidazole | Widen the RNA interhelical angle in sub-domain IIa |
| | shRNA targeting 322-340 of the 5'UTR | Inhibit ribosome assembly |
| | siRNA targeting 331-350 of the 5'UTR | Inhibit ribosome assembly |
| NS3 | Boceprevir, Telaprevir, SCH-900518, VX-813 | Inhibit protease activity |
| | Benzimidazole derivatives | Inhibit NS3 helicase activity |
| | Benzotriazole derivatives, Acridone-4-carboxylic acid derivatives | Intercalate into RNA and inhibit NS3 helicase activity |
| | Triphenylmethane derivatives (QU663) | Inhibit NTPase hydrolysis |
| | NS3 peptide (p14) | Bind to NS3 domain I |
| NS4A | ACH-806 | Inhibit NS3/NS4A protease activity |
| NS4B | Clemizole hydrochloride | Inhibit binding of NS4B to HCV negative RNA strand |
| NS5A | Thiazolidinone | Inhibit a function of NS5A domain I |
| | Piperazinyl-N-phenylbenzamides | Inhibit dimerization of NS5A |
| CyP | CsA | Inhibit PPIase activity of CyPs |
| | NIM811, Debio-025, SCY635 | Inhibit PPIase activity of CyPs |
| La | Granzyme H | Cleave La protein |
| | LAP | Competitively inhibit binding of La protein to ITAFs |
| miR-122 | SPC3649 | Silence miR-122 |

highly conserved region among HCV sub-types located in domain II of the HCV IRES. Thus, miRNAs are also attractive targets for development of new HCV inhibitors [101].

## CONCLUSIONS

The development of an *in vitro* amplification system for HCV by the Wakita group in 2005 has had a profound impact on studies of this important virus [102]. This amplification system has enabled researchers to produce viral particles in sufficient quantities to obtain a better understanding of the molecular mechanism underlying HCV infection, and has aided in the development of inhibitors of a variety of viral target molecules. The targets for anti-HCV therapeutic agents that have been discussed here can be classified into molecules involved in HCV entry (HCV receptors and p7) and in HCV replication (HCV and host cellular components) (Tables **1** and **2**). Inhibitors of NS3/4A protease or NS5B polymerase are promising anti-HCV agents among them. However, frequent mutation of HCV during proliferation has led to the emergence of drug-resistant viruses. To address this issue, numerous efforts have been paid on identification of cellular factors involved in viral replication and infection. One such promising anti-HCV agent is the LAN-modified oligonucleotide that targets the liver specific miRNA associated with HCV replication. This agent showed anti-HCV activity in chronically infected chimpanzees without apparent side effects for an extended period [100]. Very recently, monoclonal antibodies against claudin-1 prevented infection of highly variable HCV quasispecies [103]. We believe that recent progress in understanding the biology of HCV combined with advances in medicinal chemistry will lead to additional breakthroughs in anti-HCV therapy.

## ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20659012), a Health and Labor Sciences Research Grant from the Ministry of Health, labor and Welfare of Japan, and by Sasagawa Science Grants from the Japan Science Society.

## ABBREVIATIONS

| | | |
|---|---|---|
| HCV | = | hepatitis C virus |
| IFN | = | interferon |
| RBV | = | ribavirin |
| UTR | = | untranslated region |
| IRES | = | internal ribosome-entry site |
| NS | = | nonstructural protein |
| SR-BI | = | scavenger receptor class B type I |
| LDLR | = | low density lipoprotein receptor |
| LEL | = | large extracellular loop |
| eIF3 | = | eukaryotic initiation factor 3 |
| CsA | = | Cyclosporin A |
| CyP | = | cyclophilin |
| La | = | lupus autoantigen |
| PTB | = | polypyrimidine tract binding protein |
| PCBP2 | = | poly rC binding protein 2 |
| ITAFs | = | IRES-transacting factors |
| miRNA | = | microRNA |
| LNA | = | locked nucleic acid |

## REFERENCES

[1] Pileri, P.; Uematsu, Y.; Campagnoli, S.; Galli, G.; Falugi, F.; Petracca, R.; Weiner, A.J.; Houghton, M.; Rosa, D.; Grandi, G.; Abrignani, S. Binding of hepatitis C virus to CD81. *Science*, **1998**, *282*, 938-941.

[2] Scarselli, E.; Ansuini, H.; Cerino, R.; Roccasecca, R.M.; Acali, S.; Filocamo, G.; Traboni, C.; Nicosia, A.; Cortese, R.; Vitelli, A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J.*, **2002**, *21*, 5017-5025.

[3] Agnello, V.; Abel, G.; Elfahal, M.; Knight, G.B.; Zhang, Q.X. Hepatitis C virus and other flaviviridae viruses enter cells *via* low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 12766-12771.

[4] Evans, M.J.; von Hahn, T.; Tscherne, D.M.; Syder, A.J.; Panis, M.; Wolk, B.; Hatziioannou, T.; McKeating, J.A.; Bieniasz, P.D.; Rice, C.M. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature*, **2007**, *446*, 801-805.

[5] Ploss, A.; Evans, M.J.; Gaysinskaya, V.A.; Panis, M.; You, H.; de Jong, Y.P.; Rice, C.M. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature*, **2009**, *457*, 882-886.

[6] Chen, F.; Hu, Y.; Li, D.; Chen, H.; Zhang, X.L. CS-SELEX generates high-affinity ssDNA aptamers as molecular probes for hepatitis C virus envelope glycoprotein E2. *PLoS One*, **2009**, *4*, e8142.

[7] Holzer, M.; Ziegler, S.; Neugebauer, A.; Kronenberger, B.; Klein, C.D.; Hartmann, R.W. Structural modifications of salicylates: inhibitors of human CD81-receptor HCV-E2 interaction. *Arch. Pharm. (Weinheim)*, **2008**, *341*, 478-484.

[8] Labonte, P.; Begley, S.; Guevin, C.; Asselin, M.C.; Nassoury, N.; Mayer, G.; Prat, A.; Seidah, N.G. PCSK9 impedes hepatitis C virus infection *in vitro* and modulates liver CD81 expression. *Hepatology*, **2009**, *50*, 17-24.

[9] Krieger, S.E.; Zeisel, M.B.; Davis, C.; Thumann, C.; Harris, H.J.; Schnober, E.K.; Mee, C.; Soulier, E.; Royer, C.; Lambotin, M.; Grunert, F.; Dao Thi, V.L.; Dreux, M.; Cosset, F.L.; McKeating, J.A.; Schuster, C.; Baumert, T.F. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-Claudin-1 associations. *Hepatology*, **2009**, *51*, 1144-1157.

[10] Cheng, G.; Montero, A.; Gastaminza, P.; Whitten-Bauer, C.; Wieland, S.F.; Isogawa, M.; Fredericksen, B.; Selvarajah, S.; Gallay, P.A.; Ghadiri, M.R.; Chisari, F.V. A virocidal amphipathic {alpha}-helical peptide that inhibits hepatitis C virus infection *in vitro*. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*, 3088-3093.

[11] Bobardt, M.D.; Cheng, G.; de Witte, L.; Selvarajah, S.; Chatterji, U.; Sanders-Beer, B.E.; Geijtenbeek, T.B.; Chisari, F.V.; Gallay, P.A. Hepatitis C virus NS5A anchor peptide disrupts human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA*, **2008**, *105*, 5525-5530.

[12] Brooks, M.J.; Sasadeusz, J.J.; Tannock, G.A. Antiviral chemotherapeutic agents against respiratory viruses: where are we now and what's in the pipeline? *Curr. Opin. Pulm. Med.*, **2004**, *10*, 197-203.

[13] Chai, H.; Zhao, Y.; Zhao, C.; Gong, P. Synthesis and *in vitro* anti-hepatitis B virus activities of some ethyl 6-bromo-5-hydroxy-1H-indole-3-carboxylates. *Bioorg. Med. Chem.*, **2006**, *14*, 911-917.

[14] Pecheur, E.I.; Lavillette, D.; Alcaras, F.; Molle, J.; Boriskin, Y.S.; Roberts, M.; Cosset, F.L.; Polyak, S.J. Biochemical mechanism of hepatitis C virus inhibition by the broad-spectrum antiviral arbidol. *Biochemistry*, **2007**, *46*, 6050-6059.

[15] Liu, R.; Tewari, M.; Kong, R.; Zhang, R.; Ingravallo, P.; Ralston, R. A peptide derived from hepatitis C virus E2 envelope protein inhibits a post-binding step in HCV entry. *Antiviral Res.*, **2010**, *86*, 172-179.

[16] Zhang, H.; Rothwangl, K.; Mesecar, A.D.; Sabahi, A.; Rong, L.; Fong, H.H. Lamiridosins, hepatitis C virus entry inhibitors from Lamium album. *J. Nat. Prod.*, **2009**, *72*, 2158-2162.

[17] Gonzalez, M.E.; Carrasco, L. Viroporins. *FEBS Lett.*, **2003**, *552*, 28-34.

[18] Carrere-Kremer, S.; Montpellier-Pala, C.; Cocquerel, L.; Wychowski, C.; Penin, F.; Dubuisson, J. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J. Virol.*, **2002**, *76*, 3720-3730.

[19] Patargias, G.; Zitzmann, N.; Dwek, R.; Fischer, W.B. Protein-protein interactions: modeling the hepatitis C virus ion channel p7. *J. Med. Chem.*, **2006**, *49*, 648-655.

[20] Jones, C.T.; Murray, C.L.; Eastman, D.K.; Tassello, J.; Rice, C.M. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J. Virol.*, **2007**, *81*, 8374-8383.

[21] Steinmann, E.; Penin, F.; Kallis, S.; Patel, A.H.; Bartenschlager, R.; Pietschmann, T. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog.*, **2007**, *3*, e103.

[22] Sakai, A.; Claire, M.S.; Faulk, K.; Govindarajan, S.; Emerson, S.U.; Purcell, R.H.; Bukh, J. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc. Natl. Acad. Sci. USA*, **2003**, *100*, 11646-11651.

[23] Lohmann, V.; Korner, F.; Koch, J.; Herian, U.; Theilmann, L.; Bartenschlager, R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science*, **1999**, *285*, 110-113.

[24] Blight, K.J.; McKeating, J.A.; Rice, C.M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.*, **2002**, *76*, 13001-13014.

[25] Griffin, S.D.; Beales, L.P.; Clarke, D.S.; Worsfold, O.; Evans, S.D.; Jaeger,

J.; Harris, M.P.; Rowlands, D.J. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett.*, **2003**, *535*, 34-38.

[26] Griffin, S.D.; Harvey, R.; Clarke, D.S.; Barclay, W.S.; Harris, M.; Rowlands, D.J. A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. *J. Gen. Virol.*, **2004**, *85*, 451-461.

[27] Smith, J.P. Treatment of chronic hepatitis C with amantadine. *Dig. Dis. Sci.*, **1997**, *42*, 1681-1687.

[28] Zeuzem, S.; Teuber, G.; Naumann, U.; Berg, T.; Raedle, J.; Hartmann, S.; Hopf, U. Randomized, double-blind, placebo-controlled trial of interferon alfa2a with and without amantadine as initial treatment for chronic hepatitis C. *Hepatology*, **2000**, *32*, 835-841.

[29] Mangia, A.; Minerva, N.; Annese, M.; Leandro, G.; Villani, M.R.; Santoro, R.; Carretta, V.; Bacca, D.; Giangaspero, A.; Bisceglia, M.; Ventrella, F.; Dell'Erba, G.; Andriulli, A. A randomized trial of amantadine and interferon versus interferon alone as initial treatment for chronic hepatitis C. *Hepatology*, **2001**, *33*, 989-993.

[30] Berg, T.; Kronenberger, B.; Hinrichsen, H.; Gerlach, T.; Buggisch, P.; Herrmann, E.; Spengler, U.; Goeser, T.; Nasser, S.; Wursthorn, K.; Pape, G.R.; Hopf, U.; Zeuzem, S. Triple therapy with amantadine in treatment-naive patients with chronic hepatitis C: a placebo-controlled trial. *Hepatology*, **2003**, *37*, 1359-1367.

[31] Teuber, G.; Pascu, M.; Berg, T.; Lafrenz, M.; Pausch, J.; Kullmann, F.; Ramadori, G.; Arnold, R.; Weidenbach, H.; Musch, E.; Junge, U.; Wiedmann, K.H.; Herrmann, E.; Zankel, M.; Zeuzem, S. Randomized, controlled trial with IFN-alpha combined with ribavirin with and without amantadine sulphate in non-responders with chronic hepatitis C. *J. Hepatol.*, **2003**, *39*, 606-613.

[32] von Wagner, M.; Hofmann, W.P.; Teuber, G.; Berg, T.; Goeser, T.; Spengler, U.; Hinrichsen, H.; Weidenbach, H.; Gerken, G.; Manns, M.; Buggisch, P.; Herrmann, E.; Zeuzem, S. Placebo-controlled trial of 400 mg amantadine combined with peginterferon alfa-2a and ribavirin for 48 weeks in chronic hepatitis C virus-1 infection. *Hepatology*, **2008**, *48*, 1404-1411.

[33] Pavlovic, D.; Neville, D.C.; Argaud, O.; Blumberg, B.; Dwek, R.A.; Fischer, W.B.; Zitzmann, N. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc. Natl. Acad. Sci. USA*, **2003**, *100*, 6104-6108.

[34] Steinmann, E.; Whitfield, T.; Kallis, S.; Dwek, R.A.; Zitzmann, N.; Pietschmann, T.; Bartenschlager, R. Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. *Hepatology*, **2007**, *46*, 330-338.

[35] Premkumar, A.; Wilson, L.; Ewart, G.D.; Gage, P.W. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. *FEBS Lett.*, **2004**, *557*, 99-103.

[36] Griffin, S.; Stgelais, C.; Owsianka, A.M.; Patel, A.H.; Rowlands, D.; Harris, M. Genotype-dependent sensitivity of hepatitis C virus to inhibitors of the p7 ion channel. *Hepatology*, **2008**, *48*, 1779-1790.

[37] StGelais, C.; Tuthill, T.J.; Clarke, D.S.; Rowlands, D.J.; Harris, M.; Griffin, S. Inhibition of hepatitis C virus p7 membrane channels in a liposome-based assay system. *Antiviral Res.*, **2007**, *76*, 48-58.

[38] Luik, P.; Chew, C.; Aittoniemi, J.; Chang, J.; Wentworth, P., Jr.; Dwek, R.A.; Biggin, P.C.; Venien-Bryan, C.; Zitzmann, N. The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy. *Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 12712-12716.

[39] StGelais, C.; Foster, T.L.; Verow, M.; Atkins, E.; Fishwick, C.W.; Rowlands, D.; Harris, M.; Griffin, S. Determinants of hepatitis C virus p7 ion channel function and drug sensitivity identified *in vitro*. *J. Virol.*, **2009**, *83*, 7970-7981.

[40] Jackson, R.J.; Kaminski, A. Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. *RNA*, **1995**, *1*, 985-1000.

[41] Gamble, C.; Trotard, M.; Le Seyec, J.; Abreu-Guerniou, V.; Gernigon, N.; Berree, F.; Carboni, B.; Felden, B.; Gillet, R. Antiviral effect of ribonuclease conjugated oligodeoxynucleotides targeting the IRES RNA of the hepatitis C virus. *Bioorg. Med. Chem. Lett.*, **2009**, *19*, 3581-3585.

[42] Lukavsky, P.J.; Otto, G.A.; Lancaster, A.M.; Sarnow, P.; Puglisi, J.D. Structures of two RNA domains essential for hepatitis C virus internal ribosome entry site function. *Nat. Struct. Biol.*, **2000**, *7*, 1105-1110.

[43] Klinck, R.; Westhof, E.; Walker, S.; Afshar, M.; Collier, A.; Aboul-Ela, F. A potential RNA drug target in the hepatitis C virus internal ribosomal entry site. *RNA*, **2000**, *6*, 1423-1431.

[44] Spahn, C.M.; Kieft, J.S.; Grassucci, R.A.; Penczek, P.A.; Zhou, K.; Doudna, J.A.; Frank, J. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science*, **2001**, *291*, 1959-1962.

[45] Lukavsky, P.J. Structure and function of HCV IRES domains. *Virus Res.*, **2009**, *139*, 166-171.

[46] Parsons, J.; Castaldi, M.P.; Dutta, S.; Dibrov, S.M.; Wyles, D.L.; Hermann, T. Conformational inhibition of the hepatitis C virus internal ribosome entry site RNA. *Nat. Chem. Biol.*, **2009**, *5*, 823-825.

[47] Collier, A.J.; Gallego, J.; Klinck, R.; Cole, P.T.; Harris, S.J.; Harrison, G.P.; Aboul-Ela, F.; Varani, G.; Walker, S. A conserved RNA structure within the HCV IRES eIF3-binding site. *Nat. Struct. Biol.*, **2002**, *9*, 375-380.

[48] Pudi, R.; Abhiman, S.; Srinivasan, N.; Das, S. Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by specific interaction of independent regions of human La autoantigen. *J. Biol. Chem.*, **2003**, *278*, 12231-12240.

[49] Pudi, R.; Srinivasan, P.; Das, S. La protein binding at the GCAC site near the initiator AUG facilitates the ribosomal assembly on the hepatitis C virus RNA to influence internal ribosome entry site-mediated translation. *J. Biol. Chem.*, **2004**, *279*, 29879-29888.

[50] Subramanian, N.; Mani, P.; Roy, S.; Gnanasundram, S.V.; Sarkar, D.P.; Das, S. Targeted delivery of hepatitis C virus-specific short hairpin RNA in mouse liver using Sendai virosomes. *J. Gen. Virol.*, **2009**, *90*, 1812-1819.

[51] Meng, S.; Wei, B.; Xu, R.; Zhang, K.; Wang, L.; Zhang, R.; Li, J. TAT peptides mediated small interfering RNA delivery to Huh-7 cells and efficiently inhibited hepatitis C virus RNA replication. *Intervirology*, **2009**, *52*, 135-140.

[52] Romero-Lopez, C.; Diaz-Gonzalez, R.; Barroso-delJesus, A.; Berzal-Herranz, A. Inhibition of hepatitis C virus replication and internal ribosome entry site-dependent translation by an RNA molecule. *J. Gen. Virol.*, **2009**, *90*, 1659-1669.

[53] Lam, A.M.; Frick, D.N. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. *J. Virol.*, **2006**, *80*, 404-411.

[54] Gallinari, P.; Brennan, D.; Nardi, C.; Brunetti, M.; Tomei, L.; Steinkuhler, C.; De Francesco, R. Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J. Virol.*, **1998**, *72*, 6758-6769.

[55] Chen, C.S.; Chiou, C.T.; Chen, G.S.; Chen, S.C.; Hu, C.Y.; Chi, W.K.; Chu, Y.D.; Hwang, L.H.; Chen, P.J.; Chen, D.S.; Liaw, S.H.; Chern, J.W. Structure-based discovery of triphenylmethane derivatives as inhibitors of hepatitis C virus helicase. *J. Med. Chem.*, **2009**, *52*, 2716-2723.

[56] Kwong, A.D.; McNair, L.; Jacobson, I.; George, S. Recent progress in the development of selected hepatitis C virus NS3.4A protease and NS5B polymerase inhibitors. *Curr. Opin. Pharmacol.*, **2008**, *8*, 522-531.

[57] Kim, D.W.; Gwack, Y.; Han, J.H.; Choe, J. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem. Biophys. Res. Commun.*, **1995**, *215*, 160-166.

[58] Tai, C.L.; Chi, W.K.; Chen, D.S.; Hwang, L.H. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J. Virol.*, **1996**, *70*, 8477-8484.

[59] Gwack, Y.; Kim, D.W.; Han, J.H.; Choe, J. Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem. Biophys. Res. Commun.*, **1996**, *225*, 654-659.

[60] Gwack, Y.; Kim, D.W.; Han, J.H.; Choe, J. DNA helicase activity of the hepatitis C virus nonstructural protein 3. *Eur. J. Biochem.*, **1997**, *250*, 47-54.

[61] Sarno, S.; Reddy, H.; Meggio, F.; Ruzzene, M.; Davies, S.P.; Donella-Deana, A.; Shugar, D.; Pinna, L.A. Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett.*, **2001**, *496*, 44-48.

[62] Borowski, P.; Deinert, J.; Schalinski, S.; Bretner, M.; Ginalski, K.; Kulikowski, T.; Shugar, D. Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur. J. Biochem.*, **2003**, *270*, 1645-1653.

[63] Stankiewicz-Drogon, A.; Palchykovska, L.G.; Kostina, V.G.; Alexeeva, I.V.; Shved, A.D.; Boguszewska-Chachulska, A.M. New acridone-4-carboxylic acid derivatives as potential inhibitors of hepatitis C virus infection. *Bioorg. Med. Chem.*, **2008**, *16*, 8846-8852.

[64] Maga, G.; Gemma, S.; Fattorusso, C.; Locatelli, G.A.; Butini, S.; Persico, M.; Kukreja, G.; Romano, M.P.; Chiasserini, L.; Savini, L.; Novellino, E.; Nacci, V.; Spadari, S.; Campiani, G. Specific targeting of hepatitis C virus NS3 RNA helicase. Discovery of the potent and selective competitive nucleotide-mimicking inhibitor QU663. *Biochemistry*, **2005**, *44*, 9637-9644.

[65] Gozdek, A.; Zhukov, I.; Polkowska, A.; Poznanski, J.; Stankiewicz-Drogon, A.; Pawlowicz, J.M.; Zagorski-Ostoja, W.; Borowski, P.; Boguszewska-Chachulska, A.M. NS3 Peptide, a novel potent hepatitis C virus NS3 helicase inhibitor: its mechanism of action and antiviral activity in the replicon system. *Antimicrob. Agents Chemother.*, **2008**, *52*, 393-401.

[66] Huang, M.; Sun, Y.; Yang, W.; Hou, X.; Fabrycki, J.; Nie, X.; Sanchez, A.; Zhao, Y.; Phadke, A.; Deshpande, M. ACH-806: A potent inhibitor of HCV replication with a novel mechanism of action. *J. Hepatol.*, **2007**, *46*, S221.

[67] Wyles, D.L.; Kaihara, K.A.; Schooley, R.T. Synergy of a hepatitis C virus (HCV) NS4A antagonist in combination with HCV protease and polymerase inhibitors. *Antimicrob. Agents Chemother.*, **2008**, *52*, 1862-1864.

[68] Yang, W.; Zhao, Y.; Fabrycki, J.; Hou, X.; Nie, X.; Sanchez, A.; Phadke, A.; Deshpande, M.; Agarwal, A.; Huang, M. Selection of replicon variants resistant to ACH-806, a novel hepatitis C virus inhibitor with no cross-resistance to NS3 protease and NS5B polymerase inhibitors. *Antimicrob. Agents Chemother.*, **2008**, *52*, 2043-2052.

[69] Pottage, J.; Lawitz, E.; Mazur, D.; Wyles, D.; Vargas, H.; Ghalib, R.; Gugliotti, R.; Donohue, M.; Robison, H. Short-term antiviral activity and safety of ACH-806 (GS-9132), an NS4A antagonist, in HCV genotype 1 infected individuals. *J. Hepatol.*, **2007**, *46*, S294.

[70] Egger, D.; Wolk, B.; Gosert, R.; Bianchi, L.; Blum, H.E.; Moradpour, D.; Bienz, K. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.*, **2002**, *76*, 5974-5984.

[71] Elazar, M.; Liu, P.; Rice, C.M.; Glenn, J.S. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J. Virol.*, **2004**, *78*, 11393-11400.

[72] Einav, S.; Gerber, D.; Bryson, P.D.; Sklan, E.H.; Elazar, M.; Maerkl, S.J.; Glenn, J.S.; Quake, S.R. Discovery of a hepatitis C target and its pharmacol-

ogical inhibitors by microfluidic affinity analysis. *Nat. Biotechnol.*, **2008**, *26*, 1019-1027.

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

[74] Neddermann, P.; Quintavalle, M.; Di Pietro, C.; Clementi, A.; Cerretani, M.; Altamura, S.; Bartholomew, L.; De Francesco, R. Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J. Virol.*, **2004**, *78*, 13306-13314.

[75] Lemm, J.A.; O'Boyle, D., 2nd; Liu, M.; Nower, P.T.; Colonno, R.; Deshpande, M.S.; Snyder, L.B.; Martin, S.W.; St Laurent, D.R.; Serrano-Wu, M.H.; Romine, J.L.; Meanwell, N.A.; Gao, M. Identification of hepatitis C virus NS5A inhibitors. *J. Virol.*, **2010**, *84*, 482-491.

[76] Conte, I.; Giuliano, C.; Ercolani, C.; Narjes, F.; Koch, U.; Rowley, M.; Altamura, S.; De Francesco, R.; Neddermann, P.; Migliaccio, G.; Stansfield, I. Synthesis and SAR of piperazinyl-N-phenylbenzamides as inhibitors of hepatitis C virus RNA replication in cell culture. *Bioorg. Med. Chem. Lett.*, **2009**, *19*, 1779-1783.

[77] Watashi, K.; Hijikata, M.; Hosaka, M.; Yamaji, M.; Shimotohno, K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology*, **2003**, *38*, 1282-1288.

[78] Nakagawa, M.; Sakamoto, N.; Enomoto, N.; Tanabe, Y.; Kanazawa, N.; Koyama, T.; Kurosaki, M.; Maekawa, S.; Yamashiro, T.; Chen, C.H.; Itsui, Y.; Kakinuma, S.; Watanabe, M. Specific inhibition of hepatitis C virus replication by cyclosporin A. *Biochem. Biophys. Res. Commun.*, **2004**, *313*, 42-47.

[79] Chatterji, U.; Bobardt, M.; Selvarajah, S.; Yang, F.; Tang, H.; Sakamoto, N.; Vuagniaux, G.; Parkinson, T.; Gallay, P. The isomerase active site of cyclophilin A is critical for hepatitis C virus replication. *J. Biol. Chem.*, **2009**, *284*, 16998-17005.

[80] Yang, F.; Robotham, J.M.; Nelson, H.B.; Irsigler, A.; Kenworthy, R.; Tang, H. Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance *in vitro*. *J. Virol.*, **2008**, *82*, 5269-5278.

[81] Watashi, K.; Ishii, N.; Hijikata, M.; Inoue, D.; Murata, T.; Miyanari, Y.; Shimotohno, K. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell*, **2005**, *19*, 111-122.

[82] Goto, K.; Watashi, K.; Inoue, D.; Hijikata, M.; Shimotohno, K. Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor. *Cancer Sci.*, **2009**, *100*, 1943-1950.

[83] Goto, K.; Watashi, K.; Murata, T.; Hishiki, T.; Hijikata, M.; Shimotohno, K. Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. *Biochem. Biophys. Res. Commun.*, **2006**, *343*, 879-884.

[84] Paeshuyse, J.; Kaul, A.; De Clercq, E.; Rosenwirth, B.; Dumont, J.M.; Scalfaro, P.; Bartenschlager, R.; Neyts, J. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication *in vitro*. *Hepatology*, **2006**, *43*, 761-770.

[85] Hopkins, S.; Scorneaux, B.; Huang, Z.; Murray, M.G.; Wring, S.; Smitley, C.; Harris, R.; Erdmann, F.; Fischer, G.; Ribeill, Y. SCY-635, a novel non-immunosuppressive analog of cyclosporine that exhibits potent inhibition of hepatitis C virus RNA replication *in vitro*. *Antimicrob. Agents Chemother.*, **2010**, *54*, 660-672.

[86] Ali, N.; Siddiqui, A. Interaction of polypyrimidine tract-binding protein with the 5' noncoding region of the hepatitis C virus RNA genome and its functional requirement in internal initiation of translation. *J. Virol.*, **1995**, *69*, 6367-6375.

[87] Ali, N.; Siddiqui, A. The La antigen binds 5' noncoding region of the hepatitis C virus RNA in the context of the initiator AUG codon and stimulates internal ribosome entry site-mediated translation. *Proc. Natl. Acad. Sci. U S A*, **1997**, *94*, 2249-2254.

[88] Ali, N.; Pruijn, G.J.; Kenan, D.J.; Keene, J.D.; Siddiqui, A. Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation. *J. Biol. Chem.*, **2000**, *275*, 27531-27540.

[89] Blyn, L.B.; Towner, J.S.; Semler, B.L.; Ehrenfeld, E. Requirement of poly(rC) binding protein 2 for translation of poliovirus RNA. *J. Virol.*, **1997**, *71*, 6243-6246.

[90] Meerovitch, K.; Svitkin, Y.V.; Lee, H.S.; Lejbkowicz, F.; Kenan, D.J.; Chan, E.K.; Agol, V.I.; Keene, J.D.; Sonenberg, N. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J. Virol.*, **1993**, *67*, 3798-3807.

[91] Boussadia, O.; Niepmann, M.; Creancier, L.; Prats, A.C.; Dautry, F.; Jacquemin-Sablon, H. Unr is required *in vivo* for efficient initiation of translation from the internal ribosome entry sites of both rhinovirus and poliovirus. *J. Virol.*, **2003**, *77*, 3353-3359.

[92] Hellen, C.U.; Witherell, G.W.; Schmid, M.; Shin, S.H.; Pestova, T.V.; Gil, A.; Wimmer, E. A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc. Natl. Acad. Sci. U S A*, **1993**, *90*, 7642-7646.

[93] Hunt, S.L.; Hsuan, J.J.; Totty, N.; Jackson, R.J. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev.*, **1999**, *13*, 437-448.

[94] Izumi, R.E.; Valdez, B.; Banerjee, R.; Srivastava, M.; Dasgupta, A. Nucleolin stimulates viral internal ribosome entry site-mediated translation. *Virus Res.*, **2001**, *76*, 17-29.

[95] Romero, V.; Fellows, E.; Jenne, D.E.; Andrade, F. Cleavage of La protein by granzyme H induces cytoplasmic translocation and interferes with La-mediated HCV-IRES translational activity. *Cell Death Differ.*, **2009**, *16*, 340-348.

[96] Izumi, R.E.; Das, S.; Barat, B.; Raychaudhuri, S.; Dasgupta, A. A peptide from autoantigen La blocks poliovirus and hepatitis C virus cap-independent translation and reveals a single tyrosine critical for La RNA binding and translation stimulation. *J. Virol.*, **2004**, *78*, 3763-3776.

[97] Fontanes, V.; Raychaudhuri, S.; Dasgupta, A. A cell-permeable peptide inhibits hepatitis C virus replication by sequestering IRES transacting factors. *Virology*, **2009**, *394*, 82-90.

[98] Jopling, C.L.; Yi, M.; Lancaster, A.M.; Lemon, S.M.; Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science*, **2005**, *309*, 1577-1581.

[99] Pedersen, I.M.; Cheng, G.; Wieland, S.; Volinia, S.; Croce, C.M.; Chisari, F.V.; David, M. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature*, **2007**, *449*, 919-922.

[100] Lanford, R.E.; Hildebrandt-Eriksen, E.S.; Petri, A.; Persson, R.; Lindow, M.; Munk, M.E.; Kauppinen, S.; Orum, H. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science*, **2010**, *327*, 198-201.

[101] Murakami, Y.; Aly, H.H.; Tajima, A.; Inoue, I.; Shimotohno, K. Regulation of the hepatitis C virus genome replication by miR-199a. *J. Hepatol.*, **2009**, *50*, 453-460.

[102] Wakita, T.; Pietschmann, T.; Kato, T.; Date, T.; Miyamoto, M.; Zhao, Z.; Murthy, K.; Habermann, A.; Kräusslich, H.G.; Mizokami, M.; Bartenschlager, R.; Liang, T.J. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.*, **2005**, *11*, 791-796.

[103] Fofana, I.; Krieger, S.E.; Grunert, F.; Glauben, S.; Xiao, F.; Fafi-Kremer, S.; Soulier, E.; Royer, C.; Thumann, C.; Mee, C.J.; McKeating, J.A.; Dragic, T.; Pessaux, P.; Stoll-Keller, F.; Schuster, C.; Thompson, J.; Baumert, T.F. Monoclonal anti-claudin-1 antibodies prevent hepatitis C virus infection of primary human hepatocytes. *Gastroenterology*, **2010**, *139*, 953-964.

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Paper: gkr047
MANUSCRIPT CATEGORY: METHODS ONLINE



*Nucleic Acids Research, 2011, 1–8*
*doi:10.1093/nar/gkr047*

# Adenovirus vector-mediated assay system for Hepatitis C virus replication

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Received April 9, 2010; Revised January 17, 2011; Accepted January 18, 2011

## ABSTRACT

**The efficient delivery of the hepatitis C virus (HCV) RNA sub-genomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.**

## INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6 kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

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RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

## MATERIALS AND METHODS

### Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at 500 μg/ml. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% $CO_2$ atmosphere at 37°C.

### Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with EcoRI linker and digested with EcoRI/NheI, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the EcoRI-XbaI site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in $pP_1$WT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

### Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters (from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating $pP_I235$, $pP_I311$ or $pP_I412$. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

### Preparation of Ad vector expressing HCV replicon

The subgenomic HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into $pP_I235$, and then the firefly luciferase was replaced by the renilla luciferase to form $pP_I235$-HCV or $pP_I235$-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, $pP_I235$-EL, $pP_I235$-HCV and $pP_I235$-ΔGDD were digested with I-CeuI and PI-SceI, and then ligated with I-CeuI/PI-SceI-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with PacI and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). $AdP_I235$-EL, $AdP_I235$-HCV and $AdP_I235$-ΔGDD were purified by $CsCl_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

### Expression of plasmid-based HCV replicon

Huh7 cells were transfected with 0.8 μg of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000*g* for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

### Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with 0.8 μg of pPol I-HCV and 0.2 μg of pCMVβ or infected with $AdP_I235$-HCV (10 MOI) and Ad-tTA (50 MOI). After 24 or 1.5 h of transfection, the cells were treated with recombinant human interferon-α8 (IFN-α8) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque, Kyoto, Japan).

### Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with 0.1 μg of reporter plasmid ($pP_I235$-EL, $pP_I311$-EL, $pP_I412$-EL or $pP_I$WT-EL), 0.8 μg of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and 0.1 μg of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

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concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

### Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector ($AdP_I235$-EL or $AdP_I235$-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

### Western blotting

Huh7 cells were co-infected with $AdP_I235$-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

### Evaluation of NS5B-dependent replication

Huh7 cells were transfected with $AdP_I235$-HCV or $AdP_I235$-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, luciferase activities in the lysates were measured.

### Detection of a fragment of the HCV negative strand RNA

Huh7 cells were co-infected with $AdP_I235$-HCV or $AdP_I235$-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commercial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respectively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), respectively. The putative sizes of the PCR products were 242 bp for NS3 and 89 bp for GAPDH. The PCR products were separated on 2% agarose gel.

## RESULTS

### RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells (670 ± 39.1 RLU). IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

### RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene transduction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influenza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

### Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates expression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector

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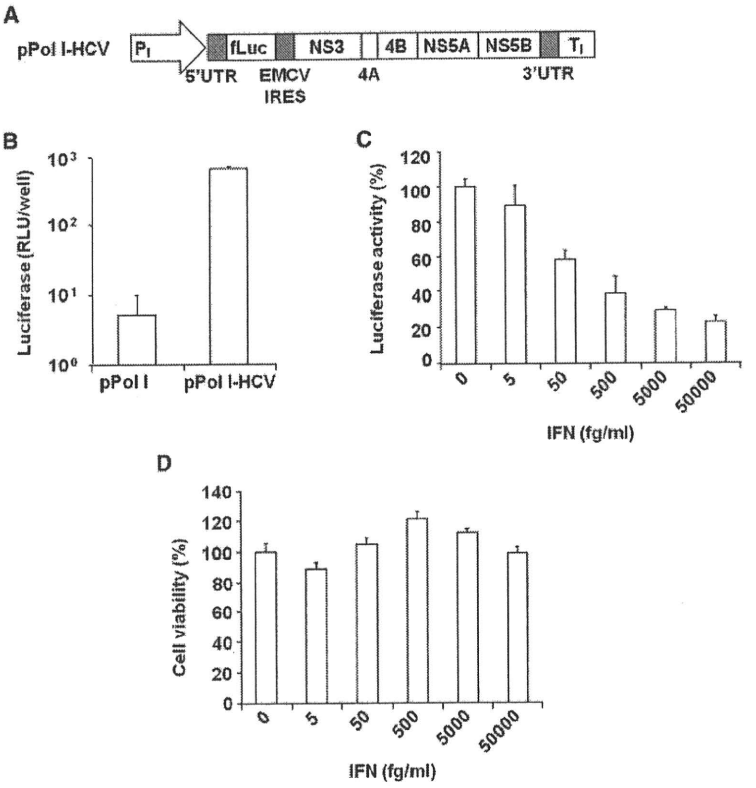


**Figure 1.** Preparation of plasmid expressing subgenomic HCV RNA driven by RNA pol I promoter. (**A**) Schematic construct of sub-genomic HCV genome-expression cassette. The HCV sub-genome gene was driven by the RNA pol I promoter ($P_I$) and terminator ($T_I$). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD ($n$ = 3). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD ($n$ = 3).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The $P_I$235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the $P_I$235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. Ad-TRE$P_I$235 (MOI of 10) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

## Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2 kb (25). The HCV replicon is ~8.9 kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8 kb (26). We were successful in preparing Ad5/35 vector particles ($9.53 \times 10^8$ IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.

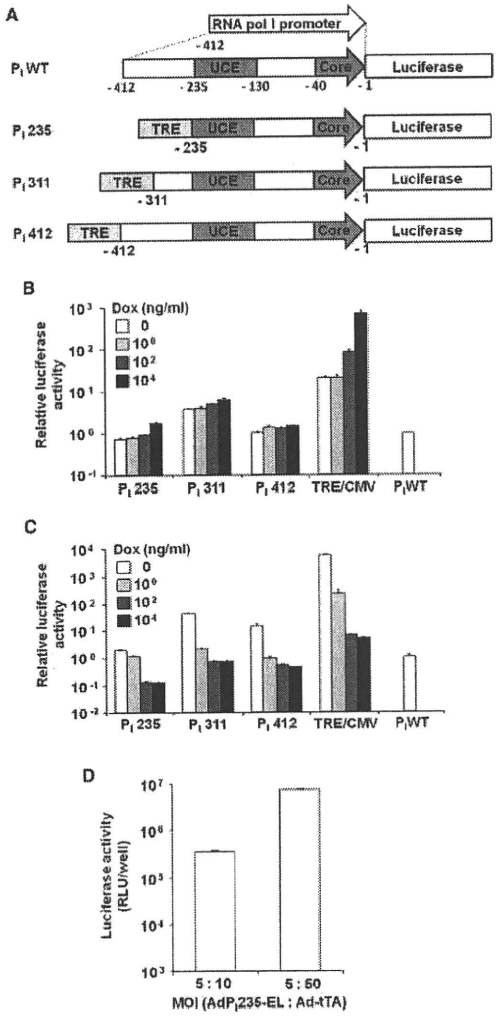


**Figure 2.** Development of tet-controllable RNA pol I promoter. (A) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in $P_I235$, $P_I311$ and $P_I412$. (**B** and **C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and transactivator plasmid [rtTA (B) or tTA (C)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of $pP_IWT$-EL-transfected cells. Data are mean ± SD ($n = 3$). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with $AdP_I235$-EL and Ad-tTA. The MOI ratio of $AdP_I235$-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD ($n = 3$).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector ($AdP_I235$-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with $AdP_I235$-HCV but not $AdP_I235$-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3E, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3F). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

## DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV sub-genome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and sub-genome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.

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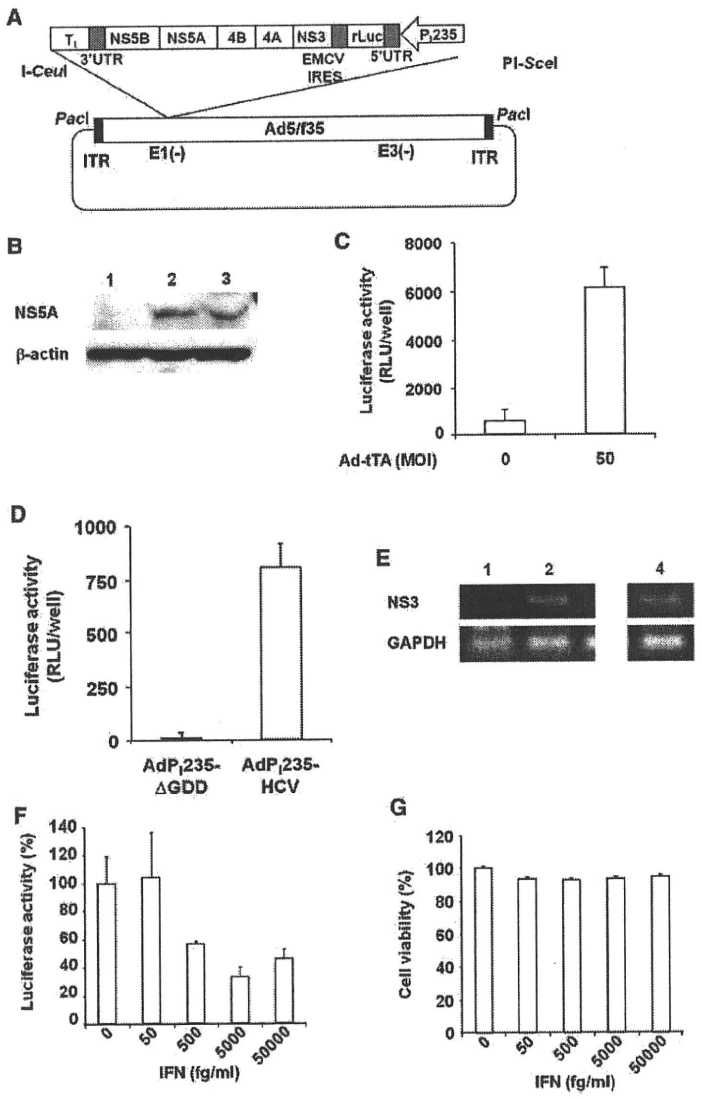


**Figure 3.** Preparation of Ad vector to monitor HCV replication. (**A**) Construct of Ad vector. The Ad vector contained the chimeric RNA pol I promoter ($P_I235$) and the HCV replicon to monitor HCV replication as the luciferase expression. (**B**) Expression of HCV NS5A protein in Huh7 cells transfected with Ad-$P_I235$-HCV. The cells were transfected with Ad-$P_I235$-HCV (10 MOI) and Ad-tTA (50 MOI). After 72 h of incubation, the cells were harvested, and the lysates (30 μg) were subjected to SDS–PAGE, followed by immunoblotting with antibody against NS5A. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7 cells infected with Ad-$P_I235$-HCV; lane 3, Huh7.5.1 1b Feo cells. (**C**) Expression of luciferase in the Ad vector-transfected cells. Huh7 cells were co-infected with Ad-$P_I235$-HCV (10 MOI) and 0 or 50 MOI of Ad-tTA. After an additional 48 h of incubation, the luciferase activity was measured. Data represent the mean ± SD ($n$ = 3). (**D**) Involvement of NS5B in expression of luciferase in the Ad vector-transfected cells. Huh7 cells were infected with Ad$P_I235$-HCV or Ad$P_I235$-ΔGDD (3 MOI) and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, the luciferase activity was measured. Data represent the mean ± SD ($n$ = 3). (**E**) Expression of minus-stranded HCV RNA in the Ad vector-transfected cells. Huh7 cells were co-infected with Ad$P_I235$-HCV or Ad$P_I235$-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then RT-PCR analysis was performed for detection of minus-stranded HCV NS3 and GAPDH. The PCR products were separated on 2% agarose gel. Huh7 cells and Huh7.5.1 1b Feo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7.5.1 1b Feo cells; lane 3, Huh7 cells infected with Ad$P_I235$-ΔGDD; lane 4, Huh7 cells infected with Ad$P_I235$-HCV. (**F** and **G**) Effect of IFN on the replication of HCV replicon. Huh7 cells were infected with Ad-$P_I235$-HCV (10 MOI) and Ad-rtTA (50 MOI). After 1.5 h of infection, the cells were treated with IFN at the indicated concentration for 72 h. Then, the luciferase activity (D) and the cell viability (E) were measured. Data represent the percentage of vehicle-treated cells. Data are the mean ± SD ($n$ = 3).

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Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1–8.2 kb (32), and the size of HCV replicon is ~8.2 kb (containing a 1.0-kb luciferase gene and a 7.2-kb fragment of HCV genome) (8). The lack of a successful preparation of Ad5 vector may be partly due to limitation of packaging transgene. Mizuguchi and Hayakawa prepared a chimeric Ad vector containing type 5 and type 35 fiber proteins, which is a package 8.8 kb of foreign gene (26). CD46 is a receptor for Ad type 35 (Ad35), and CD46 is ubiquitously expressed in human cells (33,34). The Ad5/35 chimera vector can transduce various human cells more effectively than Ad5 vectors, indicating that the Ad5/35 vector may be a better system than Ad5 (26,35). In this study, we successfully prepared an Ad5/35 vector coding a tet-regulated RNA pol I-driven HCV replicon, and we found that the Ad5/35 vectors could be applied to evaluation of anti-HCV activity.

In conclusion, to the best of our knowledge, this is the first report to establish a novel strategy for the preparation of Ad vector expressing the HCV genome by using a tet-controllable expression system. Replication-incompetent HCV particles will be a promising candidate for vaccine therapy for HCV. As mentioned above, the packaging size (8.8 kb) of Ad5/35 vector used in the present study is smaller than that of the HCV RNA genome (9.6 kb), and, therefore, the preparation of inactive HCV particles using Ad5/35 vector is impossible. Helper-dependent Ad vector (HDAd), in which all viral coding sequences are deleted, can deliver a large capacity of ~37 kb to cells (36). Tet-controllable RNA pol I HDAd vector might contribute to the development of vaccine therapy for HCV.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

We thank Y. Kawaoka (The University of Tokyo, Tokyo, Japan) and all members of our laboratory for providing us pHH21 and their helpful comments and discussion, respectively.

## FUNDING

Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20659012 to M.K.); Health and Labor Sciences Research Grant from the Ministry of Health, labor and Welfare of Japan (to M.K.); Sasagawa Science Grants from the Japan Science Society (to T.Y.). Funding for open access charge: XXX.

*Conflict of interest statement*. None declared.

## REFERENCES

1. Sarbah,S.A. and Younossi,Z.M. (2000) Hepatitis C: an update on the silent epidemic. *J. Clin. Gastroenterol.*, **30**, 125–143.
2. Carithers,R.L. Jr and Emerson,S.S. (1997) Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials. *Hepatology*, **26**, 83S–88S.
3. Hoofnagle,J.H. and di Bisceglie,A.M. (1997) The treatment of chronic viral hepatitis. *N. Engl. J. Med.*, **336**, 347–356.
4. Poynard,T., Bedossa,P., Chevallier,M., Mathurin,P., Lemonnier,C., Trepo,C., Couzigou,P., Payen,J.L., Sajus,M. and Costa,J.M. (1995) A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. *N. Engl. J. Med.*, **332**, 1457–1462.
5. Kato,N. (2001) Molecular virology of hepatitis C virus. *Acta. Med. Okayama*, **55**, 133–159.
6. Lohmann,V., Korner,F., Koch,J., Herian,U., Theilmann,L. and Bartenschlager,R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science*, **285**, 110–113.
7. Nakamura,M., Saito,H. and Hibi,T. (2008) Advances in genomic research on hepatitis C virus with a useful tool, replicon system. *Keio J. Med.*, **57**, 75–83.
8. Yokota,T., Sakamoto,N., Enomoto,N., Tanabe,Y., Miyagishi,M., Maekawa,S., Yi,L., Kurosaki,M., Taira,K., Watanabe,M. *et al.* (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep.*, **4**, 602–608.
9. Flatz,L., Bergthaler,A., de la Torre,J.C. and Pinschewer,D.D. (2006) Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA. *Proc. Natl Acad. Sci. USA*, **103**, 4663–4668.
10. Flick,R. and Pettersson,R.F. (2001) Reverse genetics system for Uukuniemi virus (Bunyaviridae): RNA polymerase I-catalyzed expression of chimeric viral RNAs. *J. Virol.*, **75**, 1643–1655.
11. Hoffmann,E., Neumann,G., Kawaoka,Y., Hobom,G. and Webster,R.G. (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl Acad. Sci. USA*, **97**, 6108–6113.
12. Neumann,G., Watanabe,T., Ito,H., Watanabe,S., Goto,H., Gao,P., Hughes,M., Perez,D.R., Donis,R., Hoffmann,E. *et al.* (1999) Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl Acad. Sci. USA*, **96**, 9345–9350.
13. Segura,M.M., Alba,R., Bosch,A. and Chillon,M. (2008) Advances in helper-dependent adenoviral vector research. *Curr. Gene Ther.*, **8**, 222–235.
14. Sharma,A., Li,X., Bangari,D.S. and Mittal,S.K. (2009) Adenovirus receptors and their implications in gene delivery. *Virus Res.*, **143**, 184–194.
15. Ozawa,M., Goto,H., Horimoto,T. and Kawaoka,Y. (2007) An adenovirus vector-mediated reverse genetics system for influenza A virus generation. *J. Virol.*, **81**, 9556–9559.
16. Mizuguchi,H. and Kay,M.A. (1999) A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum. Gene Ther.*, **10**, 2013–2017.
17. Mizuguchi,H. and Hayakawa,T. (2001) Characteristics of adenovirus-mediated tetracycline-controllable expression system. *Biochim. Biophys. Acta*, **1568**, 21–29.
18. Mizuguchi,H. and Kay,M.A. (1998) Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.*, **9**, 2577–2583.
19. Benihoud,K., Yeh,P. and Perricaudet,M. (1999) Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.*, **10**, 440–447.
20. Kovesdi,I., Brough,D.E., Bruder,J.T. and Wickham,T.J. (1997) Adenoviral vectors for gene transfer. *Curr. Opin. Biotechnol.*, **8**, 583–589.
21. Shenk,T. (1996) Adenoviridae: the viruses and their replication. In Fields,B.N., Knipe,D.M. and Howley,P.M. (eds), *In Fields Virology*, Vol. 2. Lippincott-Raven Publishers, Philadelphia, pp. 2118–2148.
22. Lee,E.R., Marshall,J., Siegel,C.S., Jiang,C., Yew,N.S., Nichols,M.R., Nietupski,J.B., Ziegler,R.J., Lane,M.B., Wang,K.X. *et al.* (1996) Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.*, **7**, 1701–1717.

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23. Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.
24. Gossen,M., Freundlieb,S., Bender,G., Muller,G., Hillen,W. and Bujard,H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766–1769.
25. Bett,A.J., Haddara,W., Prevec,L. and Graham,F.L. (1994) An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl Acad. Sci. USA*, **91**, 8802–8806.
26. Mizuguchi,H. and Hayakawa,T. (2002) Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. *Gene*, **285**, 69–77.
27. Bartenschlager,R., Ahlborn-Laake,L., Mous,J. and Jacobsen,H. (1993) Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J. Virol.*, **67**, 3835–3844.
28. Eckart,M.R., Selby,M., Masiarz,F., Lee,C., Berger,K., Crawford,K., Kuo,C., Kuo,G., Houghton,M. and Choo,Q.L. (1993) The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem. Biophys. Res. Commun.*, **192**, 399–406.
29. Hijikata,M., Mizushima,H., Akagi,T., Mori,S., Kakiuchi,N., Kato,N., Tanaka,T., Kimura,K. and Shimotohno,K. (1993) Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J. Virol.*, **67**, 4665–4675.
30. Tomei,L., Failla,C., Santolini,E., De Francesco,R. and La Monica,N. (1993) NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.*, **67**, 4017–4026.
31. Bartenschlager,R., Ahlborn-Laake,L., Yasargil,K., Mous,J. and Jacobsen,H. (1995) Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J. Virol.*, **69**, 198–205.
32. Bett,A.J., Prevec,L. and Graham,F.L. (1993) Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.*, **67**, 5911–5921.
33. Gaggar,A., Shayakhmetov,D.M. and Lieber,A. (2003) CD46 is a cellular receptor for group B adenoviruses. *Nat. Med.*, **9**, 1408–1412.
34. Seya,T., Nomura,M., Murakami,Y., Begum,N.A., Matsumoto,M. and Nagasawa,S. (1998) CD46 (membrane cofactor protein of complement, measles virus receptor): structural and functional divergence among species (review). *Int. J. Mol. Med.*, **1**, 809–816.
35. Sakurai,F., Mizuguchi,H. and Hayakawa,T. (2003) Efficient gene transfer into human CD34+cells by an adenovirus type 35 vector. *Gene Ther.*, **10**, 1041–1048.
36. Palmer,D.J. and Ng,P. (2005) Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther.*, **16**, 1–16.