厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

Ｃ型肝炎ウイルスの非構造蛋白５Ａを標的とした新規治療法の開発に関する研究

平成２３年度　総括研究報告書

研究代表者　　政木　隆博（平成23年4月～平成23年8月）
　　　　　　　村山　麻子（平成23年9月～平成24年3月）

平成２４（２０１２）年３月

厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

C型肝炎ウイルスの非構造蛋白5Aを標的とした
新規治療法の開発に関する研究

平成23年度　総括研究報告書

研究代表者　　政木　隆博（平成23年4月～平成23年8月）
　　　　　　　村山　麻子（平成23年9月～平成24年3月）

平成24（2012）年　3月

目　次

Ⅰ．総括研究報告

Ｃ型肝炎ウイルスの非構造蛋白５Ａを標的とした
新規治療法の開発に関する研究

研究代表者
政木隆博(平成 23 年 4 月〜平成 23 年 8 月)
国立感染症研究所　ウイルス第二部　主任研究官
村山麻子(平成 23 年 9 月〜平成 24 年 3 月）
国立感染症研究所　ウイルス第二部　研究員

研究要旨：本研究は、HCV のリン酸化蛋白質 NS5A を標的とした新規治療法の開発を目的としている。昨年度は、網羅的解析および HCV 培養細胞増殖系を用いた解析により HCV の生活環に関わる 3 種類のプロテインキナーゼを同定した。今年度はこれらのプロテインキナーゼが NS5A の細胞内局在を規定することにより、HCV の粒子形成過程に関与していることを明らかにした。さらに、これらのプロテインキナーゼの特異的阻害剤には抗 HCV 作用があることを示した。

A. 研究目的

　C型肝炎ウイルス(HCV)感染者は現在日本で約200万人いると推定されている。感染後は持続感染により肝炎が慢性化し、肝硬変を経て高率に肝細胞癌を発症することが知られており、公衆衛生上きわめて重要な問題となっている。肝発癌を防ぐためにはウイルス排除が必須である。現在の主な治療法はペグインターフェロンとリバビリンの併用療法であるが、その効果は血中ウイルス量、ウイルスの遺伝子型や患者自身の持つ遺伝子変異によっても大きく異なる。したがって、従来の抗HCV薬と異なる作用点をもつ新規治療法の開発は厚生労働行政上急務である。

　HCVの非構造蛋白質であるNS5Aはリン酸化蛋白質であり、NS5Aのリン酸化はウイルスゲノム複製や感染性ウイルス粒子形成に重要であることが報告されている。したがって、NS5Aのリン酸化を制御するプロテインキナーゼ(PK)の同定は、HCV生活環を理解する上で重要であり、また新たな創薬ターゲットになりうると考えられる。

　本研究では、NS5A を標的とした新規治療法の開発を目的としており、今年度は主に HCV NS5A をリン酸化する PK の HCV 生活環への関与および、そのメカニズムの解析を行った。また、PK 特異的阻害剤の抗 HCV 効果の検討も行った。

B. 研究方法

1). PK のノックダウンが HCV 侵入過程に与える影響の解析

HCV のエンベロープ蛋白質を持ったシュードウイルス(HCVpp)および、コントロールとして VSV の G 蛋白質を持ったシュードタイプウイルス(VSVpp)を用いて、PK のノックダウンが HCV の侵入過程に及ぼす影響を観察した。HuH-7 細胞に PK の siRNA を導入し、2 日後に HCVpp または VSVpp を感染させ、さらに 3 日間培養の後、細胞を回収した。HCVpp および VSVpp の侵入効率は細胞抽出液のルシフェラーゼの値を比較することにより評価した。

2). PK のノックダウンが NS5A の細胞内局在に与える影響の解析

HuH-7 細胞に PK に対する siRNA を発現するベクターを導入し、1 日後に HCV を multiplicity of infection (moi)=5 で感染させた。この siRNA 発現ベクターを細胞に導入すると、その細胞では同時に mCherry も発現するため、mCherry の発現により siRNA 発現細胞を見分けることができる。感染 3 日後に細胞を固定し、抗コア抗体および抗 NS5A 抗体で染色した。

3). PK によるリン酸化部位の同定

HCV 感染細胞および HCV 感染 PK ノックダウン細胞の細胞抽出液から NS5A 蛋白質を抗 HS5A 抗体を用いてそれぞれ精製し、質量分析法により解析した。

4). PK 阻害剤の HCV 増殖抑制効果の検討

HCV RNA を細胞に導入し、4 時間後に各

PK 阻害剤を 0-30µM の濃度で添加した。3 日後の培養上清のコア抗原量を測定し、薬剤無添加時の培養上清のコア抗原量と比較し、各 PK 阻害剤の抗 HCV 効果を評価した。

（倫理面への配慮）
各種研究材料の取り扱い及び組換えDNA実験は国立感染症研究所内のバイオリスク管理委員会、組換えDNA実験委員会等の承認を受けて行った。組換えHCVの作製は遺伝子組換え生物等の第二種使用等にあたるため「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」（平成15年法律第97号)の規定に従って申請を行い、承認を得た(大臣確認通知番号　大20-9　平成18年1月23日付17国文科振第47号、及び平成18年8月10日付18国文科振第16号)。

C. 研究結果
1). PK のノックダウンが HCV 侵入過程に与える影響の解析

昨年度の研究により、2 種類の新規プロテインキナーゼ(PK-1、PK-2)及び CK2α2 が HCV の生活環に関与する PK として同定された。また、それらのノックダウンが HCV 複製には影響しないこと、HCV 粒子形成過程に関与していることを示した。本年度は、これらの PK の HCV 侵入過程における関与について検討した。これらの PK のノックダウン細胞では VSVpp、HCVpp の感染効率はともにコントロール siRNA 導入細胞と同程度であった。比較として、HCV の受容体(CD81、Claudin-1、Occludin)のノックダウ

ンの影響を観察した。CD81をノックダウン
させた場合には、HCVppの感染効率は1/50
に低下し、Claudin-1、Occludinをノックダ
ウンさせた場合にはいずれも1/6に低下した。
これらの受容体のノックダウンはVSVppの
感染効率には影響しなかった。

2). PKのノックダウンがNS5Aの細胞内局在に与える影響の解析

PK-1、PK-2およびCK2α2のうち、ノックダウンした時に最もHCV産生に影響があったPK-1について、NS5Aの細胞内局在への影響を解析した。siRNA非導入細胞ではNS5Aは細胞内で脂肪滴周辺におけるコア蛋白質との共局在が観察された。しかし、PK-1のノックダウン細胞では、NS5Aとコアの共局在がなくなり、NS5Aの細胞内分布が脂肪滴周辺から細胞質全体へと変化していた。

3). PKによるリン酸化部位の同定

HCV感染細胞およびHCV感染PK-1ノックダウン細胞中のNS5A蛋白質をそれぞれ質量分析法により解析した。ドメインIとドメインIIの間の3箇所のセリン残基がPK-1ノックダウン細胞由来のNS5Aではリン酸化されていなかったことから、それらのセリン残基がPK-1によるリン酸化部位である可能性が示唆された。

4). PK阻害剤のHCV増殖抑制効果の検討

同定したPK(PK-1、PK-2、CK2α2)に対する阻害剤の抗HCV効果を検討した。4種類のPK阻害剤(D4476、IC261、PF670462、DMAT)において、30μM投与時のHCVコア抗原の分泌はそれぞれ1/12.7、1/2.6、1/3.2、1/8.4に低下した。また、この抗HCV効果は0-30μMの範囲で濃度依存的であり、この濃度範囲では強い細胞傷害性は認められなかった。

D. 考察

昨年度の網羅的解析およびHCV培養細胞増殖系を用いた解析により同定された3種類のPK(PK-1、PK-2、CK2α2)は、いずれもsiRNAによるノックダウンによりウイルス産生量が低下した。これらのPKの作用点解明のためにHCV生活環のそれぞれの過程について検討した結果、これらのPKのノックダウンはウイルス侵入、および複製活性には影響を与えなかった。しかし、ノックダウンによりウイルス粒子形成効率が低下したことから、これらのPKは細胞内でのウイルス粒子形成過程に関わることが示唆された。

さらに、メカニズムについて解析した。感染細胞内ではNS5Aとコア蛋白質は相互作用することにより共局在を示し、この相互作用はHCV粒子形成に重要であることが知られている。ところが、PK-1のノックダウン細胞では、NS5Aとコア蛋白質の共局在がなくなり、NS5Aの細胞内分布が脂肪滴周辺から細胞質全体へと変化していた。細胞内でのNS5Aとコアの相互作用はHCV粒子形成に重要であることから、PK-1はNS5Aとコア蛋白質の相互作用に直接的、またはNS5A

のリン酸化状態の変化などを通して間接的に関わっていると考えられる。

今回の解析で 4 種類の PK 特異的阻害剤に抗 HCV 効果があることが示された。今後、これらの PK の粒子形成過程への関与、および PK 阻害剤の抗 HCV 効果が JFH-1 株以外のウイルス株でも同様に観察されるかを検討する必要がある。また、これらの PK 阻害剤について、他の抗 HCV 薬(インターフェロン、プロテアーゼ阻害剤等)との併用効果や、薬剤の耐性変異の出現についても検討する必要がある。その後、HCV 感染マウスモデルを用いて、これらの PK 阻害剤の in vivo での効果を検討する予定である。

E. 結論

網羅的手法およびHCV培養細胞増殖系を用いた解析により感染性HCV産生に関与する3種類のプロテインキナーゼ(PK-1、PK-2、CK2α2)を同定した。これらのPKはHCVの感染性粒子形成過程に関わっており、そのメカニズムとして、PKが粒子形成に重要なNS5Aとコア蛋白質との共局在に必須であることを明らかにした。また、PKの特異的阻害剤は培養細胞でのHCVの増殖を抑制した。

本研究は、HCVゲノム複製、粒子形成機構の解明や、新たな抗HCV薬開発に貢献できると考えている。

F. 健康危険情報

特記事項なし。

G. 研究発表

1. 論文発表

1) Murayama A, Kato T, Akazawa D, Sugiyama N, Date T, Masaki T, Nakamoto S, Tanaka Y, Mizokami M, Yokosuka O, Nomoto A, Wakita T. Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1. J Virol. 86(4): 2143-2152, 2012.

2) Saeed M, Suzuki R, Watanabe N, Masaki T, Tomonaga M, Muhammad A, Kato T, Matsuura Y, Watanabe H, Wakita T, Suzuki T. Role of the Endoplasmic Reticulum-associated Degradation (ERAD) Pathway in Degradation of Hepatitis C Virus Envelope Proteins and Production of Virus Particles. J Biol Chem, 286: 37264-37273, 2011.

3) Saeed M, Shiina M, Date T, Akazawa D, Watanabe N, Murayama A, Suzuki T, Watanabe H, Hiraga N, Imamura M, Chayama K, Choi Y, Krawczynski K, Liang TJ, Wakita T, Kato T. In vivo adaptation of hepatitis C virus in chimpanzees for efficient virus production and evasion of apoptosis. Hepatology, 54: 425-433, 2011.

4) Okamoto Y, Masaki T, Murayama A, Munakata T, Nomoto A, Nakamoto S, Yokosuka O, Watanabe H, Wakita T, Kato T. Development of recombinant hepatitis C virus with NS5A from strains of genotypes 1 and

2.Biochem Biophys Res Commun. 2011 Jul 8;410(3):404-9.

5) Inoue Y, Aizaki H, Hara H, Matsuda M, Ando T, Shimoji T, Murakami K, Masaki T, Shoji I, Homma S, Matsuura Y, Miyamura T, Wakita T, Suzuki T. Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein. Virology 2011;410:38-47.

2. 学会発表

1) Okamoto Y, Masaki T, Murayama A, Wakita T, Kato T. Development of recombinant hepatitis C virus with NS5A from strains of genotypes 1 and 2: virus production and susceptibility to NS5A inhibitor. 18th International Symposium on Hepatitis C Virus and Related Viruses. September 8-12, 2011. Seattle, USA.

2) Murayama A, Sugiyama N, Yoshimura S, Ishihara- Sugano M, Wakita T, Kato T. Efficient HCV production system using HuH-7 subclone with high virus assembly efficiency. 18th International Symposium on Hepatitis C Virus and Related Viruses. September 8-12, 2011. Seattle, USA.

3) Okamoto Y, Masaki T, Murayama A, Nomoto A, Wakita T, Kato T. Strain Specific Susceptibility to The Hepatitis C Virus NS5A Inhibitor. International Union of Microbiological Societies 2011 Congress. XV International Congress of Virology. September 11-16, 2011. Sapporo, Japan.

4) Murayama A, Sugiyama N, Yoshimura S, Ishihara- Sugano M, Wakita T, Kato T. HuH-7 subclone that supports high HCV production due to high virus assembly. International Union of Microbiological Societies 2011 Congress. XV International Congress of Virology. September 11-16, 2011. Sapporo, Japan.

5) Watanabe N, Murayama A, Date T, Kato T, Aizaki H, Wakita T. Identification and analysis of envelope N-glycans required for HCV lifecycle. International Union of Microbiological Societies 2011 Congress. XV International Congress of Virology. September 11-16, 2011. Sapporo, Japan.

6) Matsumura T, Kato T, Tasaka-Fujita M, Murayama A, Masaki T, Wakita T, Imawari M. 25-hydroxy- vitamin D inhibits hepatitis C virus replication and production of the infectious viruses. The 62nd Annual Meeting of the American Association for the Study of Liver Diseases. November 4-8, 2011. San Francisco, USA.

7) 加藤孝宣、村山麻子、政木隆博、相崎英樹、脇田隆字. 国内献血検体を用いたC型肝炎ウイル

ス陽性血漿パネルの作製とウイルス量測定法の
評価. 第４７回 日本肝臓学会総会、2011年6月、
東京.

8) 加藤孝宣、<u>政木隆博</u>、脇田隆字. HCV JFH-1
キメラ株を用いた NS5A 阻害剤の株特異的抗ウ
イルス活性の評価. シンポジウム１０：Ｃ型肝炎
ウイルスの性状と治療の新たな展開 第１５回日
本肝臓学会大会、2010 年 10 月，福岡.

9) <u>村山麻子</u>、三代俊治、脇田隆字、加藤孝宣.
Ｃ型肝炎ウイルス粒子の産生効率の良い HuH-7
細胞サブクローンの分離と同定. 第１５回日本肝
臓学会大会、2010 年 10 月，福岡.

H．知的所有権の出願・取得状況
1.特許取得
　　なし。

2.実用新案登録
　　なし。

3.その他
　　なし。

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Murayama A, Kato T, Akazawa D, Sugiyama N, Date T, Masaki T, Nakamoto S, Tanaka Y, Mizokami M, Yokosuka O, Nomoto A, Wakita T.	Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1.	J Virol	86(4)	2143-2152	2012
Saeed M, Suzuki R, Watanabe N, Masaki T, Tomonaga M, Muhammad A, Kato T, Matsuura Y, Watanabe H, Wakita T, Suzuki T.	Role of the Endoplasmic Reticulum-associated Degradation (ERAD) Pathway in Degradation of Hepatitis C Virus Envelope Proteins and Production of Virus Particles.	J Biol Chem	286	37264-37273	2011
Saeed M, Shiina M, Date T, Akazawa D, Watanabe N, Murayama A, Suzuki T, Watanabe H, Hiraga N, Imamura M, Chayama K, Choi Y, Krawczynski K, Liang TJ, Wakita T, Kato T.	In vivo adaptation of hepatitis C virus in chimpanzees for efficient virus production and evasion of apoptosis.	Hepatology	54	425-433	2011
Okamoto Y, Masaki T, Murayama A, Munakata T, Nomoto A, Nakamoto S, Yokosuka O, Watanabe H, Wakita T, Kato T.	Development of recombinant hepatitis C virus with NS5A from strains of genotypes 1 and 2.	Biochem Biophys Res Commun	410	404-409	2011

Inoue Y, Aizaki H, Hara H, Matsuda M, Ando T, Shimoji T, Murakami K, Masaki T, Shoji I, Homma S, Matsuura Y, Miyamura T, Wakita T, Suzuki T.	Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein.	Virology	410	38-47	2011

Ⅲ．研究成果の刊行物・別刷

Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1

Asako Murayama,[a] Takanobu Kato,[a] Daisuke Akazawa,[a] Nao Sugiyama,[a] Tomoko Date,[a] Takahiro Masaki,[a] Shingo Nakamoto,[b] Yasuhito Tanaka,[c] Masashi Mizokami,[d] Osamu Yokosuka,[b] Akio Nomoto,[e*] and Takaji Wakita[a]

Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan[a]; Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chuo, Chiba, Japan[b]; Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan[c]; The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Chiba, Japan[d]; and Department of Microbiology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan[e]

To establish a cell culture system for chimeric hepatitis C virus (HCV) genotype 2b, we prepared a chimeric construct harboring the 5' untranslated region (UTR) to the E2 region of the MA strain (genotype 2b) and the region of p7 to the 3' UTR of the JFH-1 strain (genotype 2a). This chimeric RNA (MA/JFH-1.1) replicated and produced infectious virus in Huh7.5.1 cells. Replacement of the 5' UTR of this chimera with that from JFH-1 (MA/JFH-1.2) enhanced virus production, but infectivity remained low. In a long-term follow-up study, we identified a cell culture-adaptive mutation in the core region (R167G) and found that it enhanced virus assembly. We previously reported that the NS3 helicase (N3H) and the region of NS5B to 3' X (N5BX) of JFH-1 enabled replication of the J6CF strain (genotype 2a), which could not replicate in cells. To reduce JFH-1 content in MA/JFH-1.2, we produced a chimeric viral genome for MA harboring the N3H and N5BX regions of JFH-1, combined with a JFH-1 5' UTR replacement and the R167G mutation (MA/N3H+N5BX-JFH1/R167G). This chimeric RNA replicated efficiently, but virus production was low. After the introduction of four additional cell culture-adaptive mutations, MA/N3H+N5BX-JFH1/5am produced infectious virus efficiently. Using this chimeric virus harboring minimal regions of JFH-1, we analyzed interferon sensitivity and found that this chimeric virus was more sensitive to interferon than JFH-1 and another chimeric virus containing more regions from JFH-1 (MA/JFH-1.2/R167G). In conclusion, we established an HCV genotype 2b cell culture system using a chimeric genome harboring minimal regions of JFH-1. This cell culture system may be useful for characterizing genotype 2b viruses and developing antiviral strategies.

H epatitis C virus (HCV) is a major cause of chronic liver disease (5, 13), but the lack of a robust cell culture system to produce virus particles has hampered the progress of HCV research (2). Although the development of a subgenomic replicon system has enabled research into HCV RNA replication (15), infectious virus particle production has not been possible. Recently, an HCV cell culture system was developed using a genotype 2a strain, JFH-1, cloned from a fulminant hepatitis patient (14, 29, 32), thereby allowing investigation of the entire life cycle of this virus. However, several groups of investigators have reported genotype- and/or strain-dependent effects of some antiviral reagents (6, 17) and neutralizing antibodies (7, 25). Therefore, efficient virus production systems using various genotypes and strains are indispensable for HCV research and the development of antiviral strategies.

The JFH-1 strain is the first HCV strain that can efficiently produce HCV particles in HuH-7 cells (29). Other strains can replicate and produce infectious virus by HCV RNA transfection, but the efficiency is far lower than that of JFH-1 (24, 31). In the case of replication-incompetent strains, chimeric virus containing the JFH-1 nonstructural protein coding region is useful for analyses of viral characteristics (6, 9, 14, 23, 30, 31).

In this study, we developed a genotype 2b chimeric infectious virus production system using the MA strain (accession number AB030907) (19) harboring minimal regions of JFH-1 and cell culture-adaptive mutations that enhance infectious virus production.

MATERIALS AND METHODS

Cell culture. Huh7.5.1 cells (a kind gift from Francis V. Chisari) (32) and Huh7-25 cells (1) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions. For follow-up study, RNA-transfected cells were passaged every 2 to 5 days depending on cell status.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (29) and pMA (19). For convenience, an EcoRI recognition site was introduced upstream of the T7 promoter region of pMA by PCR, and an XbaI recognition site was introduced at the end of the 3' untranslated region (UTR). To construct MA/JFH-1, the EcoRI-BsaBI (nucleotides [nt] 1 to 2570; 5' UTR to E2) fragment of pMA was substituted into pJFH1 (Fig. 1A). Replacement of the 5' UTR was performed by exchanging the EcoRI-AgeI (nt 1 to 159) fragment. A point mutation in the core region (R167G) was introduced into MA chimeric constructs by PCR using the following primers: sense, 5'-TTA TGC AAC GGG GAA TTT ACC CGG TTG CTC T-3'; antisense, 5'-GGT AAA TTC CCC GTT GCA TAA TTT ATC CCG TC-3'. G167R substitution in the JFH-1 construct was performed by PCR using the following primers: sense, 5'-ATT ATG CAA CAA GGA ACC TAC CCG GTT TCC C-3'; antisense, 5'-GGT AGG TTC CTT GTT GCA TAA TTA ACC CCG TC-3'. Point mutations (L814S, R1012G, T1106A, and V1951A) were introduced into MA chimeric constructs by PCR using the following primers: L814S, 5'-GCT TAC GCC TCG GAC GCC GCT GAA CAA GGG G-3' (sense) and 5'-AGC GGC GTC CGA GGC GTA AGC CTG CTG CGG C-3' (antisense); R1012G, 5'-GAG GCT AGG TGG
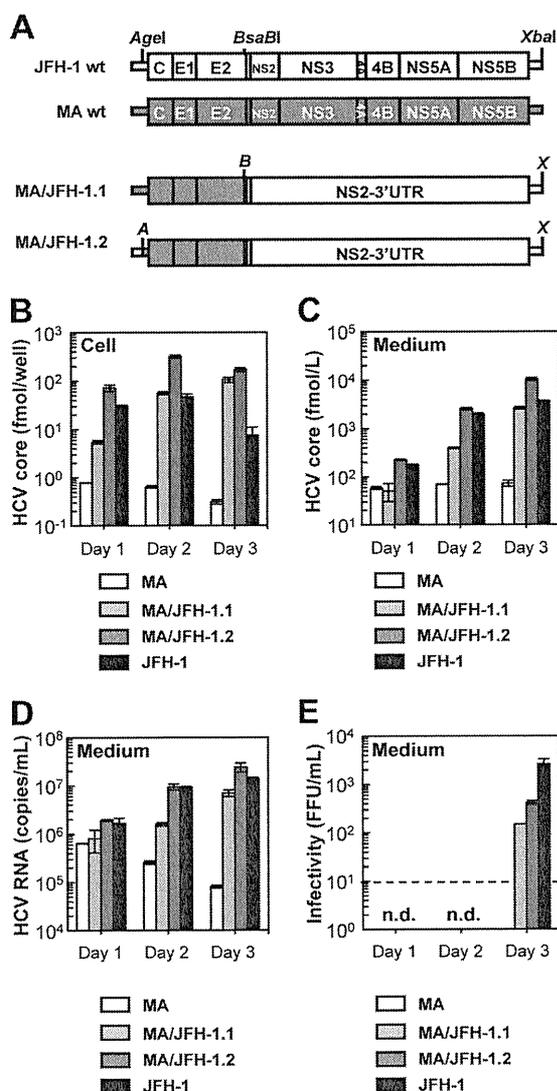
FIG 1 Replication and virus production by MA/JFH-1 chimeras in Huh7.5.1 cells. (A) Schematic structures of JFH-1, MA, and two MA/JFH-1 chimeras (MA/JFH-1.1 and MA/JFH-1.2). The junction of JFH-1 and MA in the 5′ UTR is an AgeI site, and the junction of MA and JFH-1 in the NS2 region is a BsaBI site. A, AgeI; B, BsaBI; X, XbaI. (B to E) Chimeric HCV RNA replication in Huh7.5.1 cells. HCV core protein level in cells (B) and culture medium (C) and HCV RNA levels in medium (D) and infectivity of culture medium (E) from HCV RNA-transfected Huh7.5.1 cells are shown. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and culture medium were harvested on days 1, 2, and 3. n.d., not determined. Assays were performed three times independently, and data are presented as means ± standard deviation. Dashed line indicates detection limit. wt, wild type.

GGA AGT TCT GCT CGG CCC T-3′ (sense) and 5′-AGA ACT TCC CCA CCT AGC CTC GCG GAA ACC G-3′ (antisense); T1106A, 5′-CAG ATG TAC GCC AGC GCA GAG GGG GAC CTC-3′ (sense) and 5′-CTG CGC TGG CGT ACA TCT GGG TGA CTG GTC-3′ (antisense); and V1951A, 5′-GTG ACG CAG GCG TTA AGC TCA CTC ACA ATT ACC-3′ (sense) and 5′-TGA GCT TAA CGC CTG CGT CAC GCG CAG CGA G-3′ (antisense). To construct the MA chimeric virus harboring minimal regions of JFH-1 (MA/N3H+N5BX-JFH1), ClaI (nt 3930), EcoT22I (nt 5294), and BsrGI (nt 7782) recognition sites were introduced into pMA by site-directed mutagenesis. The 5′ UTR (EcoRI-AgeI), the region of the NS3 helicase (N3H; ClaI-EcoT22I), and the region of NS5B to 3′ X (N5BX;

BsrGI-XbaI) were then replaced with the corresponding regions from JFH-1.

RNA synthesis, transfection, and determination of infectivity. RNA synthesis and transfection were performed as described previously (12, 22). Determination of infectivity was also performed as described previously, with infectivity expressed as the number of focus-forming units per milliliter (FFU/ml) (12, 22). When necessary, culture medium was concentrated 20-fold in Amicon Ultra-15 spin columns (100-kDa molecular-weight-cutoff; Millipore, Bedford, MA) in order to determine infectivity.

Quantification of HCV core protein and HCV RNA. In order to estimate the concentration of HCV core protein in culture medium, we performed a chemiluminescence enzyme immunoassay (Lumipulse II HCV core assay; Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions. HCV RNA from harvested cells or culture medium was isolated using an RNeasy Mini RNA kit (Qiagen, Tokyo, Japan) or QiaAmp Viral RNA Minikit (Qiagen), respectively. Copy number of HCV RNA was determined by real-time quantitative reverse transcription-PCR (qRT-PCR), as described previously (28).

HCV sequencing. Total RNA in culture supernatant was extracted with Isogen-LS (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA was subsequently amplified with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan). Four separate PCR primer sets were used to amplify the fragments of nt 130 to 2909, 2558 to 5142, 4784 to 7279, and 7081 to 9634 covering the entire open reading frame and part of the 5′ UTR and 3′ UTR of the MA strain. Sequences of amplified fragments were determined directly.

Immunostaining. Infected cells were cultured on Multitest Slides (MP Biomedicals, Aurora, OH) and were fixed in acetone-methanol (1:1, vol/vol) for 15 min at −20°C. After a blocking step, infected cells were visualized with anti-core protein antibody (clone 2H9) (29) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), and nuclei were visualized with 4′,6′-diamidino-2-phenylindole (DAPI).

Assessment of interferon sensitivity. Two micrograms of *in vitro* transcribed RNA was transfected into 3 × 10⁶ Huh7.5.1 cells. Four hours after transfection, cells were placed in fresh medium or medium containing 0.1, 1, 10, 100, and 1,000 IU/ml of interferon α-2b (Intron A; Schering-Plough Corporation, Osaka, Japan). Culture medium was then harvested on day 3, and HCV core levels in the cells and in the medium were measured.

Statistical analysis. Significant differences were evaluated by Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Transient replication and production of 2b/2a chimeric virus. We first tested whether the MA strain (genotype 2b) (19) was able to replicate and produce infectious virus in cultured cells. When the *in vitro* transcribed RNA of MA was transfected into Huh7.5.1 cells, a highly HCV-permissive cell line, replication and virus production were not observed (Fig. 1A to C). We then tested whether 2b/2a chimeric RNA harboring the structural region (5′ UTR to E2) of the MA strain and the nonstructural region (p7 to 3′ UTR) of JFH-1 (Fig. 1A, MA/JFH-1.1) was able to replicate in the cells. After MA/JFH-1.1 RNA transfection, time-dependent accumulation of core protein in the cells (Fig. 1B) and culture medium (Fig. 1C) was observed, indicating that MA/JFH-1.1 RNA was able to replicate in the cells autonomously. HCV RNA levels in the medium were determined by qRT-PCR, and time-dependent increases in HCV RNA level were also observed (Fig. 1D). Infectious virus production was observed on day 3, but infectivity was 17.6-fold lower than that of JFH-1 (Fig. 1E).

In order to improve the level of infectious virus production, we tested another chimeric construct, MA/JFH-1.2, which contained an additional MA-to-JFH-1 replacement of the 5′ UTR (Fig. 1A),
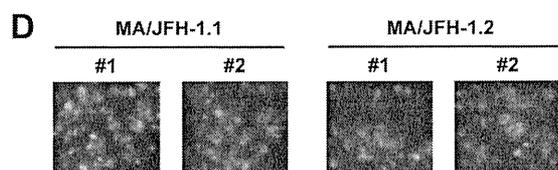
FIG 2 Long-term culture of MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells were passaged every 2 to 5 days, depending on cell status. Culture medium was collected after every passage, and HCV core protein levels were measured. Transfection was performed twice for each chimeric RNA (1 and 2 for each construct). (A) HCV core protein levels in culture medium from MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. (B) Immunostained cells at 3 days after transfection (a to d), at 21 days after transfection (e to h), and at the time
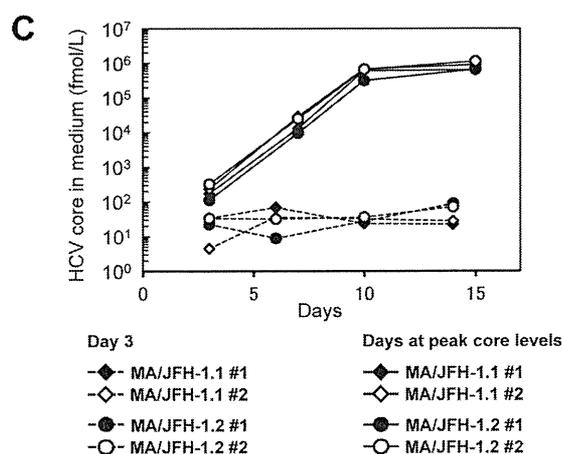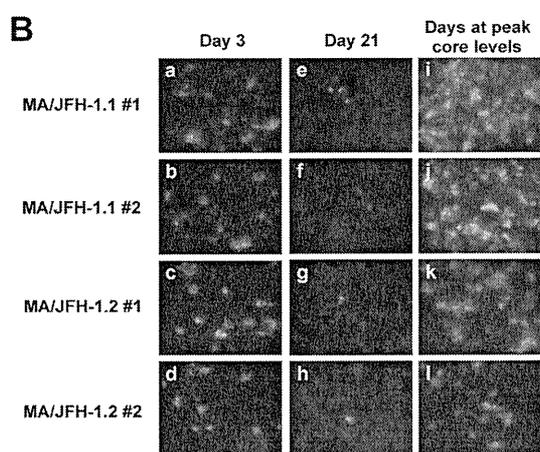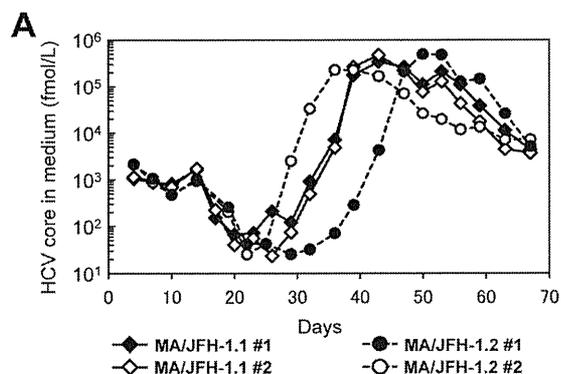
TABLE 1 HCV core protein levels and infectivity in culture medium immediately after RNA transfection (day 3) and after long-term culture (days 35 to 49)

Sample period and virus	Sample no.	Day no.[a]	HCV core (fmol/liter)	Infectivity (FFU/ml)
After transfection				
MA/JFH-1.1	1	3	1.06×10^3	5.00×10^1
	2	3	1.14×10^3	5.70×10^1
MA/JFH-1.2	1	3	2.14×10^3	7.30×10^1
	2	3	2.15×10^3	9.30×10^1
After long-term culture				
MA/JFH-1.1	1	42	3.38×10^5	1.62×10^5
	2	42	4.70×10^5	3.23×10^5
MA/JFH-1.2	1	35	2.27×10^5	1.61×10^5
	2	49	4.93×10^5	3.27×10^5

[a] For the long-term culture, the days are those of peak core protein levels.

as a 5' UTR replacement from J6CF (genotype 2a) to JFH-1 enhanced virus production of chimeric J6CF virus harboring the region of NS2 to 3' X of JFH-1 (J6/JFH-1) (A. Murayama et al., unpublished data). The core protein accumulation levels with MA/JFH-1.2 RNA-transfected cells were higher than those with MA/JFH-1.1 ($P < 0.05$) (Fig. 1B). Similarly, core protein and HCV RNA levels in the medium of MA/JFH-1.2 RNA-transfected cells were higher than those of MA/JFH-1.1 ($P < 0.05$) (Fig. 1C and D). Infectivity on day 3 was also higher than with MA/JFH-1.1 ($P < 0.05$) (Fig. 1E), indicating that the 5' UTR of JFH-1 enhanced virus production. However, infectivity of medium from MA/JFH-1.2 RNA-transfected cells on day 3 remained 6.4-fold lower than that of JFH-1 although HCV RNA levels in the medium were similar to those of JFH-1 (Fig. 1D and E).

These results indicate that 2b/2a chimeric RNA is able to replicate autonomously in Huh7.5.1 cells and produce infectious virus although infectivity remains lower than that of JFH-1.

Assembly-enhancing mutation in core region introduced during long-term culture. Because MA/JFH-1.1 and MA/JFH-1.2 replicated efficiently but produced small amounts of infectious virus, we performed long-term culture of these RNA-transfected cells in order to examine whether these chimeric RNAs would continue replicating and producing infectious virus over the long term. We prepared two RNA-transfected cell lines for each construct (MA/JFH-1.1 and MA/JFH-1.2) as both of these replicated and produced infectious virus at different levels.

Immediately after transfection, core protein levels and infectivity in culture medium were low (1.06×10^3 to 2.15×10^3 fmol/liter and 5.00×10^1 to 9.30×10^1 FFU/ml, respectively) (Fig. 2A and Table 1) although a considerable number of core protein-positive cells were observed by immunostaining (Fig. 2B, frames a to d). Subsequently, core protein levels in the culture medium decreased gradually (Fig. 2A), and core protein-positive cells were rare (Fig. 2B, frames e to h). However, at 30 to 40 days

of peak core levels (days 42 to 49). Infected cells were visualized with anti-core protein antibody (green), and nuclei were visualized with DAPI (blue). (C) Infection of naïve cells by culture medium at an MOI of 0.001. (D) Immunostained cells at 15 days after infection with medium at peak core protein levels (Fig. 2A) at an MOI of 0.001. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue).

after transfection, core protein levels in the supernatants of all chimeric RNA-transfected cells increased and reached 2.27×10^5 to 4.93×10^5 fmol/liter (Fig. 2A and Table 1). Infectivity in the culture medium also increased (1.61×10^5 to 3.27×10^5 FFU/ml) (Table 1), and at this point, most of the cells were core protein positive (Fig. 2B, frame i to l).

As the infectivity of culture supernatant of MA/JFH-1 RNA-transfected cells appeared to increase after long-term culture, we compared viral spread by infection with these supernatants on day 3 (immediately after transfection) and for each peak in core protein levels (after long-term culture). When naïve Huh7.5.1 cells were infected with supernatant on days corresponding to a peak in core protein levels at a multiplicity of infection (MOI) of 0.001, core protein levels in the medium increased rapidly and reached 0.64×10^6 to 1.13×10^6 fmol/liter by day 15 after infection (Fig. 2C). Immunostained images showed that most cells were HCV core protein positive on day 15 (Fig. 2D). When naïve Huh7.5.1 cells were infected with supernatant from day 3 at an MOI of 0.001, core protein levels in the medium did not increase under these conditions (Fig. 2C). These results indicate that both MA/JFH-1 chimeric viruses (MA/JFH-1.1 and MA/JFH-1.2) acquired the ability to spread rapidly after long-term culture.

As the characteristics of the MA/JFH-1 virus changed in long-term culture, we analyzed the possible mutations in the viral genome from the supernatant at each peak in core protein levels (Table 1, days at peak core levels). Nine- to 12-nucleotide mutations were found in the viral genome from each supernatant, and the detected mutations were distributed along the entire genome. Among these mutations, a common nonsynonymous mutation was found in the core region (Arg to Gly at amino acid [aa]167, R167G).

In order to test the effects of R167G on virus production, an R167G substitution was introduced into MA/JFH-1.2 as MA/JFH-1.2 replicated and produced infectious virus more efficiently than MA/JFH-1.1. HCV core protein levels in cells and medium of MA/JFH-1.2 with R167G (MA/JFH-1.2/R167G) were higher than with MA/JFH-1.2 ($P < 0.05$) (Fig. 3A and B). HCV RNA levels in the medium of MA/JFH-1.2/R167G RNA-transfected cells were also higher than with MA/JFH-1.2 ($P < 0.05$) (Fig. 3C). Infectious virus production was also increased by the R167G mutation ($P < 0.05$) (Fig. 3D) and was 8.7-fold higher than that of JFH-1 RNA-transfected cells on day 3 ($P < 0.05$) (Fig. 3D).

We then tested whether R167G was responsible for the rapid spread observed in culture supernatant after long-term culture by monitoring virus spread after infection of naïve Huh7.5.1 with culture medium taken 3 days after RNA transfection of MA/JFH-1.2 and MA/JFH-1.2/R167G at an MOI of 0.005. Core protein levels in medium from MA/JFH-1.2/R167G-infected cells increased with the same kinetics as levels of JFH-1 (Fig. 3E), and the population of core protein-positive cells was almost the same as with JFH-1-infected cells (Fig. 3F), indicating that MA/JFH-1.2/R167G virus spread as rapidly as JFH-1 virus. In contrast, we observed no infectious foci in the MA/JFH-1.2 virus-inoculated cells (Fig. 3F). These data suggest that the R167G mutation in the core region was a cell culture-adaptive mutation and that it enhanced infectious MA/JFH-1.2 virus production.

In order to determine whether R167G enhances RNA replication or other steps in the viral life cycle, we performed a single-cycle virus production assay (11) using Huh7-25 cells, a HuH-7-derived cell line lacking CD81 expression on the cell surface (1).
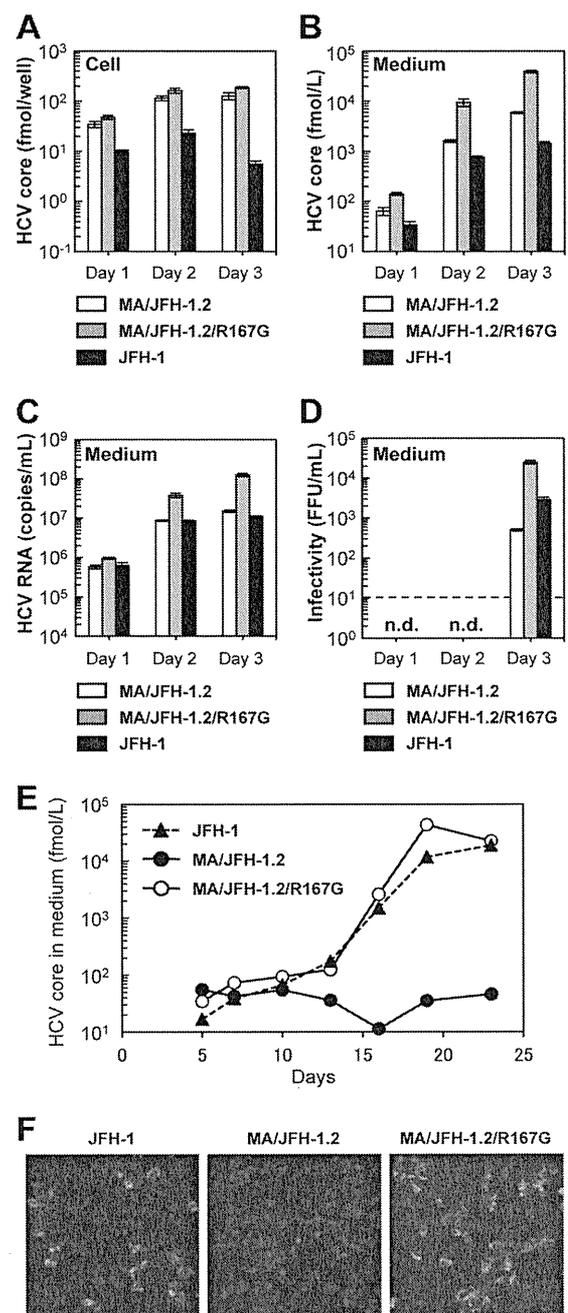

FIG 3 Effects of R167G on replication and virus production of MA/JFH-1.2 in Huh7.5.1 cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and medium were harvested on days 1, 2, and 3. HCV core protein levels in the cells (A) and culture medium (B) and HCV RNA levels in the medium (C) and the infectivity of culture medium (D) from HCV RNA-transfected Huh7.5.1 cells are shown. n.d., not determined. Dashed line indicates the detection limit. Assays were performed three times independently, and data are presented as means ± standard deviation. (E) HCV core protein levels in culture medium from cells infected with medium at 3 days posttransfection at an MOI of 0.005. (F) Immunostained cells at 19 days postinfection. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue).

This cell line can support replication and infectious virus production upon transfection of HCV genomic RNA but cannot be reinfected by progeny virus, thereby allowing observation of a single cycle of infectious virus production without the confounding ef-

fects of reinfection. R167G did not affect HCV core protein levels in the chimeric RNA-transfected Huh7-25 cells (Fig. 4A), demonstrating that R167G did not enhance RNA replication. Nevertheless, R167G increased HCV core protein levels in the medium ($P < 0.05$ on days 2 and 3) and infectivity (Fig. 4B and C). These results suggest that R167G did not affect RNA replication but affected other steps such as virus assembly and/or virus secretion.

Virus particle assembly efficiency was then assessed by determining intracellular-specific infectivity from infectivity and RNA titer in the cells, as reported previously (11). As shown in Fig. 4G, R167G enhanced intracellular-specific infectivity of MA/JFH-1.2 virus 10.2-fold. Virus secretion efficiency was also calculated from the amount of intracellular and extracellular infectious virus, but R167G had no effect (Fig. 4G).

To confirm the effects of Arg167 in other HCV strains, we tested its effects on JFH-1. As aa 167 of JFH-1 is Gly, we replaced it with Arg (G167R). HCV core protein levels in the cells were not affected by G167R (Fig. 4D), and no effects on RNA replication were confirmed. HCV core protein levels in the medium and infectivity decreased after G167R mutation (Fig. 4E and F). As the G167R mutation decreased intracellular infectious virus production of JFH-1 to undetectable levels, we were unable to determine the intracellular-specific infectivity and virus secretion efficiency of JFH-1 G167R (Fig. 4G). These results indicate that Gly is favored over Arg at core position 167 for infectious virus assembly in multiple HCV strains.

MA harboring the R167G mutation, 5′ UTR, and N3H (NS3 helicase) and N5BX (NS5B to 3′ X) regions of JFH-1 replicated and produced infectious chimeric virus. In order to establish a genotype 2b cell culture system with the MA strain with minimal regions of JFH-1, we attempted to reduce JFH-1 content in MA/JFH-1.2. We previously reported that replacement of the N3H and N5BX regions of JFH-1 allowed efficient replication of the J6CF strain, which normally cannot replicate in cells (21). Thus, we tested whether the N3H and N5BX regions of JFH-1 could also support MA RNA replication.

We prepared two chimeric MA constructs harboring the 5′ UTR and N3H and N5BX regions of JFH-1, MA/N3H+N5BX-JFH1 (Fig. 5A) and MA/N3H+N5BX-JFH1/R167G. After *in vitro* transcribed RNA was transfected into Huh7.5.1 cells, intracellular core protein levels of MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells increased in a time-dependent manner and reached almost the same levels as with MA/JFH-1.2 RNA-transfected cells on day 5 (Fig. 5B). Extracellular core protein and HCV RNA levels of MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells also increased in a time-dependent manner (Fig. 5C and D). However, they were more than 10 times lower than with MA/JFH-1.2 RNA-transfected cells although intracellular core levels were comparable on day 5 (Fig. 5B to D).

We then tested whether the medium from MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells was infectious. Infectivity of the medium from MA/N3H+N5BX-JFH1 RNA-transfected cells was below the detection limit, and that of MA/N3H+N5BX-JFH1/R167G RNA-transfected cells on day 5 was very low ($3.3 \times 10^1 \pm 2.1 \times 10^1$ FFU/ml) (Fig. 5E). To confirm infectivity, the culture media were concentrated, and their infectivity was determined. Infected foci were observed after infection with concentrated medium in MA/N3H+N5BX-JFH1/R167G RNA-transfected cells (Fig. 5F), and infectivity was found

MA/JFH-1.2
MA/JFH-1.2/R167G

JFH-1
JFH-1/G167R

G

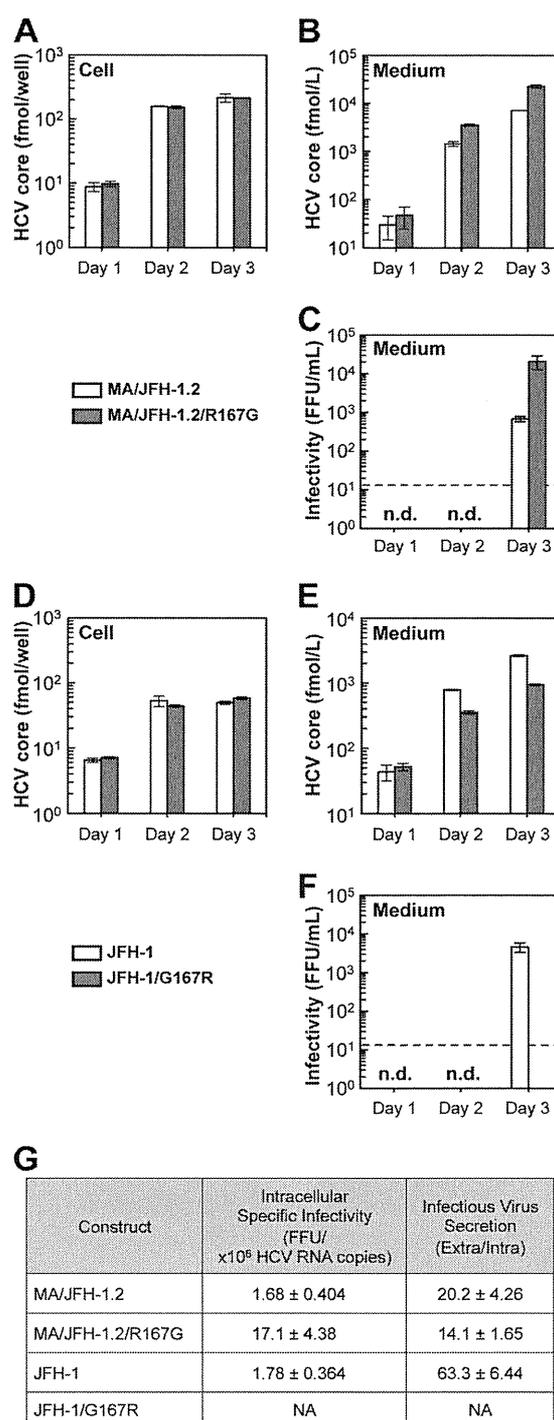| Construct	Intracellular Specific Infectivity (FFU/ $\times 10^6$ HCV RNA copies)	Infectious Virus Secretion (Extra/Intra)
MA/JFH-1.2	1.68 ± 0.404	20.2 ± 4.26
MA/JFH-1.2/R167G	17.1 ± 4.38	14.1 ± 1.65
JFH-1	1.78 ± 0.364	63.3 ± 6.44
JFH-1/G167R	NA	NA

FIG 4 Effects of R167G on replication and virus production of MA/JFH-1.2 and JFH-1 in Huh7-25 cells. Ten micrograms of HCV RNA was transfected into Huh7-25 cells, and cells and medium were harvested on days 1, 2, and 3. HCV core protein levels in cells (A and D) and in medium (B and E) were measured, and infectivity of medium (C and F) was determined. n.d., not determined. Dashed line indicates the detection limit. (G) Intracellular specific infectivity and virus secretion efficiency of chimeric HCV RNA-transfected cells. Intracellular and extracellular infectivity of day 3 samples was determined, and specific infectivity and virus secretion rate were calculated. Assays were performed three times independently, and data are presented as means ± standard deviation. NA, not available.

A

B

C
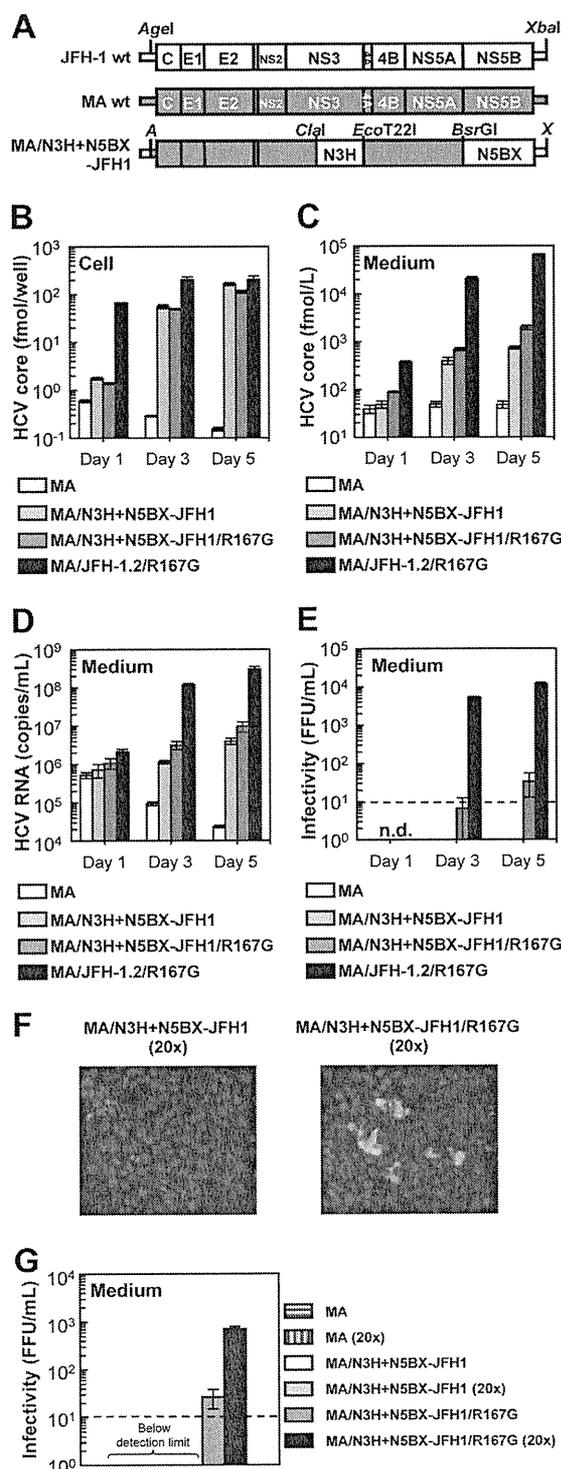

D

E

F

G

FIG 5 Replication and virus production of MA/N3H+N5BX-JFH1/R167G in Huh7.5.1 cells. (A) Schematic structures of JFH-1, MA, and MA/N3H+N5BX-JFH1. The junction of JFH-1 and MA in the 5′ UTR is an AgeI site; the junctions of MA and JFH-1 in the NS3 regions are ClaI and EcoT22I sites, and the junction in the NS5B region is a BsrGI site. A, AgeI; X, XbaI. (B to G) Chimeric HCV RNA replication in Huh7.5.1 cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and medium were harvested on days 1, 3, and 5. HCV core protein levels in cells (B) and in medium (C) and HCV RNA levels in medium (D) were measured, and infectivity of medium (E) was determined. Assays were performed three times independently, and data are presented as means ± standard deviation. n.d., not determined. Dashed line indicates the detection limit. (F) Immunostained cells. Huh7.5.1

to be $7.27 \times 10^2 \pm 7.57 \times 10^1$ FFU/ml (Fig. 5G). No infected foci were observed after infection of MA/N3H+N5BX-JFH1 RNA-transfected cells, even when medium was concentrated (Fig. 5F), although intracellular and extracellular core protein levels were comparable to those with MA/N3H+N5BX-JFH1/R167G RNA-transfected cells (Fig. 5B and C). These results indicate that replacement of the 5′ UTR and N3H and N5BX regions in JFH-1 were necessary to rescue autonomous replication in the replication-incompetent MA strain and for secretion of infectious chimeric virus. However, the secretion and infection efficiencies of the virus were low.

Cell culture-adaptive mutations enhanced infectious virus production of MA/N3H+N5BX-JFH1/R167G. Because MA/N3H+N5BX-JFH1/R167G replicated efficiently but produced very small amounts of infectious virus, we performed a long-term culture of the RNA-transfected cells in order to induce cell culture-adaptive mutations that could enhance infectious virus production. We prepared RNA-transfected cells using two constructs, MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G; both of these replicated efficiently, and MA/N3H+N5BX-JFH1/R167G produced infectious virus at low levels while MA/N3H+N5BX-JFH1 did not. Immediately after transfection, the HCV core protein levels in the medium of each RNA-transfected cell culture peaked at 3.0×10^3 fmol/liter and declined thereafter. However, the core protein level in the medium with MA/N3H+N5BX-JFH1/R167G RNA-transfected cells continued to increase and reached a peak of 2.7×10^5 fmol/liter 54 days after transfection, at which point most cells were core protein positive (Fig. 6B). The core protein level in the medium with MA/N3H+N5BX-JFH1 RNA-transfected cells did not increase and core-positive cells were scarce on day 54 (Fig. 6B). We analyzed the viral genome in the culture supernatants from day 54 for possible mutations and identified four nonsynonymous mutations in the MA/N3H+N5BX-JFH1/R167G genome: L814S (NS2), R1012G, (NS2), T1106A (NS3), and V1951A (NS4B). In order to test whether these amino acid substitutions enhance infectious virus production, L814S, R1012G, T1106A, and V1951A were introduced into MA/N3H+N5BX-JFH1/R167G, and the product was designated MA/N3H+N5BX-JFH1/5am (where am indicates adaptive mutation). On day 1, although HCV core protein levels in the MA/N3H+N5BX-JFH1/5am RNA-transfected cells were higher than those of MA/N3H+N5BX-JFH1/R167G RNA-transfected cells, they were still lower than those of MA/JFH-1.2/R167G RNA-transfected cells; however, on days 3 and 5, they reached a level comparable to that of MA/JFH-1.2/R167G RNA-transfected cells (Fig. 6C). HCV core protein and HCV RNA levels in the medium of MA/N3H+N5BX-JFH1/5am RNA-transfected cells were higher than those of MA/JFH-1.2/R167G RNA-transfected cells ($P < 0.05$, Fig. 6D and 6E, respectively). MA/N3H+N5BX-JFH1/5am, containing the four additional adaptive mutations, produced infectious virus at the same level as MA/JFH-1.2/R167G on day 5 (Fig. 6F). These results indicate that the

cells were infected with concentrated medium from RNA-transfected cells on day 5. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue). (G) Infectivity of concentrated culture medium from HCV RNA-transfected cells. Culture medium was concentrated by 20 times. Infectivities of original and concentrated culture media were determined. Dashed line indicates detection thelimit.