

図1 I型インターフェロンシステム

ウイルス感染と自然免疫

細胞にウイルスが感染するときさまざまなサイトカイン、ケモカイン等が産生され、炎症反応を惹起する。これらのなかでとくに重要なのはI型IFNである。I型IFNは複数のIFN α とIFN β からなり、ほとんどの細胞で分泌される。IFNは周囲の細胞のIFNAR1とIFNAR2からなるレセプターに結合してJanus kinase (Jak)-signal transducer and activator of transcription (STAT) 経路のシグナルを伝達し、STAT1とSTAT2がチロシンリン酸化を受ける。STAT1とSTAT2およびinterferon regulatory factor 9 (IRF 9)がIFN-stimulated gene factor 3 (ISGF 3)というヘテロ三量体の複合体を形成し、IFN-stimulated response element (ISRE)とよばれる遺伝子領域に結合してさまざまなIFN誘導遺伝子 (IFN-stimulated gene: ISG)の発現を誘導し、細胞を抗ウイルス状態へ導く(図1)。

自然免疫系は、ウイルス感染応答初期の抗ウイルス活性の誘導、さらにその後の獲得免疫の活性制御などの役割を果たす。この時に細胞はウイルス由来の核酸成分を、通常存在しない構造をもつことから、非自己として認識

すると考えられている。感染検知システムとしてはTLRによる細胞表面のシステムのほかに、retinoic acid inducible gene (RIG-1)ファミリーによる細胞質でのシステムとが知られるようになった。

ウイルス感染とTLRs

1996年、ショウジョウバエを用いた研究から、昆虫において背腹軸形成に関与するTollが、カビなどの真菌に対する免疫応答をになっているという報告がされた³⁾。その後、ヒトやネズミなどの脊椎動物にもTLRが存在することが証明された。最近まで自然免疫は、非特異的な貪食作用によって病原体を処理するだけで、獲得免疫が活性化されるまでの一時しのぎにすぎないと考えられてきた。しかし、TLRが病原微生物を認識し宿主自然免疫を惹起することで、獲得免疫の効果的な活性化に関与する重要なはたらきをになうという報告が多数され、自然免疫系は病原体の侵入を特異的に認識し、感染防御を成立させることが明らかとなった。

TLRは現在までデータベース上13種類、ヒトでは10種類(TLR1-10)が報告されており、このうち細胞外でHCV感染を認識するのはTLR3⁴⁾である。感染細胞のア

ポトシスなどによって放出された複製産物中の dsRNA がエンドサイトーシスによって取り込まれ、TLR3 によって認識される。TLR3 の細胞内ドメインには TLR domain containing adaptor inducing IFN (TRIF/TICAM-1) とよばれるアダプター分子が結合し、その下流の TANK binding kinase (TBK1), I κ B kinase (IKK) γ (別名 IKK ϵ) という2つのキナーゼにシグナルを伝達し、活性化する。活性化すると、IRF3 と IRF7 へとシグナルが伝達され、活性化することにより IFN 系が作動される。

細胞内のウイルス感染センサー

TLR は細胞表面やエンドソーム膜に発現しており、細胞外もしくはエンドサイトーシスによって取り込んだウイルスの構成成分を認識する。エンドサイトーシスによって取り込まれたウイルスは、脱核後、細胞質内に逃げ込み、複製をおこなう。また、エンベロープの融合により直接細胞質内にウイルスゲノムを注入し、複製を進行させる。最近、細胞外でのウイルス感染検知システムである TLR に対して、感染細胞内では RIG-I ファミリー(図9)が作動し、ウイルス由来の RNA を検知することがわかってきた。

RIG-I は dsRNA 刺激による IFN 遺伝子に関与する分子として、発現クローニングにより同定された dsRNA 依存性 RNA ヘリカーゼ分子である⁹⁾。N 末端に2回くり返して caspase recruitment domain (CARD) をもち、C 末端にヘリカーゼドメインをもつ特徴がある。変異体を用いた解析などから、通常 RIG-I は不活性型として神経系以外のほとんどの細胞の細胞質に局在しており、ここへウイルス由来の RNA がヘリカーゼドメインで認識されることにより活性化され、ヘリカーゼドメインで直接ウイルス由来の dsRNA と結合し、ヘリカーゼの ATPase 活性化に伴う分子内構造変化によって CARD が下流のシグナル分子と会合できるようになり、IRF-3 活性化シグナルが伝達されることがわかった。

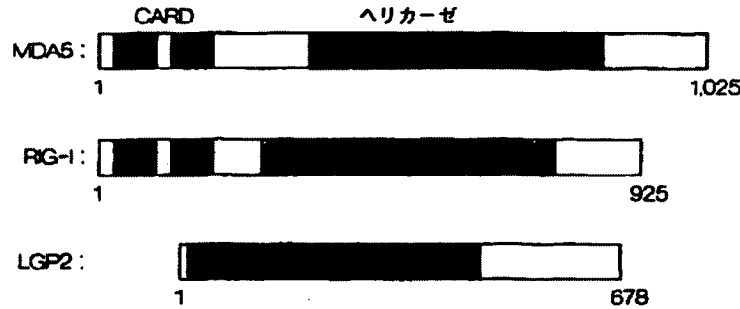
また、ヒトゲノムで RIG-I と相同性をもつ分子である melanoma differentiation-associated gene 5 (MDA5) も同様にヘリカーゼドメインで dsRNA を認識し CARD を介してシグナル伝達をおこなうことがわ

かっている⁹⁾。

最近 MDA5 欠損マウスが作製され、この解析からこれら2つのヘリカーゼは形質細胞様樹状細胞 (plasmacytoid dendritic cell: pDC) 以外の DC を含めたほとんどの細胞において、ウイルス感染センサーとしての役割をになっていることが示された。また、pDC においては、TLR がウイルス感染検知の役割をになっており、細胞種間でウイルスセンサーは異なることが明らかとなっている¹⁰⁾。さらに、欠損マウスの解析により RIG-I がパラミクソウイルス、インフルエンザウイルス、日本脳炎ウイルスといった RNA ウイルスの感染防御に必須の分子であるのに対し、MDA5 はおもにピコルナウイルス科に属するウイルス (ポリオウイルスなど) の認識に必須であることが示された。これらの役割分担を説明する明らかな報告はいまだないが、RIG-I の RNA 識別に関する報告がされ、参考に値する。すなわち、これまで RIG-I は RNA を識別するのに dsRNA 構造を識別すると考えられていたが、ここで明らかにされたのは、5' に3つのリン酸をもつような RNA を特異的に認識するという可能性である¹¹⁾。このことから、自己の RNA と非自己の RNA の区別に際し、ウイルス由来の RNA は5' に三リン酸化構造を、細胞由来の RNA は5' に Cap 構造をもつことを利用して識別されていると推測される。

一方、RIG-I と相同性をもつ第3の分子として、LGP2 が同定されている。LGP2 は RIG-I と高い相同性を示すヘリカーゼドメインをもつが、N 末端の CARD をもたない。LGP2 の強制発現は、ウイルス感染による IFN 産生シグナルを強力に抑制したことから、LGP2 が RIG-I /MDA5 を介したシグナルを抑制する負の調節因子として機能することが示唆された¹⁰⁾。

興味深いことにこれら3種のヘリカーゼ遺伝子はいずれも ISG であり、一度細胞にウイルス感染が起こるとわずかな IFN β が産生され、そのシグナルによって ISG である RIG-I や MDA5 の発現が増強される。すると、さらに大量に強力な IFN 産生シグナルが伝達されるようになると考えられる。一方 IFN β が産生されると LGP2 の発現量も上昇し、増強されたシグナルを抑制することで IFN シグナルの一過性が調節されることが予測される。



図② RIG-Iヘリカーゼとファミリー分子

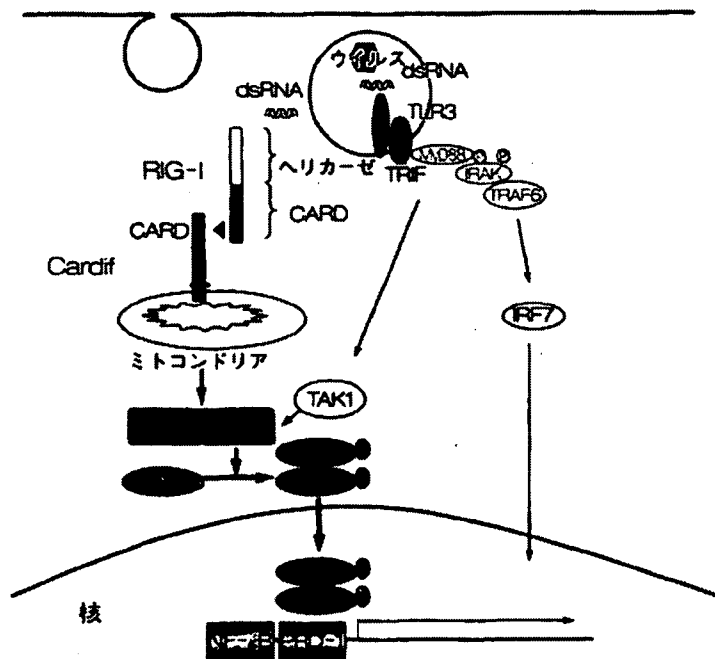
2006年、RIG-I/MDA5の下流で機能するアダプター分子として IFN β promoter stimulator-1 (IPS-1)/mitochondrial anti-viral signaling protein (MAVS)/virus induced signaling adaptor (VISA)/CARD adaptor inducing IFN β (Cardif) が4つの異なるグループから同時に報告された。IPS-1はN末端のCARDドメインとC末端のエフェクタードメインからなる。IPS-1は活性化されたRIG-IやMDA5とCARD-CARDで会合する分子であり、その機能が下流へのシグナル伝達に必須である¹⁴⁾。IPS-1を強発現すると、IFN β 、IFN α 4、IFN α 6、NF- κ Bのプロモーターを活性化し、ウイルス複製の抑制がみられ、IPS-1をRNA-iによってノックダウンすると、dsRNAを細胞質内に導入した際のI型IFN産生や、ウイルス感染によるI型IFN産生が抑制される。このように、IPS-1はRIG-IまたはMDA5依存性の抗ウイルス応答におけるアダプター分子であることが明らかとなった¹⁵⁾。MAVSの報告では、MAVSがミトコンドリア外膜に存在することが示され、シグナルの伝達にはミトコンドリアを標的とした膜貫通ドメインが必須であり、アポトーシスを含めたその生理的な意義に関心が集まっている。

■ C型肝炎ウイルス蛋白はRIG-Iを介するIFN応答を抑制する

宿主細胞はウイルス感染を検知してさまざまな方法でウイルスの増殖を抑制するが、ウイルス側も宿主細胞の自然免疫応答を確認することで有利な増殖の持続を成立させる。最近、ワクシニアウイルス (Vaccinia virus) をはじめとした多くのウイルスが、自然免疫応答のシグナルを遮断することにより、ウイルスに有利な感染を成立

させることが明らかとなった¹⁶⁾¹⁷⁾。C型肝炎ウイルス (HCV) と宿主免疫応答系の関係についてはこれまで多くの報告があり、とくに構造蛋白であるcore蛋白や、非構造蛋白であるNS5Aによる免疫応答の抑制が報告されている。NS3はHCVの多機能蛋白で、プロテアーゼとRNAヘリカーゼという2つの活性をもつ分子であり、NS4Aと会合することで自己のウイルス蛋白の修飾をおこなう。それぞれがウイルスポリプロテインの切断やウイルスRNAの複製に関与することが知られているが、最近NS3/4AがIRF3のシグナルを抑制することが報告された¹⁸⁾。さらにその活性抑制はRIG-Iを介したシグナルであることから、NS3/4AはTRIFを標的として蛋白を切ることによって、TLR3依存的な経路を障害するだけでなく、RIG-I依存的な経路も障害することがわかった¹⁵⁾¹⁹⁾。RIG-I自体はNS3/4Aによって分解されないことから、その下流に分解される未知の分子の存在が疑われていた²⁰⁾。その後、アダプター分子であるIPS-1が発見され、NS3/4Aによる分解の標的となることが示された¹⁷⁾。IPS-1の分解にあたっては、NS3/4AはIPS-1をミトコンドリア膜貫通ドメインの直上で分解することが明らかになり、IPS-1をミトコンドリア膜から切り離すことで不活化し、以下のシグナル伝達を遮断する(図③)¹⁶⁾¹⁸⁾¹⁹⁾。この段階でのシグナル遮断は、宿主免疫応答でも初期の段階にあたることから、遮断されることにより、その後幅広いひろがるサイトカイン誘導や、ISG発現、獲得免疫系などの抗ウイルス反応に影響を及ぼし、HCV増殖に有利な環境をもたらすことになる。

以上より、HCV感染によって誘導される宿主免疫応答と、感染したウイルスによる宿主免疫抑制機構とのバ



図③ HCV 蛋白による IFN 産生系の抑制機構

ランスにより HCV 感染の経過は大きく変化することが示唆され、このバランスのなかで宿主免疫応答に主導権をわたすことが HCV 感染治療につながる。近年、NS 3/4 A を標的とした抗ウイルス薬開発が進んでおり、宿主ウイルス間の反応において NS 3/4 A が大きな役割を果たすことが明らかとなった現在、著しく期待が集まっているものである。

NS 4 B は IFN 産生シグナル伝達を抑制する

われわれは数種の細胞株から樹立した HCV レプリコン細胞を用いた解析により、レプリコン発現細胞では RIG-I 依存性の二重鎖 RNA 応答が著明に低下しており、個々の HCV 非構造蛋白を強発現させた解析により、NS 3/4 A 以外に新たに NS 4 B が IFN 産生シグナル伝達系を抑制することを確認した。NS 4 B は HCV 蛋白のなかでもその機能に関する報告は少ないが、小胞体膜を貫通して存在し HCV 増殖複合体の土台を形成する。NS 4 B 変異株では HCV レプリコン増殖能が低下する、など HCV 増殖に関する報告がみられており、現在その機能を解析中である。

おわりに

最近の自然免疫系の研究や HCV 培養増殖系モデルの進歩に伴い、自然免疫応答と、HCV 増殖との関係の詳細が分子レベルで明らかになってきた。今後これらの知見をさらに深めていき、HCV 感染制御を可能とする新たな治療法の開発が期待される。



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Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA

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Abstract

We recently established a genome-length HCV RNA-replicating cell line (O strain of genotype 1b; here called O cells) using cured cells derived from sO cells, in which HCV subgenomic replicon RNA with an adaptive NS5A mutation (S2200R) is replicated. Characterization of the O cells revealed a second adaptive NS3 mutation (K1609E) required for genome-length HCV RNA replication. To clarify the role of adaptive mutation in genome-length HCV RNA replication, we newly established one and three kinds of genome-length HCV RNA-replicating cell lines possessing the cell background of sO and O cells, respectively, and found additional adaptive NS3 mutations (Q1112R, P1115L, and E1202G) required for the robust replication of genome-length HCV RNA. We further found that specific combinations of adaptive NS3 mutations drastically enhanced HCV RNA replication, regardless of the cell lines examined. These findings suggest that specific viral factors may affect the replication level of genome-length HCV RNA.

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Keywords: Hepatitis C virus; Adaptive mutation; Genome-length HCV RNA replication; HCV RNA-replicating cell line

1. Introduction

Infection with the hepatitis C virus (HCV), of the family *Flaviviridae*, frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. Since more than 170 million individuals are estimated to be infected with HCV worldwide, this disease is a global health problem (Thomas, 2000). HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993). These HCV proteins function not only in virus replication but may also affect a variety of cellular functions, including gene

expression, signal transduction, and apoptosis (Bartenschlager and Lohmann, 2000; Kato, 2001).

Although studies on the mechanism of HCV replication were for many years difficult due to the lack of efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000), such studies proliferated after the development of subgenomic HCV replicon (Con-1 of genotype 1b) that was capable of replication in human hepatoma (HuH-7) cells (Lohmann et al., 1999). The subgenomic replicon RNA is composed of the HCV 5'-untranslated region (UTR) fused to the first 12 aa of the core coding region, the neomycin phosphotransferase (*Neo*^R) gene as a selectable marker, and the HCV NS3-NS5B regions under the control of an encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), followed by 3'-UTR. After the first replicon, several additional replicons derived from H77 (1a), N (1b), 1B-1 (1b), O (1b), and JFH-1 (2a) strains were developed, and tissue, genotype, and host ranges were also expanded (Ali et al., 2004; Blight et al., 2000; Date et al., 2004; Ikeda et al., 2002; Kato and Sugiyama et al., 2003; Kato and Date et al., 2003; Kishine et al., 2002; Zhu et al., 2003). Since intracellular replicon RNAs were easily detected by Northern blot analysis and the HCV proteins produced were detected by

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Western blot analysis, these cell culture replication systems became valuable tools for basic studies of HCV, such as studies for viral replication and drug development (Bartenschlager, 2002, 2005; Lindenbach and Rice, 2005). However, in attempts to examine what happens in HCV-infected hepatocytes, subgenomic HCV replicons were insufficient because they lacked the effects of HCV structural proteins. For this reason, five kinds of genome-length HCV RNA-replicating cell lines, derived from H77 (1a), N (1b), Con-1 (1b), O (1b), and JFH-1 (2a) strains, have been established to date (Ikeda et al., 2002, 2005; Blight et al., 2002; Lindenbach et al., 2005; Pietschmann et al., 2002; Wakita et al., 2005; Zhong et al., 2005). Regarding the JFH-1 strain, the infectious virus was efficiently produced in cell culture (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

Studies in the past few years using subgenomic HCV replicon systems have revealed that most replicons possess cell culture-adaptive mutations, which enhance the efficiency of RNA replication and arise during G418 selection. Although these mutations have been found in most NS regions, they cluster in three distinct areas: the N-terminus of the NS3 helicase, two distinct positions of NS4B, and the center of NS5A (Appel et al., 2005; Blight et al., 2000, 2002, 2003; Grobler et al., 2003; Ikeda et al., 2002, 2005; Kato and Sugiyama et al., 2003; Kishine et al., 2002; Krieger et al., 2001; Lohmann et al., 2001, 2003; Yi and Lemon, 2004). To date, however, little information is available on the adaptive mutations obtained from a genome-length HCV RNA replication system. On the other hand, highly permissive cells (cured cells) for efficient RNA replication were also obtained by the elimination of replicons from the G418-selected cells by interferon (IFN) treatment (Blight et al., 2002; Kato and Sugiyama et al., 2003; Lohmann et al., 2003). These reports suggest that both viral and cellular factors determine the efficiency of RNA replication.

The sO replicon (O strain) that we developed also possesses a unique adaptive mutation (S2200R) in the center of NS5A (Kato and Sugiyama et al., 2003), and we recently established a genome-length HCV RNA-replicating cell line (O cells) by the transfection of genome-length HCV RNA with S2200R mutation into sOc cured cells, which were created by eliminating sO replicon from sO cells by IFN treatment (Ikeda et al., 2005). Sequence and functional analyses of HCV RNAs obtained from the O cells found a second adaptive mutation (K1609E) in the C-terminus of the NS3 helicase. We further found that the Oc cells, which were created by eliminating HCV RNA from O cells by IFN treatment, possessed more overwhelming advantages than the sOc cells in the replication of genome-length HCV RNA, even though the O cells were derived from sO cells (Ikeda et al., 2005). These results suggest that a second adaptive mutation, such as K1609E, is required for the robust replication of genome-length HCV RNA, and that the cell backgrounds regarding the potentials of genome-length HCV RNA replication differ greatly between Oc and sOc cells.

To evaluate these ideas, we newly established four kinds of genome-length HCV RNA-replicating cells possessing the cell background of sO or O cells, and then we characterized the genetic mutations detected in the replicating HCV RNAs. Here, we report the findings of adaptive NS3 mutations required for

the robust replication of genome-length HCV RNA, and the drastic enhancement of HCV RNA replication by the specific combination of these adaptive NS3 mutations.

2. Materials and methods

2.1. Cell culture

sOc and Oc cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum as described previously (Ikeda et al., 2005). Cells supporting genome-length HCV RNAs were cultured in the presence of G418 (300 µg/ml; Geneticin, Invitrogen, Carlsbad, CA) and passaged twice a week at a 5:1 split ratio. HCV RNA-replicating cells possess the G418-resistant phenotype because *Neo^R* was produced by the efficient replication of HCV RNA. Therefore, when HCV RNA is excluded from the cells or when its level is decreased, the cells are killed in the presence of G418.

2.2. Plasmid constructions

To introduce the mutations into the plasmid pON/C-5B (GenBank accession no. AB191333; Fig. 1; Ikeda et al., 2005), a PCR-based site-directed mutagenesis method was used. The *SpeI*-*NotI* fragment (corresponding to positions 3474–6159 of the HCV genome) and the *NotI*-*KpnI* fragment (corresponding to positions 6159–9077 of the HCV genome) of pHCV-O (Ikeda et al., 2005) were subcloned into pBluescript II (Stratagene, La Jolla, CA), resulting in pBlue/34AB and pBlue/5AB, respectively. pBlue/34AB and pBlue/5AB were used as the templates for PCR-based site-directed mutagenesis. The introduced mutations were confirmed by the sequencing of the obtained plasmids. The *SpeI*-*NotI* and *NotI*-*KpnI* fragments possessing the mutation(s) were each replaced with the corresponding region of pHCV-O. The pON/C-5B possessing mutation or mutations were generated by replacing the *EcoRI*-*SpeI* fragment of the pHCV-O, into which one or more mutations were introduced.

To construct pOR/C-5B, the *Neo^R* gene was replaced with the *Renilla luciferase* (RL) gene at *AscI* and *PmeI* sites in pON/C-5B.

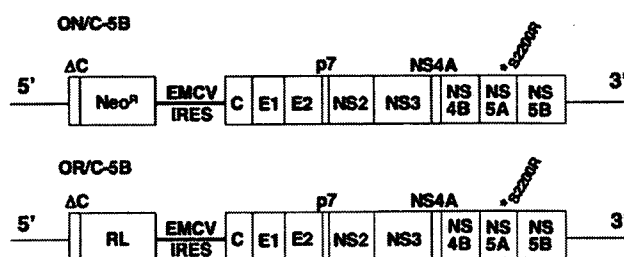


Fig. 1. Schematic gene organization of genome-length HCV RNAs used in this study. Open reading frames, untranslated regions, and EMCV IRES are depicted as open boxes, thin lines, and thick lines, respectively. ΔC indicates the 12N-terminal aa residues of the core as a part of IRES. ON/C-5B RNA and OR/C-5B RNA possess the *Neo^R* and RL genes, respectively. The asterisk indicates an adaptive mutation (S2200R) found in the sO subgenomic replicon (Kato and Sugiyama et al., 2003).

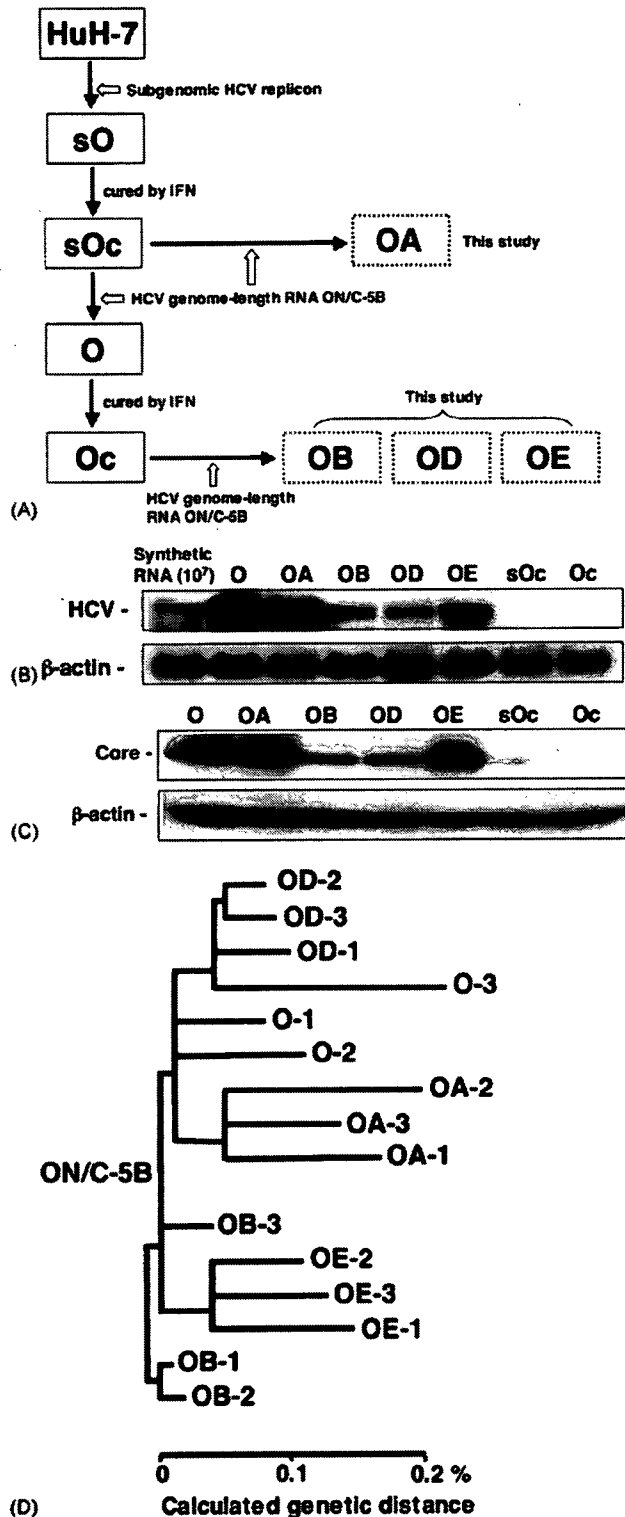


Fig. 2. Characterization of genome-length HCV RNA-replicating cell lines. (A) Lineage of genome-length HCV RNA-replicating cells. The sO and O cell lines were previously established (Ikeda et al., 2005; Kato and Sugiyama et al., 2003). (B) Northern blot analysis. Total RNAs from genome-length HCV RNA-replicating cells (O, OA, OB, OD, and OE cells), as well as total RNAs from the sOc and the Oc cells, were analyzed by Northern blot analysis using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β -actin-specific probe (lower panel). In vitro-synthesized ON/C-5B RNA (10^7 genome

2.3. RNA synthesis

Plasmid DNAs were linearized with *Xba*I and used for RNA synthesis with the T7 MEGAscript kit (Ambion, Austin, TX). Synthesized RNA was purified by lithium chloride precipitation and dissolved in nuclease-free water.

2.4. RNA transfection and selection of G418-resistant cells

RNA transfection and selection of G418-resistant cells were carried out as described previously (Ikeda et al., 2005). Briefly, for electroporation, sOc or Oc cells were suspended at 10^7 cells/ml in phosphate-buffered saline (PBS), and then RNA was mixed with 500 μ l of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad Laboratories, Hercules, CA). The mixture was immediately subjected to two electric pulses of 1.2 kV, 25 μ F, and maximum resistance. The cells were then seeded into a 10 cm diameter dish. After 24 h, the cells were selected in complete DMEM with 300 μ g/ml G418 for 3 weeks.

2.5. Northern blot analysis

Total RNA from the cultured cells was prepared using an RNeasy extraction kit (Qiagen, Hilden, Germany). Three micrograms of RNA was used to detect the HCV RNA and β -actin mRNA. Northern blotting and hybridization were carried out as described previously (Ikeda et al., 2002; Kato and Sugiyama et al., 2003). A digoxigenin-labeled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) and β -actin-specific antisense RNA probe were used to detect the HCV RNA and β -actin mRNA, respectively (Kato and Sugiyama et al., 2003; Kato et al., 2005).

2.6. Western blot analysis

The preparation of cell lysates, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a PVDV membrane were performed as described previously (Hijikata et al., 1993; Naganuma et al., 2000). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan) and β -actin (AC-15; Sigma-Aldrich, St. Louis, MO). Immuno-complexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences, Wellesley, MA).

equivalents spiked into normal cellular RNA) was used for the comparison of the expression level. (C) Western blot analysis. The orders of specimens were the same as in (B). Production of the core in these cells was analyzed by immunoblotting using anti-core antibody. β -actin was used as a control for the amount of protein loaded per lane. (D) Phylogenetic tree of HCV-O clone populations obtained from genome-length HCV RNA-replicating cells. The phylogenetic tree is depicted on the basis of nucleotide sequences of all clones obtained from the O, OA, OB, OD, and OE cells.

2.7. Preparation of cured cells

To prepare cured cells, HCV RNA-replicating cells were treated with IFN- α or IFN- γ as described previously (Abe et al., 2005; Ikeda et al., 2005; Naka et al., 2005). Briefly, the cells (each 1×10^6) were treated with IFN- α or IFN- γ (each 500 IU/ml) in the absence of G418. The treatment was continued for 3 weeks with the addition of IFN at 4-day intervals. The cured cells obtained from sO, O, OA, OB, OD, and OE cells (HCV RNA-replicating cells obtained in this study, see Fig. 2A) were named sOc, Oc, OAc, OBc, ODc, and OEc, respectively, and were cultured in DMEM supplemented with 10% fetal bovine serum in the absence of G418. RT-PCR confirmed the absence of HCV RNA in these cured cells.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

To amplify HCV RNA, RT-PCR was carried out separately in two parts as described previously (Ikeda et al., 2005). Briefly, one part covered from HCV 5'-UTR to NS3, with a final product of approximately 5.1 kb, and the other part covered from NS2 to most of HCV 3'-UTR, with a final product of approximately 6.1 kb. These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis for HCV open reading frame (ORF) after cloning into pBR322MC (Kishine et al., 2002). SuperScript II (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

2.9. cDNA cloning and sequencing

Two PCR products (5.1 and 6.1 kb) were digested with *Xba*I and then cloned into the *Xba*I site of pBR322MC, as described previously (Kato et al., 2005). Plasmid insertions were sequenced in both the sense and antisense directions using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Life Sciences) on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.10. Transient replication assays

The cells were transfected with 20 μ g RNA by electroporation as described above. After electroporation, the cells were plated onto 24-well plates at 3×10^4 cells per well. The cells were harvested with Renilla lysis reagent (Promega, Madison, WI) at 24, 48, 72, and 96 h after the electroporation, and were subjected to luciferase assay according to the manufacturer's protocol.

2.11. Molecular evolutionary analysis

Nucleotide sequences (10,972 nucleotides of HCV 5'-UTR to NS5B) of clones obtained by RT-PCRs from O, OA, OB, OD, and OE cells were analyzed by neighbor-joining analysis using the program GENETYX-MAC (Software Development, Tokyo, Japan).

3. Results

3.1. Establishment of genome-length HCV RNA-replicating cell lines

We previously established a cloned sO cell line possessing a subgenomic HCV replicon (O strain) and found an adaptive mutation (S2200R) in NS5A (Kato and Sugiyama et al., 2003). More recently we further established a cloned O cell line replicating genome-length HCV RNA by the selection with G418 treatment following the electroporation of genome-length HCV RNA with the S2200R mutation into sOc cured cells (Ikeda et al., 2005). In that study, we found a second adaptive mutation (K1609E) in the NS3 helicase region of HCV RNA-replicating in the O cells, and we also observed that the Oc cured cells showed more overwhelming advantages than the sOc cells in the replication of genome-length HCV RNA (Ikeda et al., 2005). These results suggested that the K1609E mutation was required for the robust replication of genome-length HCV RNA, and that cell backgrounds regarding the potentials of RNA replication differ greatly between sOc and Oc cells. However, we could not clarify whether or not K1609E is the unique or best adaptive mutation for RNA replication. Furthermore, we obtained no information indicating whether viral or host factors are the main contributors to the robust RNA replication.

To obtain such information, we attempted to establish additional genome-length HCV RNA-replicating cell lines by the electroporation of ON/C-5B RNA, which possesses the S2200R mutation (Fig. 1), into sOc or Oc cells (Fig. 2A). After 3 weeks of G418 selection, we obtained one G418-resistant colony derived from sOc cells and three G418-resistant colonies derived from Oc cells. These G418-resistant colonies proliferated successfully; the sOc-derived colony was named OA cell line and Oc-derived three colonies were called OB, OD, and OE cell lines (Fig. 2A), and then these cell lines were used for further analysis. To examine the replication level of genome-length HCV RNA in these cell lines, total RNAs and proteins extracted from OA, OB, OD, and OE cells were subjected to Northern and Western blot analyses, respectively. Total RNAs and proteins extracted from the O, sOc, and Oc cells were also used for the comparison. Genome-length HCV RNAs approximately 11 kb long were detected in all specimens except those from the sOc and Oc cells (Fig. 2B). The number of copies of HCV RNAs in total RNA (each 3 μ g) was estimated to be more than 10^7 by comparing these HCV RNAs with HCV RNA synthesized *in vitro*, although the levels in OB and OD cells were somewhat lower than those in O, OA, and OE cells. The core was also detected in all specimens except those from the sOc and Oc cells (Fig. 2C). The levels of the core in OB and OD cells were also somewhat lower than those in O, OA, and OE cells. These results suggest that the replication levels of HCV RNA in OA cells are equivalent to that in O cells and are higher than those in OB, OD, and OE cells. Although the expression of HCV RNAs and HCV proteins differed somewhat among these cell lines, these lines, including that of O cells, were maintained for at least several months in the presence of G418 (data not shown), suggesting the stable robust HCV RNA replication. The OA, OB, OD, and OE cells

were highly sensitive to IFN- α towards HCV RNA replication (data not shown), as were the O cells (Ikeda et al., 2005; Naka et al., 2005).

3.2. Genetic analysis of HCV RNAs replicating in the OA, OB, OD, and OE cells and comparison with that in the O cells

To learn whether or not HCV RNAs replicating in the OA, OB, OD, and OE cells possess the K1609E mutation, we performed sequence analysis of HCV RNAs replicating in these cell lines. Total RNAs extracted from these cells were subjected to RT-PCR, and then two fragments (5.1 and 6.1 kb) amplified by RT-PCR for ORF were subcloned into plasmid for sequence analysis, as described previously (Ikeda et al., 2005). The sequences of three independent clones were determined and compared with each other to avoid PCR error and to find conserved mutations. Based on the nucleotide sequence data of all clones sequenced in this study and the data obtained from the O cells (Ikeda et al., 2005), we constructed a phylogenetic tree for the HCV RNAs sequenced (10,972 nucleotides of HCV 5'-UTR to NS5B). The result (Fig. 2D) revealed that the three clones derived from each cell line were mostly clustered and located at similar genetic distances from the origin (ON/C-5B), although O-3 and OB-3 were not clustered completely in the expected positions, suggesting that O~OE are independent cell lines. In our sequence analysis, the K1609E mutation found in the O cells was not detected in the OA, OB, OD, and OE cells. However, instead of the K1609E mutation, each cell line possessed a cell line-specific conserved mutation in the NS3 protease region (Table 1). The E1202G, P1115L, Q1112R, and P1115L mutations in the NS3 protease region were detected in the OA, OB, OD, and OE cells, respectively. These results indicated that K1609E was not a representative mutation in genome-length HCV RNA-replicating in the cells. Although Q1112R, P1115L, and E1202G mutations have been detected in other subgenomic HCV replicons (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2003), E1202G mutation seems to have little impact on adaptive mutations (Lohmann et al., 2003) and there is no information on whether or not Q1112R and P1115L are adaptive mutations. Since these mutations were detected as cell line-specific conserved NS3 mutations, we estimated that these NS3 mutations are required for genome-length HCV RNA replication. However, we estimated that the D2415G mutation (the carboxyl region of NS5A) detected in the OA cells is not an adaptive mutation, because this is a naturally observed

aa substitution (Murphy et al., 2002; Tanaka et al., 1992). In addition, none of the conserved mutations in the upstream of the NS3 region were detected in the OA, OB, and OD cells or in the O cells, although three conserved mutations (I258K and Y361H in E1 and M939V in NS2) were detected in the OE cells (Table 1). Therefore, we focused on the NS3 mutations found in this analysis for further analyses described below.

3.3. Adaptive mutations found in the NS3 region are required for the robust replication of genome-length HCV RNA

To clarify whether or not conserved NS3 mutations (Q1112R, P1115L, E1202G) are adaptive mutations as are the K1609E mutation, we first carried out qualitative analysis regarding the effects of these mutations in ON/C-5B RNA on the efficiency of colony formation (ECF). The effect of the K1609E mutation derived from O cells was also examined for the comparison. We introduced ON/C-5B RNA with a single NS3 mutation into sOc and Oc cells. Since our previous study (Ikeda et al., 2005) indicated that Oc cells possessed overwhelming advantages in the replication of genome-length HCV RNA, one-twentieth of the ON/C-5B RNA used for sOc cells was used for Oc cells. The results revealed that the NS3 mutated RNA-introduced sOc and Oc cells produced a number of G418-resistant colonies, as did those in K1609E mutation-introduced cells, although no and a few G418-resistant colonies were obtained in the original ON/C-5B RNA-introduced sOc and Oc cells, respectively (Fig. 3). Although the K1609E or E1202G mutation was found in the sOc-derived O or OA cells, the effect of the K1609E or E1202G mutation was not different from that of the Q1112R or P1115L mutation found in the Oc-derived OB, OD, or OE cells. This result indicated that the effects of these mutations are not dependent on their cell origins, and that the Q1112R, P1115L, and E1202G mutations also worked as cell culture-adaptive mutations, as did the K1609E mutation in both sOc and Oc cells. Furthermore, these results indicated again that Oc cells are superior to sOc cells regarding the intracellular replication of genome-length HCV RNA, supporting the previous suggestion (Ikeda et al., 2005) that the cell backgrounds for the potentials of the RNA replication are rather different between Oc and sOc cells.

We next performed quantitative analysis whether or not the effects of these adaptive mutations on ECF are correlated with the effects in early events after RNA transfection into the cells, because of the possibility that additional adaptive mutations

Table 1
Summary of genetic analysis of HCV RNAs derived from O, OA, OB, OD, and OE cells

Cell	Cell background	Size (Nts)	Clone number sequenced	Conserved mutations
O	sOc	10972	3	K1609E (NS3)
OA	sOc	10972	3	E1202G (NS3) D2415G (NS 5A)
OB	Oc	10972	3	P1115L (NS3)
OD	Oc	10972	3	Q1112R (NS3)
OE	Oc	10972	3	I258K (E1) Y361H (E1) M939V (NS2) P1115L (NS3)

Only amino acid positions substituted in all three clones are indicated as the conserved mutations.

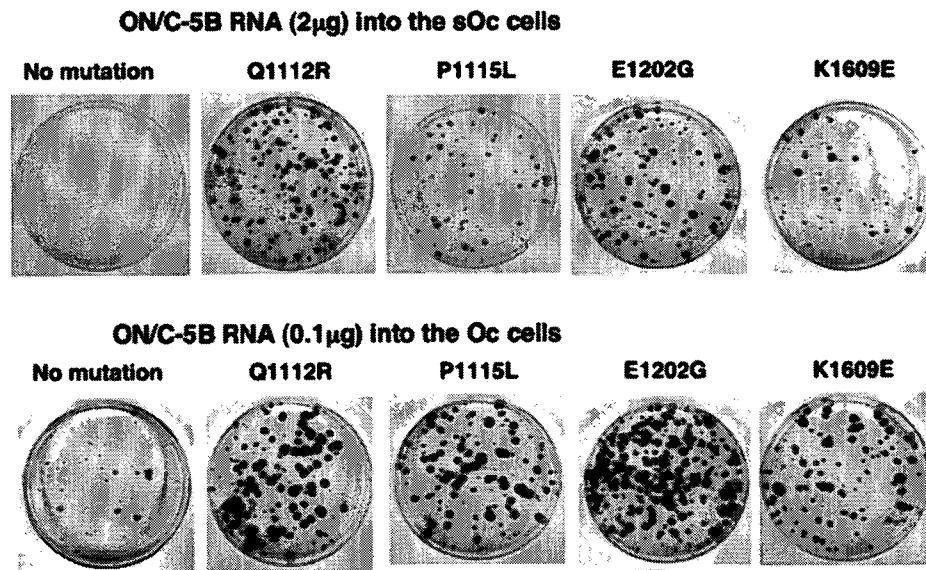


Fig. 3. Adaptive mutations found in the NS3 region show the different ECFs between the sOc and Oc cells. ONC-5B RNA or ONC-5B RNA with an additional NS3 mutation was transfected into the sOc cells (2 μ g RNA per 10 cm dish; top panel) and the Oc cells (0.1 μ g RNA per 10 cm dish; bottom panel). The panels show G418-resistant colonies that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection (Naganuma et al., 2004).

occur during G418 selection for 3 weeks. For the development of a transient replication assay using the RL reporter gene, we first constructed pOR/C-5B from pONC-5B by replacing the RL and *Neo^R* genes (Fig. 1). Next, we made OR/C-5B RNA with the Q1112R, P1115L, E1202G, or K1609E mutation, and then introduced these RNAs into the Oc cells by electroporation. OR/C-5B RNA with dGDD was used as a negative control. The results revealed that the luciferase activity increased in a time-dependent manner when OR/C-5B RNA with an adaptive NS3 mutation was transfected, whereas the activity decreased with time when OR/C-5B RNA without an adaptive NS3 mutation or with dGDD was transfected (Fig. 4A). These results suggest that genome-length HCV RNA with an adaptive NS3 mutation is efficiently able to replicate immediately after transfection. At 96 h, the luciferase activities in the cases of the adaptive NS3 mutation were approximately twice those at 24 h after RNA transfection, and no significant differences in replication efficiency among the adaptive NS3 mutations were observed (Fig. 4A). In summary, we demonstrated a good correlation between the ECF assay (Fig. 3) and luciferase reporter assay for transient replication (Fig. 4A). Therefore, we conclude that Q1112R, P1115L, E1202G, and K1609E function as cell culture-adaptive mutations, and that at least one of them is required for efficient replication of genome-length HCV RNA.

3.4. Every combination of adaptive NS3 mutations in the Oc cells caused more effective genome-length HCV RNA replication than any single adaptive NS3 mutation

According to previous reports using subgenomic HCV replicons, some combinations of adaptive mutations drastically enhance ECF (Krieger et al., 2001; Lohmann et al., 2001, 2003). However, some combinations of adaptive mutations reduced ECF drastically (Lohmann et al., 2001, 2003), suggest-

ing that some adaptive mutations are not compatible. To examine whether or not such conflicting effects of adaptive mutations are observed in genome-length HCV RNA replication, we tested the effects of combining the adaptive NS3 mutations identified in this study, using the luciferase reporter assay for transient replication. We prepared six kinds of OR/C-5B RNA with double adaptive NS3 mutations (i.e., Q1112R and P1115L), and then introduced these RNAs into the Oc cells by electroporation. OR/C-5B RNA with the K1609E mutation was used as a representative of a single adaptive NS3 mutation, and OR/C-5B RNA with dGDD was used as a negative control. The results revealed that the luciferase activities in every combination of adaptive NS3 mutations were remarkably increased, to approximately four- to nine-fold at 96 h, in comparison with the activities at 24 h after RNA transfection, although the enhancement of the luciferase activity by the K1609E mutation was only approximately two-fold (Fig. 4B). The combination of Q1112R and K1609E mutations was the most effective in the Oc cells, followed by that of Q1112R and P1115L mutations. These results suggest that all adaptive NS3 mutations identified in this study are compatible for genome-length HCV RNA replication. It is noteworthy that Q1112R and P1115L mutations are compatible, regardless of very near localization in the NS3 protease.

3.5. Specific combination of adaptive NS3 mutations drastically enhanced genome-length HCV RNA replication, regardless of cell lines

Although we found that every combination of adaptive NS3 mutations caused more effective genome-length HCV RNA replication than any single adaptive NS3 mutation and that some combinations of the mutations drastically enhanced RNA replication (Fig. 4B), the possibility remains that these findings are due to cell clonality, because a cloned Oc cell line was used

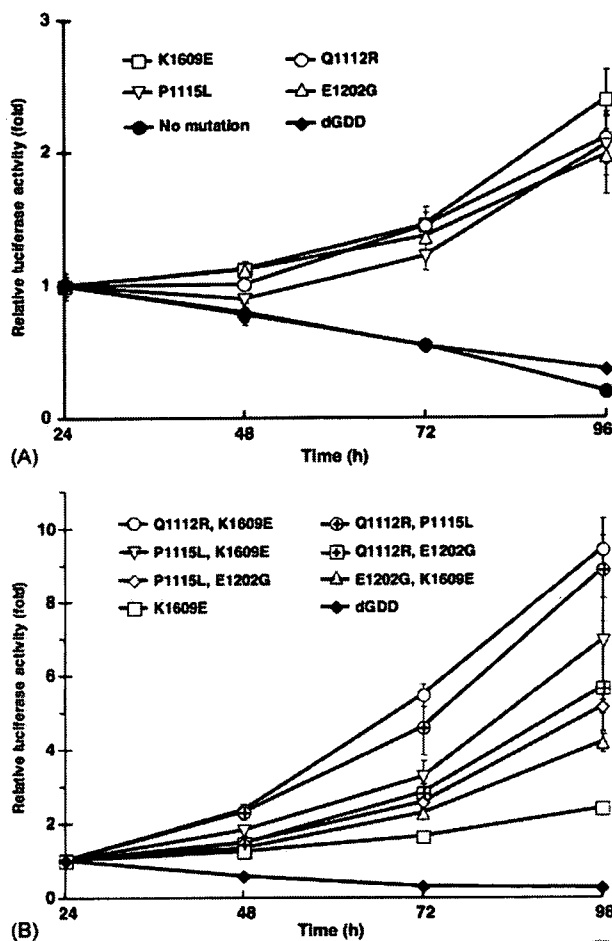


Fig. 4. Transient replication assay of genome-length HCV RNA. (A) Effects of adaptive NS3 mutations on transient replication of genome-length HCV RNA. OR/C-5B RNA or OR/C-5B RNA with an additional mutation was transfected into the Oc cells, and luciferase activity was determined in cell lysates that were prepared at given time points post-transfection. Values for each time point correspond to the mean and the error range of quadruplicate results. Note that, owing to the slightness of the variations, error bars are in some cases not viable in the graph. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 24 h after transfection. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the genome-length HCV RNA with dGDD was used as a negative control. (B) Combination effects of adaptive NS3 mutations on transient replication of genome-length HCV RNA. OR/C-5B RNAs with various combinations of adaptive NS3 mutations were transfected into the Oc cells, and luciferase activity was determined in cell lysates that were prepared at given time points post-transfection, as shown in (A). For a comparison of the results, OR/C-5B RNA with K1609E was used as a representative of a single adaptive mutation.

for the analysis. To check this possibility, we tested the effects of combining adaptive NS3 mutations in the cured OAc, OBc, ODc, and OEc cells, using the transient replication assay as described above. sOc cells, which are rather inferior to the Oc cells in RNA replication (Ikeda et al., 2005; Fig. 3), were also used for the analysis. Interestingly, in the OAc, OBc, ODc, and OEc cells also, the results were similar to those obtained in the Oc cells. The combination of Q1112R and K1609E mutations or Q1112R and P1115L mutations was the most effective on the genome-length HCV RNA replication, although the combina-

tion of P1115L and K1609E mutations or E1202G and K1609E mutations was not effective in ODc cells (Fig. 5). These results suggest that the NS3 with specific adaptive mutations is the primary determinant of the replication level of genome-length HCV RNA, regardless of cell lines. In addition, we found that OEc cells possessed the best environment for RNA replication among examined cell lines, by demonstrating that the luciferase activity in the combination of Q1112R and K1609E mutations was approximately 20-fold higher at 96 h than that at 24 h after RNA transfection (Fig. 5). On the other hand, we observed that most combinations of adaptive mutations in the sOc cells did not enhance RNA replication, although luciferase activity was enhanced approximately two-fold in the combination of Q1112R and P1115L only (Fig. 5). These results suggest that the cellular environment is also involved in the efficient replication of genome-length HCV RNA.

4. Discussion

In this study, we established four kinds of genome-length HCV RNA (O strain of genotype 1b) replicating cell lines (OA, OB, OD, and OE), which were independent from the O cell line established previously (Ikeda et al., 2005). We also found several cell culture-adaptive NS3 mutations required for the replication of genome-length HCV RNA. We further found that specific combinations of these adaptive mutations remarkably enhanced the efficiency of the RNA replication, regardless of the cell lines obtained.

To establish genome-length HCV RNA-replicating cell lines, we introduced ON/C-5B RNA with the S2200R mutation (NS5A), which was identified as the adaptive mutation for the sO replicon, into two types (sOc and Oc) of cured cells. Since the ECF of the Oc cells was higher than that of the parental sOc cells (Ikeda et al., 2005), the Oc cells were also used to facilitate the establishment of genome-length HCV RNA-replicating cell lines. We initially estimated that adaptive mutations other than K1609E (NS3 helicase region) found in the O cells would be obtained from the sOc-derived OA cell line, and that the K1609E mutation would be obtained mainly from the Oc-derived OB, OD, and OE cell lines. Although a new E1202G adaptive mutation was obtained from the sOc-derived OA cells, the K1609E mutation was not obtained from the Oc-derived OB, OD, and OE cells. Instead of the K1609E mutation, the P1115L adaptive mutation (NS3 protease region) was obtained from the OB and OE cells, and the Q1112R adaptive mutation (NS3 protease region) was obtained from the OD cells. The ECF assay and transient replication assay showed that these adaptive mutations possessed similar potentials of genome-length HCV RNA replication in the Oc cells. These results suggest that the K1609E mutation is only one of the adaptive mutations that function in the Oc cells, and suggest that the combination of an NS3 mutation (Q1112R, P1115L, E1202G, or K1609E) with the NS5A mutation (S2200R) is required for efficient replication of genome-length HCV RNA, although only the S2200R mutation is enough to efficiently replicate the subgenomic sO replicon (Kato and Sugiyama et al., 2003). Therefore, our findings suggest that viral factors, which are not required for the robust

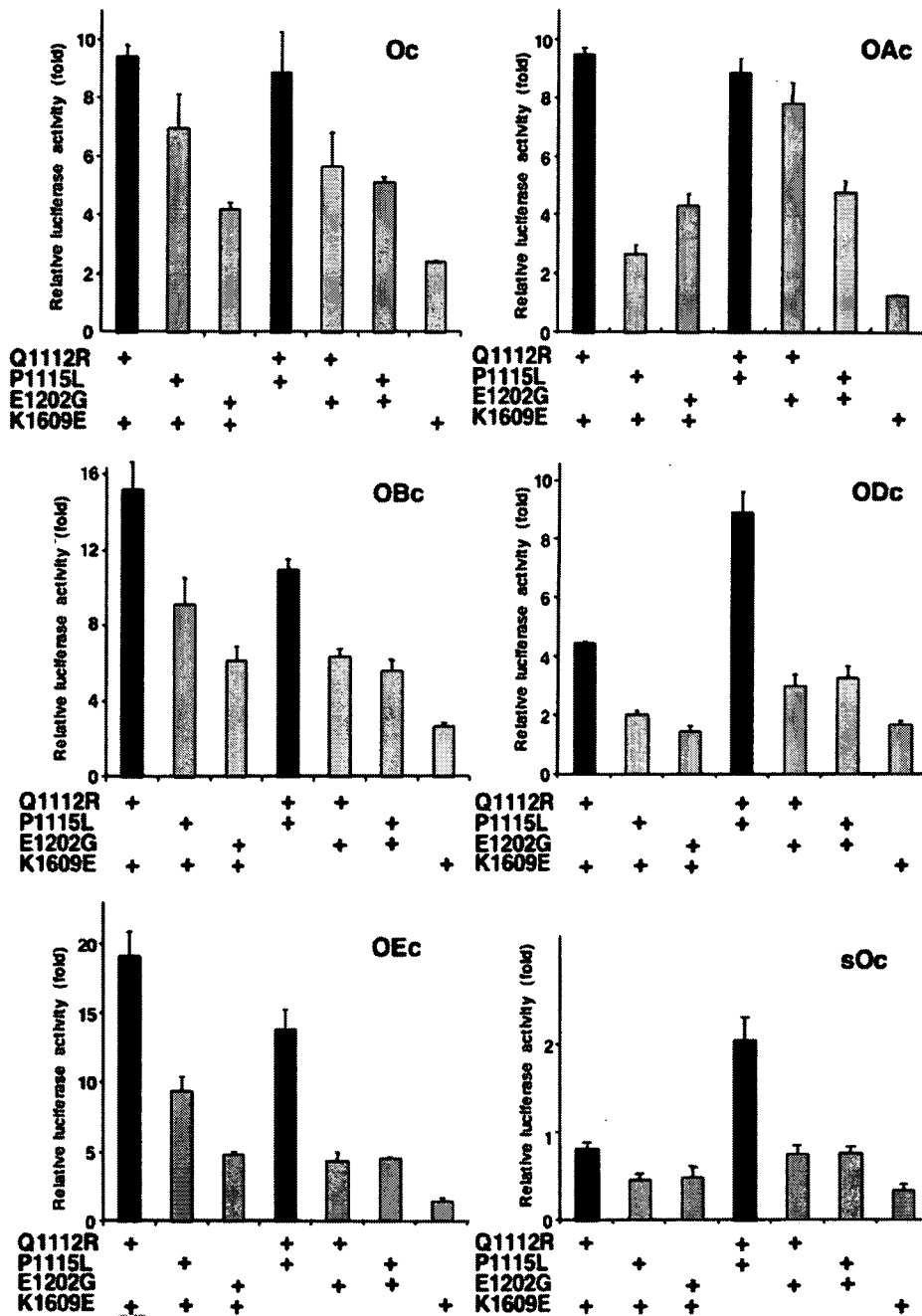


Fig. 5. Effects of combining adaptive NS3 mutations on transient genome-length HCV RNA replication in various cured cells. OR/C-5B RNAs with various combinations of adaptive NS3 mutations were transfected into various cured cells (Oc, OAc, OBc, ODc, OEc, and sOc cells), and luciferase activity was determined in cell lysates that were prepared at 96 h post-transfection, as shown in Fig. 4. Values are corrected for transfection efficiency as determined by measuring the luciferase activity 24 h after transfection.

replication of a subgenomic HCV replicon, are required for the robust replications of genome-length HCV RNA.

To date, it has been believed that cell culture-adaptive mutations that enhance HCV RNA replication do not exist in HCV-infected patients (Bartenschlager, 2005). However, Sarrazin et al. (2005) recently reported the existence of adaptive NS3 mutations in 5 of 26 HCV-infected patients. In that study, it is noteworthy that mutations (P1112R and P1115G) in positions 1112 and 1115 have been found, although Q1112R

and P1115L have not been detected, and that specific adaptive mutations have been associated with a slower initial decrease in HCV RNA concentrations during IFN- α -based antiviral therapy (Sarrazin et al., 2005). Furthermore, a search of the Hepatitis Virus Database (Nagoya City University, Japan) found Q1112R, P1115L, and E1202G in the HCV sequences (accession nos. AY460204, D84262, and AF011751, respectively) derived from HCV-infected patients. Therefore, some of the cell culture-adaptive mutations may not be artificial mutations

but may reflect some phenomena that HCV-infected patients undergo.

Although adaptive NS3 mutations found in this study were the same as those detected in subgenomic HCV replicons derived from different HCV strains (Blight et al., 2000; Kishine et al., 2002; Krieger et al., 2001; Lohmann et al., 2001, 2003), the impacts of these mutations in subgenomic HCV replicons seem to be small. The impacts of E1202G and K1609E on ECF assay and transient replication assay, respectively, were only about four- and two-fold that of the wild-type subgenomic HCV replicon (Lohmann et al., 2001, 2003), and information regarding Q1112R and P1115L mutations has yet to be reported. However, we observed that Q1112R, P1115L, E1202G, and K1609E mutations remarkably enhanced the efficiency of genome-length HCV RNA replication in both ECF and transient replication assay (Figs. 3 and 4). The discrepancy in the results might be due to the differences in subgenomic and genomic lengths of HCV RNA, in HCV strains, or in host cell lines used. Recently, we showed time-dependent genetic mutations of subgenomic HCV replicons and time-dependent expansions of their genetic diversities in long-term culture (at least 1 year) of two cell lines harboring subgenomic HCV replicons (1B-1 and O strains) (Kato et al., 2005). In that study, we observed that the expansion of the replicons' genetic diversity was associated with the enhancement of RNA replication. It is noteworthy that the P1115L mutation has been detected as a conserved mutation after 6 months in cell culture of the 1B-1-derived replicon, although this mutation's contribution to the replication is not clear (Kato et al., 2005). Genetic analysis of HCV RNAs in long-term cultures of O~OE cells will provide useful information regarding the genetic advantages of adaptive mutations found in the NS3 region. For such analysis, long-term culture (at least 1 year) of O~OE cells is in progress.

From the analyses using subgenomic HCV replicons to date, the center of NS5A has been thought to represent a hot spot for cell culture-adaptive mutations, because most mutations are found in this region. Interestingly, adaptive mutations often affect serine residues involved in hyperphosphorylation of NS5A. Although the HCV-O-derived sO replicon also possesses a unique S2200R adaptive mutation in the center of NS5A, the serine residue at position 2200 is not thought to be involved in the hyperphosphorylation of NS5A (Tanji et al., 1995). To examine whether or not S2200R mutation is required for efficient replication of genome-length HCV RNA, we tested the effect of the S2200R mutation on RNA replication by the introduction of OR/C-5B RNA with Q1112R and K1609E, in which the arginine residue in position 2200 was restored to serine residue, into Oc cells. The results revealed that the replication of genome-length HCV RNA was abolished with the restoration only in position 2200 (data not shown), suggesting that the S2200R mutation plays an important key role in HCV RNA replication in HuH-7-derived cells. However, the mechanism underlying the mutations found in NS3 causes the replication of genome-length HCV RNA is unknown, as is the mechanism underlying the great enhancement of replication by the combination of NS3 mutations. One possibility is that the NS3 mutations found in this study are able to drastically enhance the protease or helicase

activity of NS3. To evaluate this possibility, further experiments using the quantitative system that measures the NS3 protease or helicase activity will be needed.

The relation between the combination of adaptive NS3 mutations and the cloned cell lines is interesting. From information obtained in previous studies (Lohmann et al., 2003; Ikeda et al., 2005) and the present study, it clearly appeared that both viral and cellular factors contributed to HCV RNA replication in cell culture. For the replication of genome-length HCV RNA, we showed that adaptive NS3 mutations were viral factors and that the differences between sOc and Oc cells were cellular factors (Fig. 3). However, our results revealed that specific combinations of adaptive NS3 mutations (Q1112R and K1609E, or Q1112R and P1115L) were superior to the other combinations in all Oc, OAc, OBC, ODC, and OEC cell lines examined. In even sOc cells, the combination of Q1112R and P1115L, but not the combination of Q1112R and K1609E, was superior to the other combinations. These findings suggest that the effect of NS3 possessing a specific combination of mutations is superior to that of the host cell clonality. Recently, Yi et al. (2006) reported the production of infectious genotype 1a HCV in the cells transfected with genome-length HCV RNA (H77-S) possessing five adaptive mutations (two in NS3, one in NS4A, and two in NS5A), suggesting that robust replication of HCV RNA is also necessary for the production of infectious viruses (Yi et al., 2006). Therefore, identification of the best combination of adaptive mutations for efficient RNA replication may be useful in the development of a system to produce infectious genotype 1b HCV, and for understanding the HCV replication mechanism.

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Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication

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Abstract

HuH-7 is a highly differentiated hepatoma cell line and the only cell line that supports robust RNA replication of the hepatitis C virus (HCV). HuH-7 cells cause cell death in serum-free culture condition. However, the effect is reversed by supplementation with selenium. Serum-free cell cultures are advantageous for vaccine development and experimental reproducibility. However, HCV RNA replication in HuH-7 cells in serum-free medium had not yet been achieved. Therefore, we tried to develop a system for robust HCV RNA replication in a serum-free cell culture. Although HuH-7 cells grew in serum-free medium in the presence of selenium, HuH-7 cells under these conditions did not support HCV RNA replication in long-term culture. Among the supplements tested, serum-free medium with lipid-rich albumin (LRA) was found to yield robust HCV RNA replication. HCV proteins were detected for more than 9 months in serum-free medium supplemented with LRA. This is the first report to demonstrate a long-term, serum-free cell culture that successfully maintained robust HCV RNA replication. This cell culture system is expected to be a useful tool for vaccine development, as well as for further investigation of cellular factors that are essential for HCV RNA replication.
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Keywords: HCV; Serum-free cell culture; Selenium; Lipid-rich albumin; Vaccine

1. Introduction

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (Feld and Hoofnagle, 2005). HCV, a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1996). This polyprotein is processed by a combination of host and viral proteases into at least 10 proteins: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993).

The discovery of the HCV subgenomic replicon in 1999 was a turning point for HCV RNA replication in cultured cells (Lohmann et al., 1999). Furthermore, genome-length HCV RNA replication systems were developed using N, Con1, and H strains (Blight et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6) as an effective screening system (Ikeda et al., 2005). The development of infectious virus-producing cells has been a remarkable breakthrough in the fields of virology (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HuH-7 is a highly differentiated hepatoma cell line that is commonly used for replication and infection studies. However, these cells have been reported by a number of groups to be heterogeneous, and the replication efficiency of this cell line differed among subclonal HuH-7 cells. Parental HuH-7 cells showed low capacity for HCV RNA replication and low susceptibility for HCV infection, but Huh7.5, Huh-Lunet, and our recently developed the RSc cells efficiently support HCV RNA replication and infection (Blight et al., 2002; Pietschmann et al., 2006; Ikeda et al., in preparation). In addition to these sub-

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clonal HuH-7 cell features, fetal bovine serum (FBS) may be another factor that affects HCV RNA replication and infection, as FBS is a pooled material containing unknown factors from different origins. Therefore, different FBS lots may affect the reproducibility of experiments conducted by different research groups. Furthermore, pathogens contained in FBS may introduce additional problems during the development of an HCV vaccine.

To resolve these issues, in this study, we investigated whether or not HCV RNA could replicate in serum-free cell culture. As HuH-7 cells produce an autocrine growth factor, hepatoma-derived growth factor, additional supplementation with growth factor seemed to be unnecessary (Nakamura et al., 1989, 1994). HuH-7 cells cause cell death in serum-free culture condition. However, when HuH-7 cells were cultured in serum-free medium supplemented with selenium, they produce a number of plasma proteins and liver-specific enzymes essential for their survival (Nakabayashi et al., 1982, 1984). Therefore, the serum-free culture of HuH-7 cells can be maintained by the addition of selenium alone. However, HCV RNA replication was not yet maintainable under these conditions.

In the present study, we found that HCV RNA replicates robustly for more than 9 months in serum-free medium supplemented with selenium and lipid-rich albumin (LRA). These results indicate the requirement of the lipid for HCV RNA replication. This cell culture system is expected to be a useful tool for the development of an HCV vaccine, and will also enhance the reproducibility of experiments, including those that evaluate anti-HCV reagents.

2. Materials and methods

2.1. Reagents

Sodium selenite (Na_2SeO_3), insulin, linoleic acid, oleic acid, IFN- α , and cyclosporine A (CsA) were purchased from Sigma–Aldrich (St. Louis, MO). Fluvastatin (FLV) and low-density lipoprotein (LDL) were purchased from Calbiochem (San Diego, CA). Lipid-rich albumin (ALBUMAX ITM) was purchased from Invitrogen and is referred to as LRA in this study.

2.2. Cell cultures

The OR6 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin (referred to as 10% FBS medium in this study), and G418 (300 μg per ml; Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C. The cells were supplied with fresh medium twice a week at a 5:1 split ratio. The serum-free medium was DMEM containing 100 nM sodium selenite (Na_2SeO_3 , Sigma–Aldrich) with LRA. The cells were cultured on six-well plates in 10% FBS medium or the serum-free medium. The cells cultured in the serum-free medium were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis.

2.3. Cell count

To examine cell growth in selenium-containing medium with 10% FBS, 2 mg per ml of LRA, or no supplementation, OR6 cells were seeded at a density of 1×10^5 cells per well onto six-well plates in the absence of G418. Then, the number of the cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

2.4. Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis with a polyvinylidene difluoride membrane were performed as described previously (Kato et al., 2003). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, UK) and β -actin (AC-15; Sigma–Aldrich). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

2.5. Luciferase reporter assay

A luciferase reporter assay was performed as described previously (Ikeda et al., 2006). Briefly, 2×10^4 cells were plated onto 24-well plates and cultured in 10% FBS or the serum-free medium, at least in triplicate for each assay, and the cells were cultured for 24 h. Then, the cells were treated with human IFN- α , CsA, or FLV at several concentrations for 72 h. The cells were then harvested and subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). The cells were washed twice with phosphate-buffered saline and were then extracted with 100 μl of *Renilla* lysis reagent. The relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

3. Results

3.1. Efficiency of HCV RNA replication in HuH-7 cells with supplements in serum-free medium

At the early stage of the establishment of the HuH-7 cells, the serum-free cell culture was examined; the HuH-7 cells were found to replicate continuously for more than 9 months in a chemically defined medium containing selenium (Nakabayashi et al., 1982). Furthermore, the HuH-7 cells were maintained for a period of more than 3 years in improved serum-free medium containing additional supplements, i.e., oleic acid, linoleic acid, and insulin (Nakabayashi et al., 1984). We first investigated whether these serum-free conditions would support HCV RNA replication using the OR6 reporter system. The OR6 cells supported the replication of genome-length HCV-O RNA, into which the luciferase gene had been introduced (Ikeda et al., 2005). Using this OR6 system, we were able to monitor the level of HCV RNA

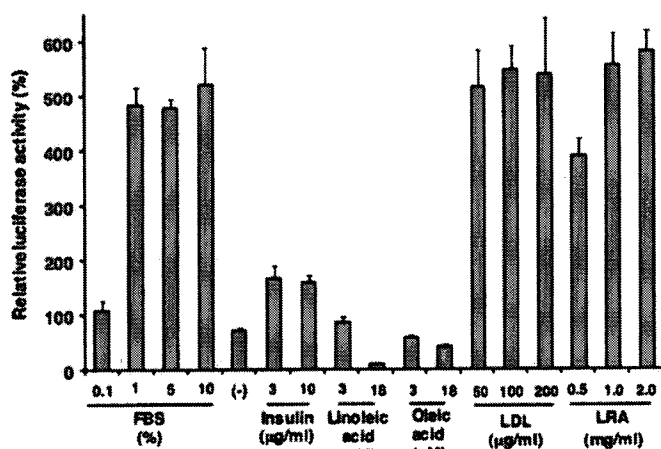


Fig. 1. HCV RNA replication in OR6 cells under different cell culture conditions. The OR6 cells were cultured in DMEM containing 100 nM sodium selenium with FBS (0.1, 1, 5, and 10%), insulin (3 and 10 µg per ml), linoleic acid (3 and 18 nM), oleic acid (3 and 18 nM), LDL (50, 100, and 200 µg per ml), or LRA (0.5, 1.0, and 2.0 mg per ml). The cells were harvested at 24 and 96 h and were subjected to luciferase assay as described in Section 2. Relative luciferase activities (%) were obtained from the value at 96 h, when the value at 24 h was assigned as 100%. The data indicate means \pm standard deviations (S.D.s) from three independent experiments. (–) indicates culture in DMEM containing 100 nM sodium selenium.

replication by measuring the activity of luciferase. Luciferase activity at 96 h was five times higher than that at 24 h in 10% FBS medium (Fig. 1). However, HCV RNA replication was reduced when the OR6 cells were cultured in serum-free medium containing only selenium (Fig. 1). Serum-free medium supplemented with insulin reduced HCV RNA replication to about one-third of that observed in cultures maintained in 10% FBS medium supplemented with selenium. When used in combination with linoleic acid or oleic acid, the serum-free medium with selenium remarkably reduced HCV RNA replication (Fig. 1). However, in the serum-free medium with selenium in combination with LDL (50, 100, or 200 µg per ml) or LRA (1 or 2 mg per ml), HCV RNA replication was supported at the same level as that in 10% FBS medium, although the replication of HCV RNA was slightly low level in serum-free medium with selenium and LRA supplement at 0.5 mg per ml. These results suggest that chemically conditioned serum-free medium supplemented with selenium is not sufficient to support HCV RNA replication, but the addition of either LDL or LRA restored HCV RNA replication to almost the same level as that observed in 10% FBS medium. Thus, some of the elements essential for HCV RNA replication may be contained in LDL and LRA.

3.2. Cell growth of HuH-7 cells in selenium-containing medium supplemented with FBS or LRA

As HCV RNA replication depends on cell growth (Guo et al., 2001; Pietschmann et al., 2001), we next determined the number of cells at 24, 48, 72, and 96 h of culture. The doubling time of the OR6 cells was estimated to be approximately 29, 43, and 64 h in selenium-containing medium with 10% FBS, or 0.5, 1.0, and 2 mg per ml of LRA, or no supplementation, respectively

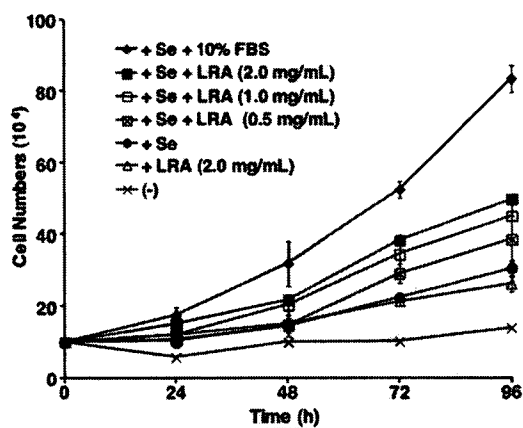


Fig. 2. Cell growth of HuH-7 cells in serum-free medium with LRA or in 10% FBS medium. The OR6 cells were plated at 1×10^5 cells per well onto six-well plates in triplicate. The cells were cultured in DMEM containing 100 nM sodium selenium with 10% FBS or 0.5, 1.0 and 2.0 mg per ml LRA or no supplement and were harvested at 24, 48, 72, and 96 h. The cells were cultured in DMEM or DMEM with LRA in the absence of selenium and were harvested at indicated time points.

(Fig. 2). Selenium or LRA containing medium enhanced the growth of OR6 cells and the combination of the selenium with LRA further enhanced the cell growth in a LRA dose-dependent manner (Fig. 2). Interestingly, the cell culture in serum-free medium supplemented with 2 mg per ml of LRA and selenium supported HCV RNA replication as efficiently as did that supplemented with 10% FBS and selenium (Fig. 1), although the cell growth of the culture in the medium with LRA and selenium was slower than that in the medium supplemented with 10% FBS and selenium. These results indicate that LRA may contain factors that enhance HCV RNA replication, and these LRA-derived factors appear to function in a manner that is not dependent on the cell growth factor.

3.3. Expression of HCV proteins in HuH-7 cells at 1 month of cell culture under various medium conditions

We continued to maintain the culture of OR6 cells for 1 month in different types of conditioned media. NS3 and Core HCV proteins were detected in the OR6 cell culture for 1 month in medium containing selenium with 10% or 5% FBS, but not with 1% or 0.5% FBS (Fig. 3A). The protein expression levels were higher in the cell culture with 10% FBS medium than in that with the 5% FBS medium. HCV proteins were not detected in the OR6 cells cultured in serum-free medium containing selenium alone (Fig. 3A). In contrast, HCV proteins were detected in LRA- and selenium-containing cell cultures. The levels of expression of HCV proteins were almost equal to those in the cell culture with selenium and 5% FBS (Fig. 3A). To further confirm the results, we performed luciferase assay for the OR6 cells cultured for 1 month after RNA transfection (Fig. 3B). These results indicated that HCV RNA replication was not maintained for more than 1 month in low concentrations (less than 1%) of FBS with selenium. However, the cell culture in serum-free medium with selenium and LRA at concentrations of 0.5, 1, or 2 mg per ml did efficiently support HCV RNA replication for more than