2. 実用新案登録：なし
3. その他：なし

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）
分担研究報告書

GFP 挿入ウイルスを用いた HCV 感染細胞検出系の確立

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研究要旨　C型肝炎ウイルス(HCV)の培養細胞への感染を簡便に検出できる系の構築のため、HCV のゲノムに挿入し、細胞感染後に GFP を発現するレポーターウイルスシステムの構築を試みた。これまでにレポーターウイルスの効率的な生成が可能な GFP 挿入部位を同定し、さらに感染力価の高いウイルスを作製するための適応変異を同定した。本年度はヒト肝細胞移植マウスを用い、in vivo でのこのレポーターウイルスの感染を検討した。さらに、HCV の細胞内増殖能に依存しない HCV 感染の検出のためのレポーター細胞構築も試みた。

A. 研究目的

JFH-1株を用いたC型肝炎ウイルス(HCV)の感染増殖系の開発により、このウイルスのライフサイクルが培養細胞で再現できるようになり、抗ウイルス剤の評価が培養細胞中で可能となった。しかし現行の方法では、操作が煩雑で時間もかかることから多くの薬剤のスクリーニングには不向きである。そこで、さらに簡便なHCVの感染検出系の構築のため、GFP遺伝子をHCVのゲノムに挿入し、細胞感染後にGFPを発現するレポーターウイルスシステムの構築を試みた。このシステムでは、HCV感染後に生細胞中でGFPが発現するため、HCVの感染増殖が可能な細胞が生きたまま分離でき、感染増殖に影響を与える宿主因子が同定可能である。

これまでキメラウイルスや適応変異の導入などウイルス株を評価し、また全長レプリコンやコア領域N末端にGFPを挿入したウイルスなどGFPの挿入部位、方法を変えることで効率の良いレポーターウイルスの同定をおこなってきた。

その結果、これらのコンストラクトの中でJ6/JFH-1キメラの全長レプリコンが最も高いウイルス生成効率と感染性を示すことがわかった。

さらにJFH-1株NS5a領域にGFPを挿入したウイルスの長期培養により適応変異を誘導し、高いウイルス生成効率と感染力価を持ったウイルス株を同定しようと試みたが、培養細胞中ではGFP遺伝子挿入ウイルスが非常に不安定であり、長期培養することでGFP遺伝子の欠失が頻繁に起こることが判明した。従って、JFH-1ウイルスで用いられるような長期培養により多くのウイルスを貯留し、濃縮する方法では高力価のGFP挿入ウイルスを作製することは難しいと考えられた。

本年度は、これまでの検討において最も効率的にウイルス生成が可能であったJ6/JFH-1キメラ全長レプリコンを用い、ヒト肝細胞移植マウスに接種することでin vivoでの感染性を検討した。さらにHCVの細胞内増殖能に依存しないHCV感染の検出のためのレポーター細胞の構築も試みた。

B. 研究方法

1．GFP 挿入全長レプリコンウイルスのヒト幹細胞移植マウスへの接種

全長レプリコンの薬剤耐性遺伝子部分を GFP に置換した GFP 全長レプリコンの RNA を Huh7.5.1 細胞に導入し、得られたウイルスを濃縮した後に HCV RNA 量と感染力価を測定した。ヒト肝細胞移植マウスには 1×10^6 copy / head のウイルスを接種した。

2．HCV 感染検出のためのレポーター細胞構築

HCV の細胞内増殖能に依存せず HCV 感染が検出可能なレポーター細胞の構築のため、CAG プロモータの下流に loxP 配列で挟まれたネオマイシン耐性遺伝子および polyA 配列と、さらにその下流に GFP 遺伝子配列を持つコンストラクトを作製した。このコンストラクトを培養細胞に導入し、Cre 酵素を作用させると loxP 配列によりネオマイシン耐性遺伝子が除去され、GFP

が発現するようになる。Cre 酵素を持ったウイルス作製のためコア領域N末端にCre遺伝子を挿入したJ6/JFH1 ウイルス（Genotype 2a）と CG1b ウイルス（Genotype 1b）を作製した。さらにCreを持たないウイルスの感染の検出のため、HCV の NS3 で切断される IPS-1 の膜結合領域と切断部位の配列を Cre 遺伝子と結合したコンストラクトも準備した。

（倫理面への配慮）

本研究で使用するヒト由来試料はすでに樹立された細胞株であり倫理面での問題はないと考えられる。各種組換え DNA を用いた組換えウイルス感染実験は、大臣確認申請し承認を受けた。

C. 研究結果

1．GFP 挿入全長レプリコンウイルスのヒト幹細胞移植マウスへの接種

GFP 全長レプリコンウイルス 1 x 10^6 copy をヒト幹細胞移植マウスへ接種した。投与二週後にマウス血中のウイルス量が 1.1 x 10^7 copy/mL まで上昇し、接種したウイルスの感染が確認された。さらにその二週後には血中ウイルス量は 6.3 x 10^7 copy/mL まで上昇し、感染したウイルスがマウスの移植肝細胞で増殖していると考えられた。しかし血清中に存在している HCV の RNA を抽出し、GFP 領域に設定したプライマーを用い RT-PCR 法で検出を行ったところ、GFP 領域のフラグメントの増幅は認められなかった。さらに、感染肝細胞のパラフィンブロック包埋標本を蛍光顕微鏡で観察したが、GFP の蛍光は確認できなかった。また GFP の蛍光観察よりもさらに高感度な GFP の免疫染色も試みたが、GFP 陽性所見は得られなかった。

2．HCV 感染検出のためのレポーター細胞構築

現在、培養細胞への感染が確認されているのは Genotype 2a の JFH-1 株と Genotype 1b の H77S 株のみである。他の genotype の他の株でも培養細胞において感染性ウイルス粒子の生成が可能と考えられるが、他の株では感染細胞で検出可能なレベルの HCV 蛋白の発現を認めないため感染を確認できないと考えられる。そこで、Cre-loxP システムを用い、感染細胞内での複製に依存せず HCV 感染の検出が可能なシステムの構築を試みた。まず、loxP-GFP コンストラクトを Huh7.5.1 細胞に導入しレポーター細胞を作製した。その後コア領域N末端に Cre 遺伝子を挿入した J6/JFH1 キメラウイルスと CG1b ウイルスを作製し感染させたところ、J6/JFH1 キメラウイルスでは GFP 陽性細胞を認め感染が確認された。CG1b ウイルスの感染では、ごく少数の GFP 陽性細胞を認め感染が起こっていると考えられたが、その感染効率は J6/JFH1 キメラウイルスに比べかなり低いと考えられた。さらに Cre-IPS-1 コンストラクトを loxP-GFP コンストラクトと共に Huh7.5.1 細胞に導入し、Cre を持たない HCV 感染の検出を試みたが、これはこの二つのプラスミドの導入のみで GFP の発現を認め、バックグラウンドが高く HCV 感染の検出は不可能であった。

D. 考察

今年度の検討により、GFP を挿入したウイルスは培養細胞中のみならず生体中でも不安定であり、感染成立時には高頻度に GFP の欠失が起こることが明らかとなった。従って、生体での HCV の感染をリアルタイムでモニターするためには他の方法でのレポーター遺伝子挿入が必要であると考えられた。また Cre-loxP システムを用い、HCV の複製に依存しない感染検出システムの構築を試みた。このシステムにおいて、Cre 遺伝子をゲノム内に挿入した HCV の感染検出は可能であったが、Cre-IPS-1 コンストラクトを用いた通常の HCV 株の感染検出は不可能であった。

E. 結論

これまでの研究により、GFP 遺伝子を HCV のゲノムに挿入したレポーターウイルスは、培養細胞中生体中ともに非常に不安定であり、このシステムにより安定して HCV 感染を検出することは難しいと考えられた。今後は Cre-loxP システムを用いた感染検出システムなど、細胞側にレポーター遺伝子を導入することで、培養細胞中での複製能が低い HCV 株でも感染が検出できるようなシステムの構築を目指したい。

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G. 知的所有権の出願・登録状況

なし

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）
分担研究報告書

HBVpseudotype 作製の試みとレセプターの分離・同定に関する研究

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研究要旨　HBV 膜粒子にレトロウイルスゲノムをもつ HBV pseudotype の作製に成功し、感染を指標にしたヒト肝細胞 cDNA ライブラリーの発現による HBV レセプターの同定を遂行中である。実験の経過上 cDNA ライブラリーの導入操作で本 pseudotype の感染性が著しく向上することが解った。

A．研究目的

HBV レセプターは未だ不明で有効な感染系が確立されていない。これまで試みられなかった HBV pseudotype（HBVp）による生物学的アッセイ法で本レセプターを分離・同定し感染系を構築し、病態解明、治療法の開発に役立てる。

B．研究方法

Gfp-hygR を含むレトロウイルスコア粒子を HBV 膜粒子で覆った HBVp の作製を試みた。本 HBVp を用いてヒト肝細胞 cDNA ライブラリーをスクリーニングした。

（倫理面への配慮）

現在のところ特に関係しないものと思われる。

C．研究結果

培養上清中に出現すると想定される HBVp を抗 HBs 抗体による免疫沈降し RNA 抽出、GFP 遺伝子を標的とした RT-PCR から HBVp 産生されたと考えられた。また超遠心法による物理化学的性質も HBVp の産生を示唆した。本 HBVp を用いてヒト肝細胞 cDNA ライブラリーのスクリーニング中にある処理で感染性が向上することが観察された。

D．考察

HBVp の作製は可能であり、本ウイルスを用いて HBV レセプターの分離･同定に生物学的アッセイが可能になった。ある処理で感染性が向上することは HBV レセプターの性情を示唆すると思われた。

E．結論

HBVp を用いて感染性を指標に HBV レセプターの分離・同定を促進させ得る。

F．健康危険情報

特に無し

G．研究発表

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Ⅲ．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

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Ⅳ．研究成果の刊行物・別冊

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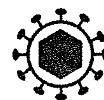

RESEARCH **Open Access**

Impaired antiviral activity of interferon alpha against hepatitis C virus 2a in Huh-7 cells with a defective Jak-Stat pathway

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Abstract

Background: The sustained virological response to interferon-alpha (IFN-α) in individuals infected with hepatitis C virus (HCV) genotype 1 is only 50%, but is about 80% in patients infected with genotype 2-6 viruses. The molecular mechanisms explaining the differences in IFN-α responsiveness between HCV 1 and other genotypes have not been elucidated.

Results: Virus and host cellular factors contributing to IFN responsiveness were analyzed using a green fluorescence protein (GFP) based replication system of HCV 2a and Huh-7 cell clones that either possesses or lack a functional Jak-Stat pathway. The GFP gene was inserted into the C-terminal non-structural protein 5A of HCV 2a full-length and sub-genomic clones. Both HCV clones replicated to a high level in Huh-7 cells and could be visualized by either fluorescence microscopy or flow cytometric analysis. Huh-7 cells transfected with the GFP tagged HCV 2a genome produced infectious virus particles and the replication of fluorescence virus particles was demonstrated in naïve Huh-7.5 cells after infection. IFN-α effectively inhibited the replication of full-length as well as sub-genomic HCV 2a clones in Huh-7 cells with a functional Jak-Stat pathway. However, the antiviral effect of IFN-α against HCV 2a virus was not observed in Huh-7 cell clones with a defect in Jak-Stat signaling. HCV infection or replication did not alter IFN-α induced Stat phosphorylation or ISRE promoter-luciferase activity in both the sensitive and resistant Huh-7 cell clones.

Conclusions: The cellular Jak-Stat pathway is critical for a successful IFN-α antiviral response against HCV 2a. HCV infection or replication did not alter signaling by the Jak-Stat pathway. GFP labeled JFH1 2a replicon based stable cell lines with IFN sensitive and IFN resistant phenotypes can be used to develop new strategies to overcome IFN-resistance against hepatitis C.

Background

Hepatitis C virus (HCV) is the most common blood-borne infection affecting the liver. Chronic HCV infection often leads to the development of liver cirrhosis and cancer [1]. HCV infection often does not present early symptoms and thus can go undetected while significant liver damage sets in over the course of 10-20 years. There are 180 million people currently infected with HCV worldwide [2,3]. The incidence of new HCV infection is increasing each year, creating a significant public health problem. The standard treatment for chronic HCV infection is interferon with ribavirin, but many patients infected with certain viral strains develop resistance to treatment [4,5]. The mechanisms of interferon action and resistance in chronic HCV infection are currently not well understood. Development of efficient HCV cell culture systems for all major HCV strains is required to understand the role of host-virus interaction in the IFN-antiviral response.

HCV, a member of the *Flaviviridae*, is an enveloped virus containing a single-stranded positive sense RNA genome approximately 9600 nucleotides in length [6,7]. The nucleotide sequences of HCV genomes isolated in different parts of world vary considerably and are quite

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heterogeneous. There are six major genotypes and numerous sub-types of HCV that have been described from all over the world [8-10]. Genotype 1 (subtype 1a and 1b) is the most common in the United States, followed by genotype 2 and 3 [10,3]. Genotype 3 is most common in the Indian subcontinent [8]. Genotype 4 is the most common genotype in Africa and the Middle East [11]. Genotypes 5 and 6 are most common and predominant in South Africa and Southeast Asia [12].

In spite of high sequence variability among different HCV genotypes, the genomic organization of all HCV strains starts with a highly conserved untranslated sequences (called 5' UTR), followed by a large open reading frame, and terminating with 3'-untranslated sequences. The large polyprotein is processed by cellular and viral proteases into structural proteins (core, E1, and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The nonstructural proteins NS3 to NS5B are essential for RNA replication and have distinct functions in the HCV life cycle. The 5' and 3' UTR sequences of HCV contain numerous cis-acting signals that are absolutely required for RNA translation and replication as shown by *in vitro* experiments using the cell culture system. Despite the high nucleotide sequence homology of the 5' and 3' UTRs among all genotypes of HCV, the efficiency of RNA genome replication of different HCV strains in the cell culture varies significantly [13]. Some strains of HCV with adaptive mutations replicate efficiently in the cell culture, whereas others do not require any adaptive mutations. The best example is the JFH1 clone of HCV 2a strain that replicates to a higher level in cell culture and generates more infectious virus particles compared to all other full-length infectious clones [14-16]. These observations suggest that HCV genetics and host cellular environments are the two major determinants of the efficacy of HCV replication and its response to antiviral therapy.

Interferon alpha (IFN-α) along with ribavirin has been widely used as a standard treatment option for patients with chronic HCV infection all over the world [3]. However, the sustained virological response to IFN-α in individuals infected with HCV genotype 1 is only 50% as compared with 80% in patients infected with genotype 2 to 6 viruses [17]. Molecular mechanisms explaining why certain genotype viruses respond better to IFN-α than others have not been elucidated. We have shown that IFN-α effectively inhibits the IRES mediated translation of all HCV strains in the cell culture, indicating that differential resistance is not due to IRES sequence heterogeneity [18-20]. To gain an insight into the mechanisms of IFN resistance in the HCV cell culture model, we have developed Huh-7 cell lines in which the HCV 1b Con1 strain is resistant to IFN, after prolonged IFN-α treatment of a low inducer Huh-7 replicon cell line [21,22]. We demonstrated that phosphorylation of Stat1 and Stat2 proteins in the IFN-resistant replicon cell lines is blocked due to reduced phosphorylation of Jak1 and Tyk2 proteins [21,22]. These studies provided direct evidence that a defective Jak-Stat pathway makes HCV replication resistant to interferon treatment in a replicon cell line, and indicated that cellular factors are important for determining the response of HCV to IFN-α treatment. To extend our observations, we have examined the replication and anti-viral response of an IFN-sensitive HCV 2a virus clone in a Huh-7 clone with a defective Jak-Stat pathway. For this purpose, we first developed a chimeric clone between GFP and a highly efficient HCV 2a virus. Insertion of the GFP coding sequences into HCV 2a allowed us to study a high level replication of the virus in Huh-7 cells directly by fluorescence microscopy or flow cytometric analysis. We also determined that replication of HCV 2a can only be inhibited by IFN-α in a dose dependent manner in Huh-7 cells with a functional Jak-Stat pathway. Replication of the full-length and sub-genomic clone of a HCV 2a strain was not inhibited by IFN-α in Huh-7 cell clones with a defective Jak-Stat pathway. Infection with full-length virus or stable replication of sub-genomic HCV RNA did not alter the state of Jak-Stat signaling or interferon sensitivity in these two different Huh-7 clones. We have now developed multiple GFP tagged HCV sub-genomic replicon cell clones in which replication of HCV are totally resistant to IFN-α. We believe that these cell clones can be used to understand the cellular basis of IFN-resistance in a cell culture as well as develop alternative strategies to overcome mechanisms of resistance.

Materials and methods

Cell culture

Huh-7.5 cells, obtained from the laboratory of Dr. Charles M. Rice (Center for the Study of Hepatitis C, The Rockefeller University, New York), were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum, under 5% CO_2 conditions. Interferon resistant (R-24/1) replicon cells were generated in our laboratory by prolonged treatment of low inducer replicon cell lines (Con-15, Con-17, and Con-24) with IFN-α as described previously [21,22]. A cured Huh-7 cell line with defective Jak-Stat pathway (R-Huh-7) was prepared from IFN-α resistant replicon cell line (R-24/1) after repeated treatment with cyclosporine-A (1 μg/ml) as described previously [22]. Interferon sensitive cured Huh-7 cells (S-Huh-7) were derived from the 5-15 replicon cell line after treatment with IFN-α.

Interferon sensitive and interferon resistant phenotypes in the cured S-Huh-7 and R-Huh7 cells were examined by measuring their ability to activate the ISRE-luciferase promoter in the presence of exogenous IFN-α. The expression of functional Jak-Stat signaling proteins in these cells after IFN-α treatment was examined by western blot analysis of phosphorylated Stat1 and Stat2. All the resistant cell lines have defects in the phosphorylation of Stat1 and Stat2 protein, whereas the S-Huh-7 clone showed a high level of phosphorylation of Stat1 and Stat2 proteins within 30 minutes of IFN-α treatment [22]. All Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 5% fetal bovine serum.

Construction of full-length and sub-genomic JFH1 2a chimeric clones

The JFH1 full-length cDNA clone of HCV 2a strain which was isolated from a chronically infected Japanese fulminant hepatitis patient was obtained from Wakita and his coworkers [14]. Chimeric clones between JFH1 and enhanced green fluorescent protein (EGFP) were constructed in our laboratory by the standard overlapping PCR amplification and cloning methods. The coding sequence of GFP was amplified from pEGFP-N1 plasmid and inserted in-frame of the NS5A coding sequence of the JFH1 cDNA clone between 2394 and 2395 amino acids position (between 417 and 418 amino acids of the NS5A protein). The PCR amplification of recombinant DNA and cloning was performed in four different steps. In the first step, the 228 bp (F1) recombinant DNA fragment containing 70 amino acids of NS5A (nts.7339-7546) fusion with the first 6 amino acids of EGFP-N1 was amplified using a sense primer (S/7336-7360/HCV-5'-CCTCCCCCAAGGAGACGCCGGACA-3') and antisense primer (AS/7529/HCV- 5'CTCGCCCTTGCTCACCATG GGGGGCATAGAGGAGGC-3'). In the second step, the 719 bp (F2) recombinant DNA fragment containing the total EGFP-N1 open reading frame (ORF) fused with the N- and C-termini of HCV NS5A was amplified using sense and anti-sense overlapping primers (S/7529/GFP- 5'-GCCTCCTCTATGCCCCCCATGGTGAGC AAGGGCGAG-3' and (AS/7547-7564/GFP 5'-TCCAGGCTCCCCCTCGAGCTTGTACA GCTCGTCCAT-3'). In the third step, the recombinant 531 bp DNA fragment (F3) containing last 6 amino acids of EGFP-N1 and 177 amino acids of NS5A (nt. 7547-8077) was amplified by using sense primers (S/7547/HCV- 5'-ATGGACGAGCTGTACAAG CTCGAGGGGGAGCCTGGA-3') and anti-sense primer (AS/8059-8077/HCV-5'-GTCTTCCAGGAGGTCCTTCCACAC-3'). In fourth step, the F1, F2 and F3 PCR fragments were assembled into the 1478 bp DNA fragment through overlapping PCR. In the final step, the recombinant DNA was digested with restriction enzyme *RsrII* and *HpaI*, gel purified and then ligated with pJFH1 clone using the unique *RsrII* and *HpaI* restriction sites present in the NS5A gene. The resulting plasmid was named pJFH1-GFP. The recombinant plasmid was amplified and the construction was confirmed by sequence analysis. A full-length pJFH1-GFP plasmid clone was prepared with a GDD to GND mutation in the NS5B gene to use as a control (pJFH1-GND-GFP) in the replication assay. A full-length pJFH1-GFP plasmid was also prepared with a deletion in the E1-E2 gene (pJFH1-ΔE1E2-GFP) to use as a control in the infectivity assay. To generate a sub genomic GFP replicon clone of HCV 2a, the recombinant plasmid containing the NS5A and EGFP-fusion was excised from full-length pJFH1-GFP plasmid using the *NsiI* and *HpaI* enzyme and re-cloned into the pSGR replicon [23]. This chimeric clone was named pSGR-GFP. As a control, we created a mutant construct pSGR-GND-GFP clone with a point mutation that changes a GDD motif to GND, abolishing the RNA polymerase activity of the NS5B protein. All the recombinant plasmids constructs were confirmed by DNA sequence analysis.

In-vitro RNA synthesis

Full-length (pJFH1-GFP) and sub-genomic replicon (pSGR-GFP) plasmids were linearized with *XbaI* restriction enzyme and purified by phenol chloroform extraction and precipitated by ethanol. The HCV full length and sub-genomic RNAs were transcribed from *XbaI* linearized plasmid DNA templates using the MEGA-script T7 kit (Ambion, Austin, TX, USA). *In vitro* transcribed RNA was treated with DNase I to eliminate any residual plasmid DNA, extracted with phenol and chloroform, and then precipitated with absolute ethanol. The RNA pellet was re-suspended in nuclease free water and 10 μg aliquots of this RNA were stored at -80°C. The integrity of *in vitro* transcribed RNA was verified by agarose gel electrophoresis.

RNA transfection

Huh-7.5, S-Huh-7 and R-Huh-7 cells were electroporated with *in vitro* transcribed HCV RNA using a standard protocol described previously [17]. Briefly, cells were harvested using trypsin-EDTA, pelleted by centrifugation and washed in 10 ml of phosphate buffered saline (PBS). The cell pellet was suspended in PBS (10^7 cells per ml). Ten micrograms of *in vitro* transcribed RNA was mixed with 400 μl of Huh-7 cell suspension in a cuvette (0.4 cm, Bio-Rad) and subjected to an electric pulse at 960 μF and 270 volts using a gene pulser

apparatus (Bio-Rad). Following electroporation, cells were diluted in 10 ml of complete medium and plated in a 100-mm diameter cell culture dish.

Replication assay

To study replication of full-length HCV-GFP chimeric RNA, the electroporated Huh-7 cells were cultured in a 100-mm plate with regular growth medium. The expression of GFP in the transfected Huh-7 cells was recorded at 0, 24, 48, 72 and 96 hours post-transfection. To study the replication of HCV sub-genomic RNA, stable Huh-7 cells replicating sub-genomic RNA were prepared. Cured Huh-7 cells derived from interferon sensitive (S-Huh-7) and resistant replicon cell lines (R-Huh-7) in our laboratory were used. Huh-7 cells electroporated with sub-genomic RNA were cultured in a growth medium supplemented with 500 μg/ml G-418 drug. These cells were maintained with a regular medium change at every three days for 3-4 weeks until distinct G-418 resistant cell colonies were developed. To make a stable cell line replicating HCV 2a sub-genomic RNA, multiple G-418 resistant cell clones were isolated and cultured in medium supplemented with 1 mg/ml G-418. In these stable cell lines absence of HCV plasmid DNA integration was confirmed by direct PCR followed by Southern blot analysis for the neo gene (sense 5'-ATCGAATTCATCGTGGCTGGCCA-3'; anti-sense 5'-CTAGAATTCGGCGCGAGCCCCTG-3'; probe 5'-GCTTGGTGGTCGAATGGGCAG GTAGCCGGA-3'.

Infectivity assay

An infectivity assay for HCV was performed using a published protocol [15]. Huh-7.5 cells were transfected with 20 μg of *in vitro* transcribed full-length JFH1-GFP RNA by electroporation method. After 72 h, cells were collected by scraping and then lysed by four rounds of freeze-thaw cycles. The cell lysates were clarified by centrifugation at 3400 rpm for five minutes. The clear supernatant was collected and the titer of HCV in the supernatant was determined by real-time RT-PCR using a primer set targeted to the 5'UTR. A tissue culture infective dose (TCID50) was determined using 10-fold serial dilution of the virus containing supernatant on 2-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, New York). Briefly, Huh-7.5, S-Huh-7 and R-Huh-7 cells were seeded on a 2-well glass chamber slide at a density of 1×10^4 cells per well. The next day, the culture medium was removed and 1-ml of serial dilutions of culture supernatant containing infectious virus was added to the wells. The cells were incubated overnight at 37°C. On the following day the culture medium was removed, and the cells were washed once using PBS and then incubated in fresh complete medium. After 96 hours post-transfection, the cells were removed, washed in PBS, fixed in 4%-parformaldehyde for 30 minutes and then counter stained with Hoechst dye (H33342, Calbiochem, Darmstadt, Germany) for 15 minutes at 37°C. Cells were examined at 484 nm using a fluorescence microscope (Olympus) for expression of green fluorescence. Cells were then examined at 340 nm for blue nuclear staining. For each area, two sets of pictures were taken. The final image was generated by superimposing blue nuclear fluorescence of Hoechst dye with green fluorescence of GFP using Abode Photoshop software (V 7.0). The numbers of green positive cells in ten different fields were counted and the percentage of green fluorescence positive cells in the culture was determined. The dilution of virus-containing supernatant that showed 50% GFP positive cells 96 hours after infection in the culture (called the TCID50) was determined. The MOI of the infectious culture supernatants was determined by dividing the TCID50 with the cell number used in the infectivity assay.

Interferon treatment

To study the effect of interferon on the full-length HCV 2a clone, transfected or infected Huh-7 cells were treated with increasing concentrations of IFN-α(Intron A, Schering-Plough, NJ, USA). The antiviral effect of IFN-α against HCV using different Huh-7 clones was confirmed by observing GFP expression under a fluorescence microscope or by flow cytometric analysis, and HCV RNA levels was measured by RPA.

Ribonuclease protection assay (RPA)

Total RNA was prepared from the cell pellet by the GITC method and subjected to RPA for the detection of genomic positive-strand HCV RNA. For RPA experiments, 25 μg of the total RNA was mixed with a negative-strand RNA probe targeted to the 5'UTR of HCV (1×10^6 cpm) in a 10 μl hybridization solution, denatured for 3 minutes at 95°C and then hybridized overnight at 50°C. RNase digestion was performed in 200 μl of RNase digestion buffer (10 mM Tris, pH 7.5, 5 mM EDTA and 0.3 M NaCl) containing RNaseA/T1 cocktail at 1:100 dilutions (Ambion Inc., Austin, TX) for an hour at 37°C. Then the sample was treated with 2.5 μl of 25% SDS and 10 μl of proteinase K (20 mg/ml) for 15 minutes. Samples were extracted with phenol and chloroform and then precipitated after addition of 2.5 volumes of absolute ethanol. The pellet was obtained by centrifugation for 30 minutes at 12,000 rpm. The RNA pellet was washed with 70% ethanol, suspended in 8 μl of gel loading buffer, boiled for one minute and separated on a 6% polyacrylamide TBE-Urea gel (Invitrogen, Carlsbad, CA). The gel was dried and exposed to X-ray film (Kodak Biomax-XAR, Rochester, NY). We prepared a plasmid construct called pCR-II (2a), which contained

the 79-297 nucleotides of the 5'UTR sequence of the JFH1 clone (pCR-II NT-218) (Invitrogen). This plasmid was linearized with *HindIII* restriction enzyme and a positive strand RNA probe was prepared using T7 RNA polymerase in the presence of 32p labeled CTP. Likewise, this plasmid was linearized with *XbaI* restriction enzyme and Sp6 RNA polymerase was used to prepare a negative strand RNA probe for the detection of a positive strand HCV RNA. The same amounts of the RNA extracts were subjected to RPA for GAPDH mRNA. We used a linearized pTRI-GAPDH-human anti-sense control template to prepare a probe to detect GAPDH mRNA using Sp6 RNA polymerase (Ambion Inc., Austin, TX). The appearance of 218 (HCV 2a) and 317 nts protected fragments indicated the presence of the HCV positive-strand and the GAPDH mRNA, respectively.

Flow analysis

The percentage of Huh-7 cells expressing GFP after transfection with full-length GFP-RNA transfected cells was analyzed by flow cytometric analysis. Cells were transfected with 10 μg of *in vitro* transcribed RNA in 6-well plates, and harvested by treatment with trypsin-EDTA at 24, 48, 72 and 96 hours post-transfection. The cells were pelleted by centrifugation at 500 rpm in a refrigerated centrifuge. The cell pellet was resuspended in 4% paraformaldehyde for 30 minutes, and washed twice in 10 ml of PBS using centrifugation. After this step, the cell pellet was resuspended in 1 ml of PBS and analyzed by flow cytometer (BD-Biosciences). The percentage of GFP expressing cells in the replicon culture was determined by flow analysis using the identical procedure. Stable replicon cells after interferon treatment were harvested by trypsin-EDTA treatment and analyzed by flow cytometry.

Real-time RT-PCR

Real time RT-PCR was performed to quantify HCV RNA levels in the infected cell culture using a published protocol [24]. The 243 bp HCV DNA was amplified from the RNA extract by reverse transcription polymerase chain reaction using the outer sense (OS) primer 5'-GCAGAAAGCGCCTAGCCATGGCGT-3' (67-90) and outer anti-sense (OAS) primer 5'-CTCGCAAGCGCCCTATCAGGCAGT-3' (287-310). First the complementary DNA synthesis was performed from positive strand HCV-RNA using an outer anti-sense primer (OAS) targeted to the highly conserved 5'UTR region of HCV in 20 μl volume. Briefly, 2 μgm of total cellular RNA were mixed with 1 μl OAS primer (200 ng/μl), denaturized at 65°C for 10 minutes and annealed at room temperature. Avian myeloblastosis virus (AMV) reverse transcriptase (10 U) (Promega, Madison, WI) was added and incubated at 42°C for 60 minutes in the presence of 50 mmol/L Tris, pH 8.3, 50 mmol/L ethylenediaminetetraacetic acid (EDTA), 500 nmol/L dNTP, 250 nmol/L spermidine, and 40 U RNasin (Promega). The cDNA was stored at -20°C until use. SYBR Green real time PCR amplification was performed in 20 μl of volume containing 10 μl of SYBR Green ER qPCR SuperMix, 1 μl (250 ng/ul) of sense and antisense primer with 4 μl of cDNA and 4 μl of distilled water. All samples were run in triplicate. The amplification was carried out using the standard program recommended by Bio-Rad Laboratory that includes: 50°C for 2 minutes, 95°C for 8 minutes, then additional 50 cycles wherein each cycle consists of a denaturation step at 95°C for 10 seconds, and annealing and extension step at 60°C for 30 seconds. At the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (0.1°C/s) up to 95°C. Amplification, data acquisition, and analysis were performed on CFX96 Real Time instrument (Bio Rad) using CFX manager software (Bio Rad).

Results

High-level replication of pJFH1-GFP chimera clone in Huh-7.5 cells

Replication of the full-length HCV 2a genome is possible due to the availability of the JFH1 cDNA clone. However, the highly sensitive RT-PCR and immunodetection methods are most often needed to detect replication of HCV in the transfected cells. To overcome the technical difficulties associated with the detection of the full-length viral RNA replication, we constructed chimeric clones of the JFH1 clone and GFP so that replication of whole viral genome in the transfected cells could be examined by fluorescence microscopy. Previous reports suggest that heterologous sequences can be inserted into the HCV genome without altering its ability to replicate [25-28]. The coding sequence of GFP was inserted into C-terminus of the NS5A protein of HCV at the 2394 amino acid position. Chimeric clones of GFP and full-length, and a sub-genomic replicon of HCV 2a were prepared (Fig. 1). The N-terminal and C-terminal fusion of HCV NS5A with EGFP protein was confirmed by sequence analysis. To study the replication of full-length virus, *in vitro* transcribed RNA derived from wild type and GND-mutant clone were electroporated into Huh-7.5 cells. The expression of GFP was recorded in a kinetic study. The replication of full-length JFH1-GFP chimera in the transfected Huh-7.5 cells was seen as early as 24 hour post-transfection and the number of GFP positive cells in the culture increased gradually at 48, 72 and 96 hours (Fig 2A). In contrast, replication of the JFH1-GND-GFP mutant RNA in Huh-7.5 cells was not observed at 48, 72 or 96 hours post-transfection, while only a very faint GFP

signal was seen in Huh-7.5 cells at 24 hours post-transfection (Fig. 2B). The efficiency of replication of chimeric clones in Huh-7.5 cells after RNA transfection was observed in approximately 8% of cells as examined by flow cytometry (Fig. 2C and 2D). Replication of full-length JFH1-GFP chimera clone was confirmed by examining HCV positive and negative strand RNA levels by RPA assay. The levels of HCV RNA in the full-length transfected cells and GND mutant transfected cells were clearly different (Fig 3A). As expected, the levels of mutant RNA dropped below the input level and remained undetected at 48, 72 and 96 hours post-transfection. The level of HCV positive strand RNA seen in the RPA assay appeared to be higher at an earlier time point in the full-length transfected cells at a later time point. This may be due to an input RNA carryover during the transfection step. There was a good correlation between the amount of HCV RNA and expression of GFP at later time points.

HCV is a positive strand RNA virus that replicates via the synthesis of negative strand RNA. To demonstrate that the replication of transfected RNA resulted in the production of negative strand RNA in the transfected cells, RPA for negative strand HCV RNA was performed in the transfected cells at 0, 24, 48, 72 and 96 hours post-transfection. Negative strand HCV RNA was not detectable at the zero-time point but appeared at 24

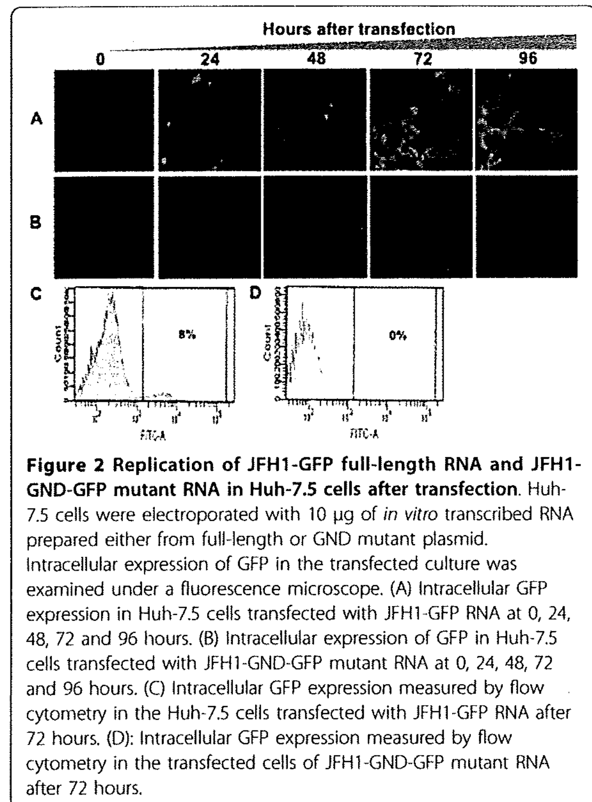

Figure 2 Replication of JFH1-GFP full-length RNA and JFH1-GND-GFP mutant RNA in Huh-7.5 cells after transfection. Huh-7.5 cells were electroporated with 10 μg of *in vitro* transcribed RNA prepared either from full-length or GND mutant plasmid. Intracellular expression of GFP in the transfected culture was examined under a fluorescence microscope. (A) Intracellular GFP expression in Huh-7.5 cells transfected with JFH1-GFP RNA at 0, 24, 48, 72 and 96 hours. (B) Intracellular expression of GFP in Huh-7.5 cells transfected with JFH1-GND-GFP mutant RNA at 0, 24, 48, 72 and 96 hours. (C) Intracellular GFP expression measured by flow cytometry in the Huh-7.5 cells transfected with JFH1-GFP RNA after 72 hours. (D): Intracellular GFP expression measured by flow cytometry in the transfected cells of JFH1-GND-GFP mutant RNA after 72 hours.

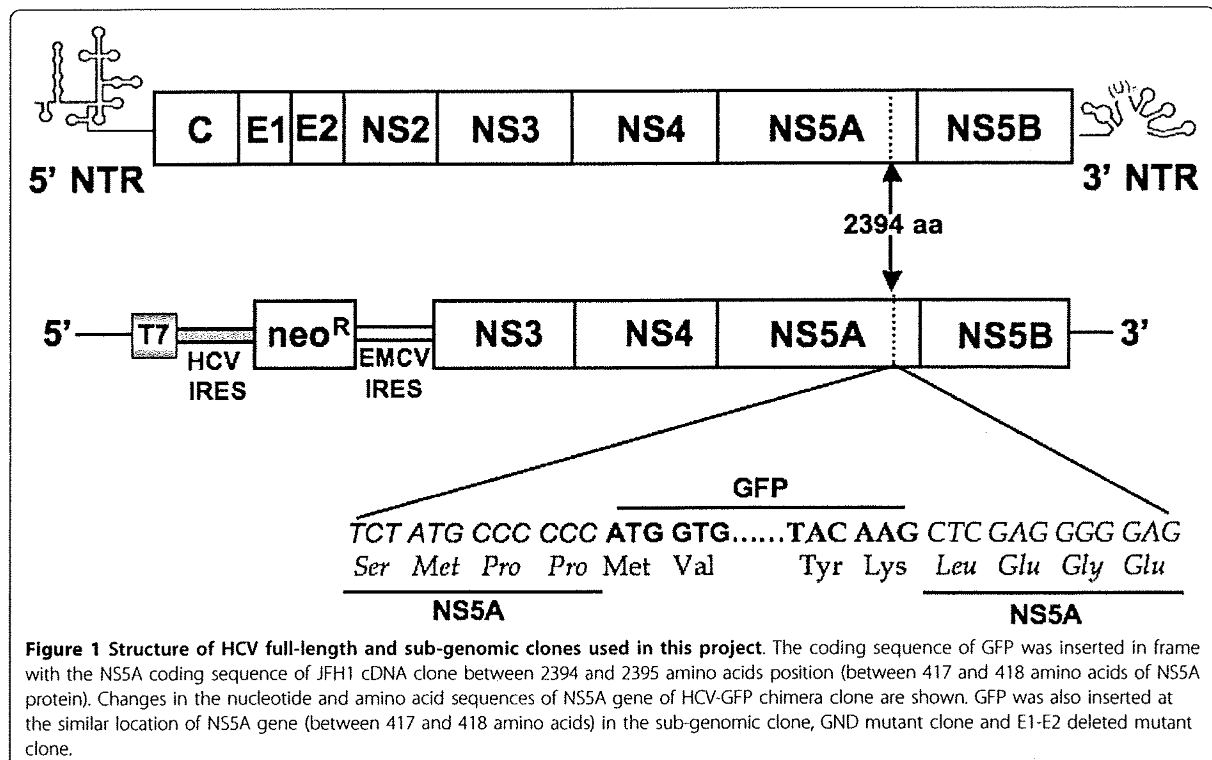

Figure 1 Structure of HCV full-length and sub-genomic clones used in this project. The coding sequence of GFP was inserted in frame with the NS5A coding sequence of JFH1 cDNA clone between 2394 and 2395 amino acids position (between 417 and 418 amino acids of NS5A protein). Changes in the nucleotide and amino acid sequences of NS5A gene of HCV-GFP chimera clone are shown. GFP was also inserted at the similar location of NS5A gene (between 417 and 418 amino acids) in the sub-genomic clone, GND mutant clone and E1-E2 deleted mutant clone.

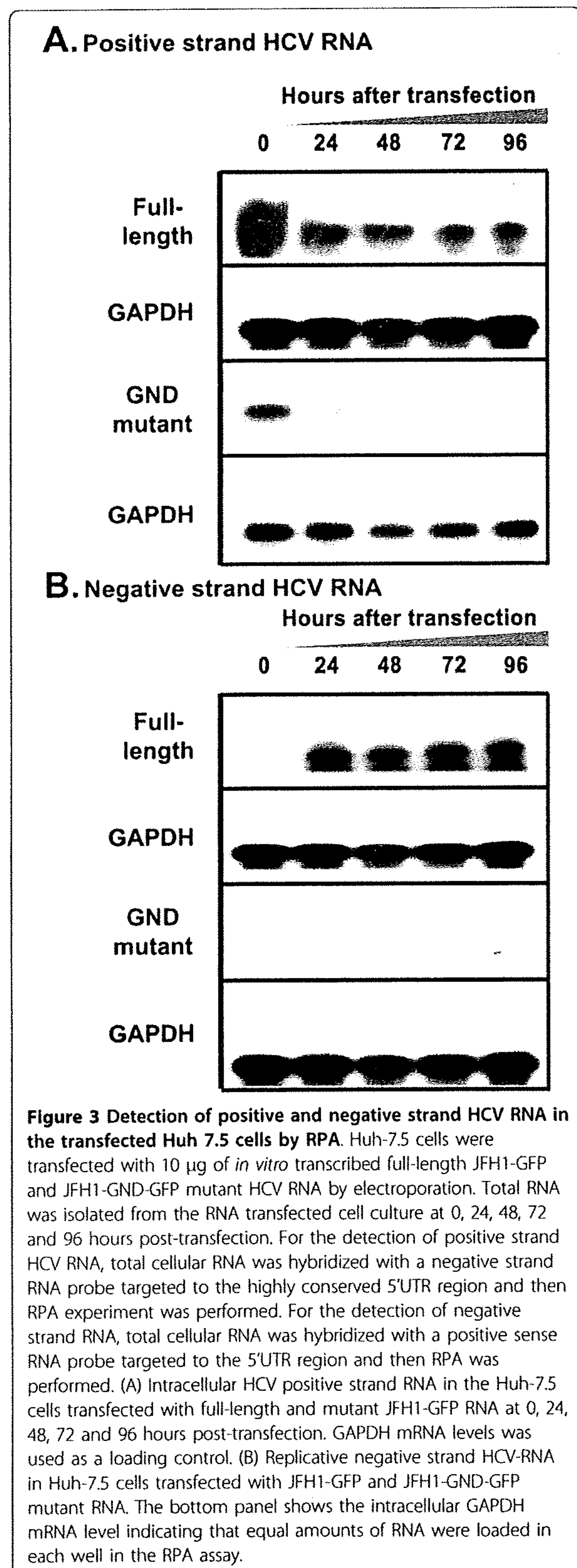

Figure 3 Detection of positive and negative strand HCV RNA in the transfected Huh 7.5 cells by RPA. Huh-7.5 cells were transfected with 10 μg of *in vitro* transcribed full-length JFH1-GFP and JFH1-GND-GFP mutant HCV RNA by electroporation. Total RNA was isolated from the RNA transfected cell culture at 0, 24, 48, 72 and 96 hours post-transfection. For the detection of positive strand HCV RNA, total cellular RNA was hybridized with a negative strand RNA probe targeted to the highly conserved 5'UTR region and then RPA experiment was performed. For the detection of negative strand RNA, total cellular RNA was hybridized with a positive sense RNA probe targeted to the 5'UTR region and then RPA was performed. (A) Intracellular HCV positive strand RNA in the Huh-7.5 cells transfected with full-length and mutant JFH1-GFP RNA at 0, 24, 48, 72 and 96 hours post-transfection. GAPDH mRNA levels was used as a loading control. (B) Replicative negative strand HCV-RNA in Huh-7.5 cells transfected with JFH1-GFP and JFH1-GND-GFP mutant RNA. The bottom panel shows the intracellular GAPDH mRNA level indicating that equal amounts of RNA were loaded in each well in the RPA assay.

hour post-transfection (Fig. 3B). Negative strand RNA was undetectable in Huh-7.5 cells transfected with GND mutant RNA. The presence of negative strand HCV RNA in the full-length transfected cells confirmed active replication of virus in the culture. Based on the results of these experiments we conclude that the chimeric JFH1-GFP clone is replication competent.

To examine infectious virus particle production from cells transfected with JFH1-GFP chimera RNA, an infectivity assay was performed. Culture supernatants were collected from transfected cells, clarified by centrifugation and inoculated to Huh-7.5 cells. The infectivity of HCV was confirmed by direct examination of infected cells under a fluorescence microscope and HCV RNA levels were measured by real-time RT-PCR assay. Infectivity of culture supernatants from cells transfected with full-length and E1-E2 deleted mutant clone was determined by measuring intracellular GFP expression. There was an increase in the number of GFP positive cells after 24, 48 and 72 hours suggesting the replication of HCV RNA after natural infection (Fig. 4A). No GFP expression was observed in Huh-7.5 cells infected with supernatants derived from cells transfected with E1-E2 deleted mutant HCV RNA (Fig. 4B). To confirm that the expression of HCV in the infected cells is associated with the increase in viral RNA, the titer of HCV positive strand RNA was measured using a real-time RT-PCR. The level of HCV RNA in the infected cell cultures was increased from 24 to 72 hours suggesting the replication of HCV-RNA genome in the infected culture (Fig. 4C). Thus, JFH1-GFP-tagged HCV RNA genome is able to replicate in Huh-7.5 cells after transfection and generates an infectious virus.

High-level replication of GFP labeled sub-genomic RNA of HCV 2a clone

Since the JFH1 2a clone replicates to a high level in a cell culture without adaptive mutations, we attempted to develop stable replication competent Huh-7 cells containing GFP labeled sub-genomic HCV RNA. The availability of these cell lines allowed us to reliably quantify the antiviral effect of IFN-α. A chimeric clone combining GFP and sub-genomic clone was prepared. As a control, GND mutant (pSGR-GND-GFP) for the replicon clone was also prepared. The full-cycle replication of pSGR-GFP RNA and unmodified pSGR-RNA in Huh-7 cells were compared for their ability to form cell colonies when cultured in the presence of a medium containing G-418 (500 μg/ml). In this assay, the cells supporting HCV RNA replication survived G-418 drug selection and formed cell colonies. No noticeable differences in the efficiency of replication of the sub-genomic RNA with or without GFP insertion in the NS5A region were observed based on the number of G-418 resistant