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A recombinant replication-competent hepatitis C virus expressing Azami-Green, a bright green-emitting fluorescent protein, suitable for visualization of infected cells

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ABSTRACT

The hepatitis C virus (HCV) production system consists of transfecting the human hepatoma cell line Huh7 with genomic HCV RNA (JFH1). To monitor HCV replication by fluorescence microscopy, we constructed a recombinant HCV clone expressing Azami-Green (mAG), a bright green fluorescent protein, by inserting the mAG gene into the nonstructural protein 5A (NS5A) gene; the resultant clone was designated JFH1-hmAG. The Huh-7.5.1 (a subclone of Huh7) cells transfected with JFH1-hmAG RNA were found to produce cytoplasmic NS5A-mAG, as readily visualized by fluorescence microscopy, and infectious virus, as assayed with the culture supernatant, indicating that JFH1-hmAG is infectious and replication-competent. Furthermore, the replication of this virus was inhibited by interferon alpha in a dose-dependent manner. These results suggest that JFH1-hmAG is useful for studying HCV life cycle and the mechanism of interferon's anti-HCV action and for screening and testing new anti-HCV drugs.

Hepatitis C virus (HCV), a positive-strand RNA virus, which belongs to *Flaviviridae*, causes serious chronic hepatitis that results in cirrhosis and hepatocellular carcinoma. Recently, cell culture systems supporting HCV replication have been developed with the complementary DNA clones of the genotype 2a isolate JFH1 [1–7] and the genotype 1a isolate H77S [8]. These systems have been shown to greatly contribute to the studies of HCV biology and the development of novel antiviral strategies against HCV [9–24]. However, the procedures for quantitative analysis of HCV are considerably complicated, because the yield of HCV in cell cultures is fairly low. Moreover, observation of living infected cells has been impossible. For solving these problems, development of recombinant HCV carrying a reporter gene such as *Renilla* luciferase or red/green fluorescent protein gene has been attempted [25–27].

A green-emitting fluorescent protein, Azami-Green (AG, tetrameric) has been identified in a stony coral, *Azami-Sango* [28]. Its monomeric derivative (mAG) is stably brilliant independently of pH and thus suitable for labeling proteins or subcellular structures in mammalian cells [28]. Most importantly, as far as we know, mAG is the brightest among the monomeric forms of green-emitting fluorescent proteins; for example, it is approximately 1.23-fold brighter than EGFP from *Aequorea victoria* [28,29] (brightness is calculated as a product of molar extinction coefficient and fluorescence quantum yield). In this study, inserting an mAG gene with humanized codon usage (hmAG) into the nonstructural protein 5A (NS5A)-coding sequence, we generated a novel replication-competent HCV clone. The brightness of the mAG allowed us to visualize infected cells with high sensitivity and ease.

Materials and methods

HCV plasmid construction. The DNA fragment encoding a monomeric Azami-Green (mAG) with the *Xho*I sites (C′TCGAG) at both ends was obtained by PCR using phmAG1-MC1 (MBL, Tokyo, Japan) as a template, digested with *Xho*I, and inserted into the *Abs*I site (5′-CCTCGAGG-3′) of pJFH1 [1] (GenBank Accession No. AB047639). The integrity of the resulting plasmid, pJFH1-hmAG was verified by DNA sequencing (Fig. 1).

RNA synthesis. We followed previously developed methods [30]. In brief, we cut pJFH1-hmAG with *Xba*I and treated it with Mung

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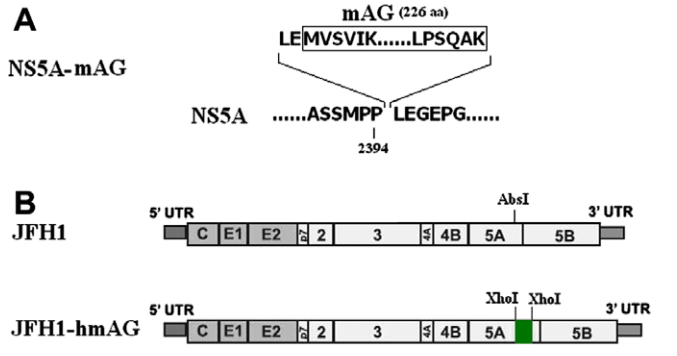

Fig. 1. HCV plasmids. (A) Sites and amino acid sequences of the mAG insertion in NS5A. (B) Schematic diagrams of JFH1 and its derivative JFH1-hmAG containing the hmAG gene within the NS5A gene.

Bean nuclease (New England Biolabs) to remove the 3′-protruding four nucleotides. With this DNA, we synthesized HCV RNA by using a MEGAscript™ T7 kit (Applied Biosystems/Ambion, TX). We treated the synthesized RNA with DNaseI (Promega, WI) at 37 °C for 15 min, and extracted it with acid phenol to remove remaining template DNA.

Cell cultures and transfection. Huh-7.5.1 cells [2], which are highly permissive to HCV RNA replication, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, MO) supplemented with 10% fetal bovine serum (Invitrogen Corporation/Gibco, CA), 100 μg/mL of kanamycin (Sigma), and non-essential amino acids (Invitrogen Corporation/Gibco). The synthesized RNA of JFH1-hmAG was delivered to cells by electroporation or lipofection. We performed electroporation with a Gene Pulser II apparatus (Bio-Rad Laboratories, CA) as described [1], and lipofection with UniFECTOR reagent (B-Bridge International, Tokyo, Japan); a mixture of 6 μg of JFH1-hmAG RNA and 36 μl of UniFECTOR was subjected to 10^6 cells in a 100-mm culture dish.

Immunofluorescence analysis. Intracellular staining was performed as described [2]. In brief, a anti-Core mouse monoclonal antibody (IgG1, clone Hyb-K0811B, Cosmo Bio, Tokyo, Japan) was used at a dilution of 1:200 followed by incubation with a 1:400 dilution of Alexa Fluor 594-conjugated donkey anti-mouse antibody (Invitrogen Corporation/Molecular Probes, CA). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were acquired by using Biozero fluorescence microscopy (Keyence, Tokyo, Japan) or with a confocal microscope Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany).

Infection. Huh-7.5.1 cells were seeded 24 h before infection at a density of 2×10^4 cells/well in a 8-well culture slide (BD BioCoat™, poly-D-lysine coated 8-well CultureSlide, BD, NJ). The cells were inoculated with the culture supernatant obtained from the cells transfected with JFH1-hmAG RNA for 3 h, washed three times with PBS, then cultured in 0.5 mL/well of the complete culture medium.

Reverse transcription (RT) PCR. RNA was extracted from cell culture supernatant with ISOGEN-LS (Nippon GENE, Tokyo, Japan) or from cells with ISOGEN (Nippon GENE). Complementary DNA was generated by a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to manufacturer's instructions. PCRs targeting the different regions along the JFH1-hmAG genome were performed. The primers for PCR were as follows: for the 5′-untranslated region (5′UTR), 5′-TCTGCGGAACCGGTGAGTAC-3′ and 5′-TCAGGCAGTACCACAAGGCC-3′); for the nonstructural protein 3 (NS3) region, 5′-CTTTGACTCCGTGATCGACC-3′ and 5′-CCCTGTCTTCCTCTACCTG-3′; and for the NS5A-hmAG junction, 5′-CTGGCCATCAAGACCTTTG-3′ and 5′-GCTTGAAGTAGTCCTGGATG-3′.

Interferon inhibition. Huh-7.5.1 cells were incubated for 12 h with various concentrations of interferon alpha (IFNα, Universal Type I Interferon, PBL InterferonSource, NJ), then incubated with the culture supernatant containing JFH1-hmAG virus for 3 h, washed twice with PBS, and further incubated for 3 days. The copy numbers of intracellular HCV RNA were determined by quantitative RT-PCR (RT-qPCR). RT-qPCR with a LightCycler 2.0 Instrument (Roche) allowed us to determine relative copy numbers by normalization with that of GAPDH mRNA. The primers for RT-qPCR were as follows: for HCV, 5′-TCTGCGGAACCGGTGAGTA-3′ (sense) and 5′-TCAGGCAGTACCACAAGGC-3′ (antisense); and for GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense) and 5′-GAAGATGGTGATGGGATTTC-3′ (antisense), as described previously [2,31].

Results and discussion

Construction of pJFH1-hmAG and direct visualization of NS5A-mAG by fluorescent microscopy

We constructed a novel HCV clone (JFH1-hmAG) containing the hmAG sequence fused with NS5A at the amino acid 418 of NS5A (see Materials and methods for detail. Fig. 1). Three days after transfection of Huh-7.5.1 cells with JFH1-hmAG RNA, the green signal of the NS5A-mAG fluorescence was strong enough to be readily visualized in the cytoplasm as bright dots in a reticular pattern surrounding the nucleus by confocal microscopy (Fig. 2A). To further confirm viral protein production, we stained the cells with anti-Core antibody (Fig. 2B, upper right) and found the signal in a pattern similar to that of NS5A-mAG. Indeed, merging the NS5A-mAG and Core images (Fig. 2B, lower right), we observed partial colocalization of NS5A-mAG and Core, shown as yellow signals. Since the wild-type JFH1 has been reported to show the same colocalization pattern [25,32], JFH1-hmAG seems to inherit this property from the wild-type JFH1. Furthermore, RT-PCR with the RNA extracted from these transfected cells showed that the NS5A-hmAG junction in the JFH1-hmAG genome was stably retained (data not shown). Further studies seem necessary to determine the stability of NS5A-mAG over more extended passages indicator.

Inoculation of naïve Huh-7.5.1 cells with the supernatant of transfected cells

To test whether the reporter construct JFH1-hmAG can produce and release infectious virus particles, we inoculated naïve Huh-7.5.1 cells with the culture supernatant obtained from the transfected cells 30 days after transfection with JFH1-hmAG RNA. Two days after inoculation, NS5A-mAG signal was visualized in infected cells by fluorescence microscopy while not in uninfected cells (Fig. 2C). Immunostaining showed the same colocalization configuration of NS5A-mAG and Core proteins in inoculated cells (Fig. 2D) as in the transfected cells (Fig. 2B). Therefore, we considered that the new recombinant virus replicates in the same manner as the parental JFH1, even though NS5A was truncated by insertion of hmAG. Moreover, the RNA in cells and supernatants was considered to keep integrity without loss of the inserted mAG because all the RT-PCRs targeting the 5′UTR, NS3, and NS5A-hmAG junction regions yielded the products with predicted sizes (Fig. 3).

Taken altogether, we concluded that the JFH1-hmAG clone is infectious and replication-competent. In the transfected cells, JFH1-hmAG RNA produced NS5A-mAG/Core proteins, replicated, and released HCV particles that were infectious to Huh-7.5.1 cells. Though the culture supernatants of transfected cells at different time points were capable of infecting and re-infecting Huh-7.5.1 cells (data not shown), further studies seem necessary to

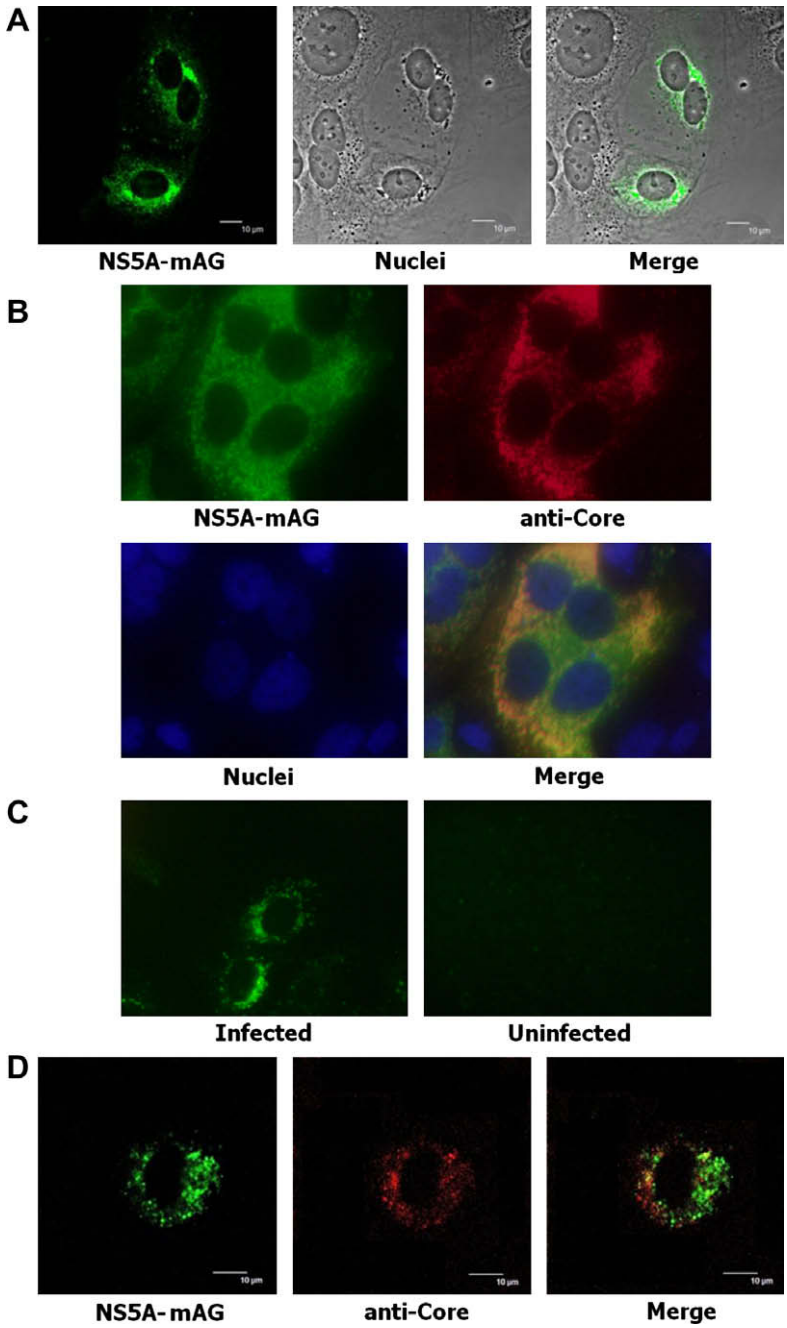

Fig. 2. Cell Images by fluorescence microscopy. (A) Huh-7.5.1 cells were analyzed by confocal microscopy 3 days after transfection with JFH1-hmAG RNA. The mAG emitted green signals. (B) Subcellular localization of NS5A-mAG and Core proteins in transfected cells. Huh-7.5.1 cells transfected with JFH1-hmAG RNA were grown in a 8-well chamber for 3 days. Cells were stained with monoclonal anti-Core mouse antibody and Alexa Fluor 594-conjugated donkey anti-mouse antibody. The localization patterns of NS5A-mAG and Core were shown in green and red, respectively. The merged images were also shown. Nuclei were stained with DAPI (blue). (C) NS5A-mAG was directly visualized in Huh-7.5.1 cells by fluorescence microscopy after inoculation with the culture supernatant obtained from the transfected cells 30 days after RNA transfection. (D) Subcellular localization of NS5A-mAG and Core protein in infected cells. NS5A-mAG and Core proteins were visualized 2 days after inoculation as described in (B).

determine the infectivity and integrity of JFH1-hmAG virus for a longer period, since genetic mutations frequently appeared in an persistent HCV infection in vitro [33].

Inhibition of JFH1-hmAG infection by interferon (IFN)

The current standard therapy to chronic hepatitis C includes IFN [34]. JFH1 replication has been found to be sensitive to IFN [2]. To test IFN-sensitivity of JFH1-hmAG, we examined the viral infection in the presence of IFNα. Huh-7.5.1 cells were pretreated for 12 h with IFNα and then were incubated with the culture supernatant containing JFH1-hmAG virus. Three days after infection, the levels of intracellular HCV RNA were determined by RT-qPCR (Fig. 4). IFNα pretreatment inhibited JFH1-hmAG RNA accumulation in a dose-dependent manner (Fig. 4). Thus, the results imply that JFH1-hmAG inherited IFN-sensitivity from JFH1 and that this new infection system is useful for studying mechanisms of IFN actions and viral resistance to IFNs.

In conclusion, we have developed a novel infectious HCV clone (JFH1-hmAG) containing *hmAG* in the NS5A region of the genome,

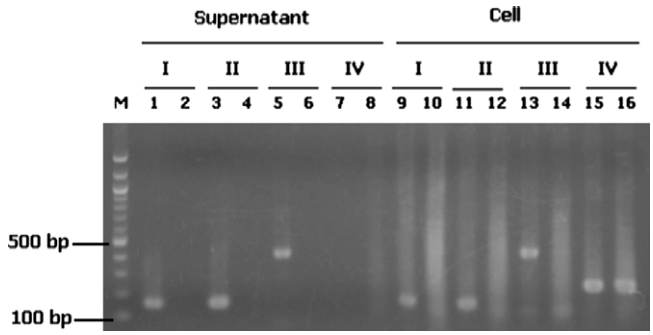

Fig. 3. Reverse transcription (RT) PCR. Huh-7.5.1 cells were inoculated with the supernatant containing JFH1-hmAG virus. Two days after infection, RNA was extracted from cell culture supernatant and from harvested cells. RT-PCRs targeting 5′UTR (group I), NS3 (group II), NS5A-hmAG junction (group III) regions in JFH1-hmAG genome were performed, respectively. RT-PCR targeting GAPDH (group IV) mRNA was also performed simultaneously as a control. Reactions with RNA extracted from culture supernatants (lanes 1, 3, 5, and 7) or cells (lanes 9, 11, 13, and 15) with infection, and from culture supernatants (lanes 2, 4, 6, and 8) or cells (lanes 10, 12, 14, and 16) without infection were shown, respectively.

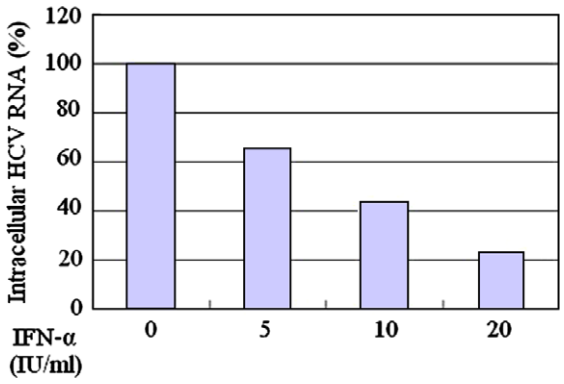

Fig. 4. Inhibition of JFH1-hmAG replication by interferon alpha (IFNα). Huh-7.5.1 cells in duplicate were treated with 0, 5, 10, and 20 U/mL IFNα followed by incubation with the culture supernatant containing with JFH1-hmAG virus. Three days after infection, the relative numbers of intracellular HCV RNA molecules were determined by RT-qPCR (see Materials and methods). Each bar represents the mean number of intracellular HCV RNA molecules expressed as a percentage of that in the control infection without IFN pretreatment.

and have been able to readily observe living infected cells by green-emitting fluorescence. Its replication was restricted by IFNα in a dose-dependent manner. Our results suggested that this new recombinant virus replicated as properly as the parental JFH1 virus, and that this new reporter virus is useful in investigating the HCV biology and the anti-HCV action of IFNs. The JFH1-hmAG virus may provide us with a new system useful for readily screening anti-HCV drugs.

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