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Hepatitis C virus NS5A protein interacts with 2',5'-oligoadenylate synthetase and inhibits antiviral activity of interferon in an ISDR-independent manner

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ABSTRACT

The nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) has been implicated in inhibition of antiviral activity of interferon (IFN). While previous studies suggested interaction between NS5A and double-stranded RNA-dependent protein kinase (PKR), the possibility still remains that interaction with another molecule(s) is involved in the NS5A-mediated inhibition of IFN. In the present study, we investigated possible interaction between NS5A and 2',5'-oligoadenylate synthetase (2-5AS), another key molecule for the antiviral activity. We observed that NS5A physically interacted with 2-5AS in cultured cells, with an N-terminal portion of NS5A (amino acid [aa] 1 to 148; NS5A[1-148]) and two separate portions of 2-5AS (aa 52 to 104, and 184 to 275) being involved in the interaction. Single point mutations at residue 37 of NS5A affected the degree of the interaction with 2-5AS, with a Phe-to-Leu mutation (F37L) augmenting, and a Phe-to-Asn mutation (F37N) diminishing it. Virus rescue assay revealed that the full-length NS5A (NS5A-F) and NS5A(1-148), the latter of which does not contain the IFN sensitivity-determining region (ISDR) or the PKR-binding domain, significantly counteracted antiviral activity of IFN. Introduction of the F37N mutation into NS5A(1-148) impaired the otherwise more significant IFN-inhibitory activity of NS5A(1-148). It was also found that the F37N mutation was highly disadvantageous for the replication of an HCV RNA replicon. Taken together, our results suggest the possibility that NS5A interacts with 2-5AS and inhibits antiviral activity of IFN in an ISDR-independent manner.

INTRODUCTION

Hepatitis C virus (HCV) is a single stranded, positive sense RNA virus, which easily causes persistent infection in infected patients and is the most common pathogen of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in industrialized countries (Lauer & Walker, 2001). HCV-infected patients are treated with interferon-alpha (IFN- α) alone or in combination with ribavirin, but the IFN treatment is effective in only a half or less of the treated patients (McHutchison *et al.*, 1998). The HCV genome exhibits a considerable degree of sequence variation and HCV is now classified into at least 6 genotypes and more than 60 subtypes (Mellor *et al.*, 1995; Doi *et al.*, 1996; Robertson *et al.*, 1998). Clinico-pathological features, such as the severity of liver injury, development of hepatocellular carcinoma, viremia titres and responsiveness to IFN treatment, are likely to vary with different subtypes (Bruno, *et al.*, 1997; Noursbaum *et al.*, 1995) and even with different strains of the same subtype (Enomoto *et al.*, 1996; Song *et al.*, 1999; Lusida *et al.*, 2001; Ogata *et al.*, 2002, 2003). The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is processed by the signal peptidase of the host cell and virally encoded proteases to generate at least 10 viral proteins (Reed & Rice, 2000).

The nonstructural protein 5A (NS5A) of HCV has been reported to be multifunctional. NS5A is localised in the cytoplasmic, perinuclear region despite the presence of a functional nuclear localisation signal (Ide *et al.*, 1996; Song *et al.*, 1999, 2000) and represents two

forms with different degrees of phosphorylation, p56 and p58 (Kaneko *et al.*, 1994; Tanji *et al.*, 1995; Song *et al.*, 1999; Reed & Rice, 2000). Mutation in the central region of NS5A (aa 237 to 276) was demonstrated to be correlated with responsiveness to IFN treatment in patients chronically infected with HCV subtype 1b (HCV-1b) and, therefore, this region has been designated as IFN sensitivity-determining region, or ISDR (Enomoto *et al.*, 1996). We also reported that mutations in ISDR inversely correlated with HCV RNA titres in patients infected with HCV-1b, HCV-1c and HCV-2a (Lusida *et al.*, 2001). In experimental settings, NS5A has been demonstrated to rescue encephalomyocarditis virus (EMCV) replication in IFN-treated cell cultures (Polyak *et al.*, 1999; Song *et al.*, 1999). It was also reported that NS5A inhibited antiviral activity of IFN by binding to double-stranded RNA-dependent protein kinase (PKR) through ISDR and its adjacent region, called the PKR-binding region (aa 237 to 302) (Gale *et al.*, 1997, 1998). However, apparently controversial observations were reported that ISDR sequence variation did not account for different IFN resistance in patients (Duverlie *et al.*, 1998) and also in an HCV subgenomic RNA replicon system (Guo *et al.*, 2001). Moreover, expression of NS5A or entire HCV polyprotein was reported to counteract the antiviral effect of IFN in PKR-independent, ISDR-independent manner (Francois *et al.*, 2000; Podelvin *et al.*, 2001). The possibility, therefore, still remains that another molecule(s) than PKR is involved in the NS5A-mediated inhibition of IFN.

In addition to PKR, antiviral effects of IFN are executed through the function of 2',5'-oligoadenylate synthetase (2-5AS), RNase L, Mx proteins, etc. (Staeheli & Pavlovic, 1991; Hassel *et al.*, 1993; Sen & Ransohoff, 1993; Li *et al.*, 1998). As for human MxA protein,

its role in regulating HCV infection might be marginal (Frese *et al.*, 2001). In the present study we investigated possible interaction between NS5A and 2-5AS. We report here that an N-terminal one-third of NS5A (NS5A[1-148]), which does not contain ISDR or the PKR-binding domain, physically interacted with 2-5AS and counteracted antiviral activity of IFN. Introduction of a point mutation (Phe to Asn) to residue 37 of NS5A(1-148) significantly reduced 2-5AS-binding activity and negated the otherwise more significant IFN-inhibitory activity of NS5A(1-148). The same mutation introduced to an HCV subgenomic RNA replicon abolished its replication competence. These results collectively suggest that NS5A interacts with 2-5AS and inhibits antiviral activity of IFN in an ISDR-independent manner.

METHODS

Plasmid construction. The FLAG peptide-coding sequence was introduced to pcDNA3.1(-)/myc-His expression plasmid (Invitrogen) to generate pcDNA-FLAG vector. A full-length NS5A of HCV-1bJk strain (Song *et al.*, 1999, 2000) was subcloned into pcDNA-FLAG in frame to the FLAG peptide and the resultant plasmid was designated as pcDNA-FLAG-NS5A-F. A 0.7-kb *NheI* fragment of pcDNA-FLAG-NS5A-F encoding FLAG-tagged N-terminal 232 residues of NS5A was ligated to *NheI*-treated pcDNA3.1(-)/myc-His in proper orientation to generate pcDNA-FLAG-NS5A(1-232) (Fig. 1A). Plasmids for other deletion mutants of NS5A were constructed by cloning the PCR products, that had been amplified using appropriate sets of primers (Table 1), into pcDNA-

← Fig. 1

← Table 1

FLAG in frame to the FLAG peptide. Various point mutations were introduced to pcDNA-FLAG-NS5A(1-148) using appropriate primers and QuickChange Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's protocol, so that residue 37 of NS5A(1-148) was mutated from Phe to Leu, Asn, Ser and Tyr (F37L, F37N, F37S and F37Y, respectively). The above plasmids were used for transient expression, as described below. The sequences for the full-length NS5A, NS5A(1-148) and NS5A(1-148)F37N were each subcloned into pCAGGS expression vector (Niwa *et al.*, 1991) to generate pCAGGS-NS5A-F, pCAGGS-NS5A(1-148) and pCAGGS-NS5A(1-148)F37N, respectively, and used to establish stable transformants, as described below.

The plasmid pMA25 containing the entire coding sequence except the first three amino acid residues for murine 2-5AS (DDBJ/EMBL/GenBank accession number, X04958) was a kind gift from Dr. Y. Sokawa, Kyoto Institute of Technology, Kyoto, Japan. The mouse 2-5AS belongs to the isoform 1a since its sequence is 99.5% identical to a standard sequence of the isoform 1a (accession number BC013715). The sequence was fused in frame to the influenza virus HA epitope and cloned into pSG5 expression vector (Stratagene Cloning Systems). The resultant plasmid was designated as pSG-HA-2-5AS-F.

The coding sequence for glutathione S-transferase (GST) in pGEX-4T-1 (Pharmacia Biotech, Uppsala, Sweden) was cloned into the unique *Sma*I site of pBlueScript II SK⁻ (Stratagene). This vector plasmid was designated as pBS-GST*Bam*. pBS-GST*Bam* was digested with *Bam*HI, filled-in by treatment with Klenow fragment of *Escherichia coli* DNA polymerase I and then self-ligated to create an in-frame stop codon after the GST sequence.

This plasmid, designated as pBS-GST, was used to express control GST. The full-length coding sequences for NS5A and 2-5AS were each subcloned in frame to the GST sequence into the unique *Bam*HI site of pBS-GST*Bam* to generate pBS-GST-NS5A-F and pBS-GST-2-5AS-F, respectively. Plasmids for various deletion mutants of GST-tagged 2-5AS (see Fig. 1B) were constructed by cloning the PCR products, that had been amplified using appropriate sets of primers (Table 1), into the unique *Bam*HI site of pBS-GST*Bam*.

We also used the entire coding sequence for human 2-5AS isoform 1 (DDBJ/EMBL/GenBank accession number, D00068) in pHE25AS (a kind gift from Dr. Y. Sokawa). The human 2-5AS sequence was fused in frame to GST in pBS-GST*Bam* and pcDNA vectors to generate pBS-GST-Hu-2-5AS-F and pcDNA-GST-Hu-2-5AS-F, respectively.

The plasmid pFK5B2884Gly containing an HCV subgenomic RNA replicon with a cell culture-adaptive Arg-to-Gly mutation at residue 2884 (Lohmann *et al.*, 2001) was a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany. The F37L and F37N mutations were introduced to pFK5B2884Gly by site-directed mutagenesis, as described above.

The sequences of all the plasmids constructed were verified by sequence analysis.

Transient and stable expression. Transient expression was performed as described previously (Muramatsu *et al.*, 1997). In brief, HeLa cells, maintained in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum, were infected

with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) for 1 h and then transfected with the expression plasmids using Lipofectin reagents (Life Technologies, Inc.). After cultivation for 12-16 h, the cells were analysed for the expression of the respective proteins and their possible interaction, as described below.

To establish cell clones stably expressing NS5A, mouse fibroblast L929 and human hepatoma Huh-7 cell lines were used. The cells were cotransfected with a selection plasmid, pSV2neo, and either pCAGGS-FLAG-NS5A-F, pCAGGS-FLAG-NS5A(1-148), pCAGGS-FLAG-NS5A(1-148)F37N or the pCAGGS vector by using FuGene 6 transfection reagents (Roche). After cultivation in the presence of G418 (1 mg/ml) for 2 to 3 weeks, resultant colonies were cloned using cloning cylinders.

Huh-7 human hepatoma cells harboring HCV subgenomic RNA replicon were also generated by transfecting RNA that had been transcribed *in vitro* from pFK5B2884Gly (Lohmann *et al.*, 2001), followed by G418 selection.

GST pull-down assay. HeLa or Huh-7 cells were transiently transfected with the expression plasmids for GST-tagged NS5A and HA-tagged 2-5AS, or GST-tagged 2-5AS and FLAG-tagged NS5A, as described above. After 12-16 h, the cells were washed once with phosphate-buffered saline (PBS) and lysed in NETN buffer consisting of 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 0.5% NP40. The lysates were centrifuged at 14000 rpm for 5 min and the supernatants were mixed with 20 µl of glutathione-conjugated Sepharose beads at 4°C for 90 min. The beads were washed 5 times with NETN buffer and

possible association between NS5A and 2-5AS was analysed by immunoblotting using appropriate antibodies, as described below. To verify comparable amounts of the proteins being analysed, the cell lysates were directly (without pull-down) subjected to immunoblotting.

Immunoblotting analysis. Samples dissolved in a solution consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically blotted onto a polyvinylidene difluoride filter (Bio-Rad). After blocking in PBS containing 5% nonfat dry milk, the filters were incubated with mouse monoclonal antibodies against NS5A (a kind gift from Dr. I. Fuke, Research Institute for Microbial Diseases, Kan-Onji Branch, Osaka University, Kan-Onji, Kagawa, Japan), the FLAG (F-3165, Sigma) or HA peptide (16B12, BabCO). After being washed 5 times with PBS containing 0.5% Tween 20, the filters were incubated with peroxidase-labelled goat anti-mouse IgG (MBL). After being washed 5 times, the protein bands were visualised by an enhanced chemiluminescence method (ECL; Amersham Pharmacia). The intensity of the signals was quantified by using NIH image 1.62 software.

Coimmunoprecipitation analysis. HeLa cells transiently transfected with the expression plasmids were lysed with NETN buffer and the lysates were clarified by centrifugation. The resultant supernatants were incubated at 4°C for 3 h with 0.5 µg of anti-HA rabbit

polyclonal antibody (Y-11, Santa Cruz Biotech., Santa Cruz, CA, USA) to immunoprecipitate HA-tagged 2-5AS. Normal rabbit IgG served as a control. The mixtures were then incubated with 10 μ l of protein G-coupled Sepharose (Amersham Pharmacia). After being washed 6 times with NETN buffer, the immunoprecipitates were subjected to immunoblot analysis using anti-FLAG antibody to detect NS5A.

Immunofluorescence analysis. HeLa cells transiently transfected with the expression plasmids were fixed with 95% ethanol and double-stained with anti-FLAG mouse monoclonal antibody and anti-HA rabbit polyclonal antibody. After being washed 5 times with PBS, the cells were incubated with Texas-Red-conjugated anti-mouse IgG (Amersham Life Science) and FITC-conjugated anti-rabbit IgG (MBL). After being washed 5 times with PBS, the cells were analysed by confocal laser scanning microscopy (MRC-1024; Bio-Rad).

IFN antiviral activity assay. Cells were seeded in 6-well tissue culture plates at a density of 2×10^5 cells/well and cultivated for 24 h. The cells were treated with recombinant mouse IFN- α A (PBL Biomed. Lab., New Brunswick, NJ, USA) or human IFN- α 2a (Roche, Nutley, NJ, USA) at concentrations of 5 and 25 U/ml or left untreated for another 24 h, and then inoculated with 50–60 p.f.u. of EMCV (strain DK-27) (Dan *et al.*, 1995; Song *et al.*, 1999) per well. After 1 h with intermittent rocking, fresh medium containing 1% methylcellulose was added to each well of the plates. After cultivation for 2 days, the plates were stained

with crystal violet and the number of plaques in each well was counted. Percent plaque numbers on IFN-treated cells compared to those on untreated cells were calculated.

RESULTS

NS5A-F colocalises and physically interacts with 2-5AS in mammalian cells.

We first investigated subcellular localisation of NS5A and 2-5AS. FLAG-tagged NS5A-F and HA-tagged 2-5AS-F were transiently expressed in HeLa cells using vaccinia virus-T7 hybrid expression system. Consistent with our previous observations and others (Ghosh *et al.*, 2000; Song *et al.*, 1999, 2000), both NS5A-F and 2-5AS-F were localised in the cytoplasmic, perinuclear region. Confocal laser scanning microscopic analysis revealed that NS5A partially colocalised with 2-5AS (Fig. 2A).

← Fig. 2

We then examined possible complex formation between NS5A and 2-5AS in mammalian cells by GST pull-down assay and coimmunoprecipitation analysis. In the GST pull-down assay, GST-tagged 2-5AS-F or the control GST was expressed with or without NS5A-F in HeLa cells, and pulled down by glutathione-conjugated Sepharose beads, which were then subjected to immunoblotting using anti-NS5A monoclonal antibody. The result clearly demonstrated that GST-tagged 2-5AS-F, but not the control GST, pulled down NS5A-F (Fig. 2B, left panel). Similarly, GST-tagged NS5A-F or the control GST was coexpressed with HA-tagged 2-5AS or the control HA, pulled down by glutathione Sepharose beads, and probed with anti-HA monoclonal antibody to detect 2-5AS. The

result showed that GST-tagged NS5A-F, but not the control GST, pulled down HA-tagged 2-5AS-F (Fig. 2B, right panel).

N-terminal 148 residues of NS5A is involved in the interaction with 2-5AS.

Various deletion mutants of FLAG-tagged NS5A (see Fig. 1A) were coexpressed with GST-tagged 2-5AS-F to determine a region responsible for the interaction with 2-5AS. All of the NS5A deletion mutants tested were localised in the cytoplasm (data not shown).

Immunoblot analysis using anti-FLAG antibody revealed that FLAG-tagged NS5A(1-232) and NS5A(1-148), but not NS5A(1-109) or NS5A(27-148), were efficiently pulled down by

GST-tagged 2-5AS-F (Fig. 3A, upper panel). It should be noted that NS5A(1-232) and NS5A(1-148) bound to 2-5AS-F more efficiently than did NS5A-F. Comparable degrees of expression of NS5A-F and the deletion mutants (Fig. 3A, lower panel) and GST-tagged 2-5AS-F (data not shown) in each transfected cell culture were verified. NS5A(1-67) or NS5A(1-87) was not pulled down by GST-tagged 2-5AS-F (data not shown). ← Fig. 3

We also performed coimmunoprecipitation analysis to confirm the complex formation between NS5A and 2-5AS. HA-tagged 2-5AS-F was expressed in HeLa cells with or without FLAG-tagged NS5A(1-148). The cell lysates were immunoprecipitated by using anti-HA antibody or control antibody, and probed with anti-FLAG antibody. As shown in Fig. 3B, anti-HA antibody (directed against HA-tagged 2-5AS-F), but not the control antibody, coimmunoprecipitated FLAG-tagged NS5A(1-148) from the lysates of cells expressing both NS5A(1-148) and 2-5AS-F (lane 9); the same anti-HA antibody did not

coimmunoprecipitate FLAG-tagged NS5A(1-148) from the lysates of cells expressing NS5A(1-148) alone or 2-5AS-F alone (lanes 3 and 6). NS5A-F was also coimmunoprecipitated with HA-tagged 2-5AS-F (data not shown). These results thus confirmed that NS5A formed a complex with 2-5AS in mammalian cells.

Two separate portions of 2-5AS are independently involved in the interaction with NS5A.

To determine a region(s) of 2-5AS responsible for the interaction with NS5A, various deletion mutants of GST-tagged 2-5AS (see Fig. 1B) were coexpressed with FLAG-tagged NS5A(1-148), pulled down by glutathione-conjugated Sepharose beads and subjected to immunoblot analysis using anti-FLAG antibody. GST-tagged 2-5AS(1-104), 2-5AS(52-144) and 2-5AS(184-275), but not 2-5AS(1-60), 2-5AS(184-235) or the control GST, pulled down NS5A(1-148) (Fig. 3C, upper panel). Comparable degrees of expression of FLAG-tagged NS5A(1-148) (Fig. 3C, lower panel), GST-tagged 2-5AS-F and the deletion mutants (data not shown) in each transfected cell culture were verified. Collectively, these results suggested that two separate regions of 2-5AS (aa 52 to 104, and aa 184 to 275) were independently involved in physical interaction with NS5A(1-148).

Mutation of residue 37 of NS5A affects interaction with 2-5AS.

We previously noticed that single-point mutation of NS5A at residue 37 might be correlated with serum HCV RNA titres (data not shown). Therefore, we were interested in testing

possible effects of NS5A mutations at residue 37 (F37L, F37N, F37S and F37Y) on the interaction with 2-5AS. FLAG-tagged mutants of NS5A(1-148) were coexpressed with GST-tagged 2-5AS-F or the control GST in HeLa cells. The cell lysates were subjected to GST pull-down assay using glutathione-conjugated Sepharose beads, followed by immunoblot analysis using anti-FLAG antibody. F37L mutation of NS5A significantly augmented complex formation with 2-5AS whereas F37N mutation significantly decreased it (Fig. 4A, upper panel). F37S or F37Y mutations did not significantly affect the complex formation. A comparable degree of expression of NS5A(1-148) and the single-point mutants (Fig. 4A, lower panel) in each transfected cell culture was verified. Mean values of relative degrees of complex formation between each NS5A mutant and 2-5AS obtained from 4 independent experiments are shown in Fig. 4B. ← Fig. 4

NS5A-F and NS5A(1-148) counteract antiviral activity of IFN.

To test possible inhibitory effects of NS5A on antiviral activity of IFN, we established L929 cell clones stably expressing NS5A-F, NS5A(1-148) or NS5A(1-148)F37N as well as the non-expressing control. The cells were treated with IFN or left untreated and challenged with 50 p.f.u. of EMCV. Consistent with our previous observation (Song *et al.*, 1999), the numbers of plaques formed on the cells that had not been treated with IFN were practically the same among the cell clones tested, irrespective of NS5A expression and NS5A mutation (data not shown). IFN at concentrations of 5 and 25 U/ml suppressed EMCV replication in the non-expressing control cells by 70% and 95% (as judged by the relative virus titres of

30% and 5%), respectively (Fig. 5A). The antiviral activity of IFN against EMCV was significantly reduced in cells expressing NS5A-F or NS5A(1-148). ← Fig. 5

NS5A(1-148)F37N, which was shown to interact with 2-5AS only weakly (see Fig. 4), was less effective in counteracting antiviral activity of IFN, compared with the wild-type (WT) NS5A(1-148) (Fig. 5B). This result suggested that the F37N mutation negated an otherwise more evident inhibitory effect of NS5A(1-148) on IFN antiviral activity.

The possible inhibitory effects of NS5A on antiviral activity of IFN were assessed using Huh-7 human hepatoma cells as well. We first confirmed the physical interaction between NS5A and human 2-5AS in the cell. As shown in Fig. 6A, NS5A(1-148), NS5A(1-148)F37L and NS5A(1-148)F37N physically interacted with GST-tagged human 2-5AS. NS5A-F was also shown to interact with human 2-AS (data not shown). We then tested antiviral activity of IFN in Huh-7 cells stably expressing NS5A-F or NS5A(1-148) and the non-expressing control. The result obtained showed that antiviral activity of IFN was significantly counteracted by NS5A-F and NS5A(1-148) (Fig. 6B), the result being consistent with that obtained with the L929 mouse cell system. ← Fig. 6

Significant counteraction of IFN antiviral activity by an HCV protein(s) was observed also in Huh-7 cells harboring an HCV subgenomic RNA replicon or a mutant replicon with the F37L mutation (Fig. 7A). It should be noted that another mutant replicon possessing the F37N mutation did not generate any colony after selection in G418-containing medium although the wild-type HCV replicon and the F37L mutant generated substantial numbers of G418-resistant colonies (Fig. 7B). Similar results were reproducibly obtained, suggesting ← Fig. 7

that the F37N mutation is highly disadvantageous for replication of the HCV genome.

DISCUSSION

Antiviral effects of IFN are executed by a variety of antiviral proteins, such as PKR, 2-5AS, RNase L, Mx proteins, etc. (Staeheli & Pavlovic, 1991; Hassel *et al.*, 1993; Sen & Ransohoff, 1993; Li *et al.*, 1998). HCV NS5A has been reported to bind to, and inhibit the function of, PKR through ISDR of NS5A (Gale *et al.*, 1997, 1998). However, PKR-independent, ISDR-independent IFN inhibition by NS5A has also been suggested (Duverlie *et al.*, 1998; Francois *et al.*, 2000; Guo *et al.*, 2001; Podelvin *et al.*, 2001). Moreover, NS5A was reported to up-regulate interleukin 8 expression, which counteracted IFN activity possibly through inhibiting 2-5AS (Polyak *et al.*, 2001; Girard *et al.*, 2002). As for the role for 2-5AS in HCV infection, controversial observations in clinical settings were reported that 2-5AS activity in peripheral blood mononuclear cells or liver cells correlated well with IFN responsiveness (Grander *et al.*, 1996; Podelvin *et al.*, 1997) whereas serum 2-5AS activity did not (Murashima *et al.*, 2000). On the other hand, 2-5AS and, in turn, RNase L were shown to be activated by HCV RNA (Han & Barton, 2002). It was also reported that the 2-5AS gene was transcriptionally activated by HCV core protein (Naganuma *et al.*, 2000). Thus, the significance of possible inhibition and activation of 2-5AS in HCV infection is yet to be clarified and, to our knowledge, direct interaction between 2-5AS and HCV proteins has not been documented so far.

In the present study we demonstrated that HCV NS5A physically interacted with 2-5AS, with an N-terminal region of NS5A (aa 1 to 148) and two separate regions of 2-5AS (aa 52 to 104, and aa 184 to 275) being involved in the interaction (Figs. 2 and 3). We used two different 2-5AS molecules; the isoform 1a (p42) of mouse 2-5AS and the isoform 1 (p40) of human 2-5AS. While the overall sequence similarity between them is 68%, the NS5A-binding portions show even higher sequence similarities (83% and 85%, respectively) between mouse and human 2-5AS. Our results also demonstrated that NS5A(1-148) inhibited antiviral activity of IFN against EMCV, as did the full-length NS5A-F, in both mouse and human cell culture systems (Figs. 5 and 6). Since NS5A(1-148) does not harbor ISDR or the PKR-binding domain, the IFN inhibition by NS5A(1-148) is likely independent of ISDR and PKR. The major phosphorylation sites of NS5A (Reed & Rice, 1999; Katze *et al.*, 2000) and residues undergoing adaptive mutation in NS5A of HCV replicons (Blight *et al.*, 2000; Krieger *et al.*, 2001; Lohmann *et al.*, 2001), which are located in the central and C-terminal portions of NS5A, are unlikely to be involved in the interaction with 2-5AS or IFN inhibition since NS5A(1-148) does not contain those residues. We could not confirm, however, the inhibition of 2-5AS enzymatic activity in NS5A-expressing cells (data not shown). We assume that the possible inhibition might have been masked due to a technical limitation since 2-5AS in lysates of the cells, either NS5A-expressing cells or the control, is unavoidably activated to some extent during the experimental procedures.

In our previous study on NS5A sequence diversity among HCV isolates in Indonesia (Lusida *et al.*, 2001), we noticed that mutation at residue 37 of NS5A might be correlated

with HCV viremia titres (data not shown). In a hope that we could find correlation between the mutation and IFN inhibition, we introduced various point mutations; F37L, F37N, F37S and F37Y. Interestingly, our present result revealed that NS5A(1-148)F37L interacted with 2-5AS twice more strongly than did the wild-type NS5A(1-148) (Fig. 4). On the other hand, interaction between another mutant NS5A(1-148)F37N and 2-5AS was much weaker than that between the wild-type NS5A(1-148) and 2-5AS. The weaker interaction may account for the weaker inhibitory effect of NS5A(1-148)F37N on antiviral activity of IFN compared with the wild-type NS5A(1-148). Moreover, our result suggested that the F37N mutation was highly disadvantageous for the replication of HCV subgenomic RNA replicon (Fig. 7B). It should be noted that, while Phe, Leu and Tyr are hydrophobic and found at this position in clinical isolates of HCV, Asn is hydrophilic and has not been found so far at this position in clinical isolates.

There are a number of functional domains in the N-terminal half of NS5A. The N-terminal ~30 residues of NS5A were reported to include a membrane-anchor domain that determines cytoplasmic localisation of NS5A (Sato *et al.*, 2000; Song *et al.*, 2000; Brass *et al.*, 2002). It was also demonstrated that an N-terminal half of NS5A (aa 1 to 224) bound to apolipoprotein A1 and colocalised with HCV core protein on lipid droplets (Shi *et al.*, 2002). Moreover, a region of NS5A spanning from aa 105 to 162 formed a complex with NS5B and modulated its RNA-dependent RNA polymerase activity (Shirota *et al.*, 2002). The 2-5AS-binding region of NS5A determined in the present study (aa 1 to 148) overlaps those domains. As for 2-5AS, we identified two independent regions that are responsible for the interaction

with NS5A; one spanning from aa 52 to 104, and the other from aa 184 to 275. The former region contains an ATP-binding motif (P-loop) followed by an Asp⁷⁶-Ala-Asp⁷⁸ sequence (D-box) while the latter contains a region with high contents of Lys and Arg (KR-rich region). The P-loop, D-box and KR-rich region are important for the enzymatic activity of 2-5AS and mutations in these motifs impair the enzymatic activity (Yamamoto *et al.*, 2000). It is likely, therefore, that NS5A interferes with 2-5AS functions by binding to the active sites.

Whether NS5A(1-148) or its equivalent(s) is actually generated in the cell would be an interesting issue to address. We observed that NS5A was cleaved to generate a cleavage product of 19 kDa in FL cells undergoing apoptosis and that the cleavage was inhibited by the caspase inhibitor Z-VAD (data not shown). An NS5A cleavage product of ~19 kDa in apoptotic cells was previously reported by Satoh *et al.* (2000), with the estimated cleavage site being residue 154. Goh *et al.* (2001) also reported NS5A cleavage by a caspase-like protease(s) that was activated by coexpressed HCV core protein. Collectively, these results suggest the possibility that NS5A(1-148) or its equivalent(s) is generated in the cell under certain conditions, which interferes with 2-5AS functions more strongly than does the full-length NS5A.

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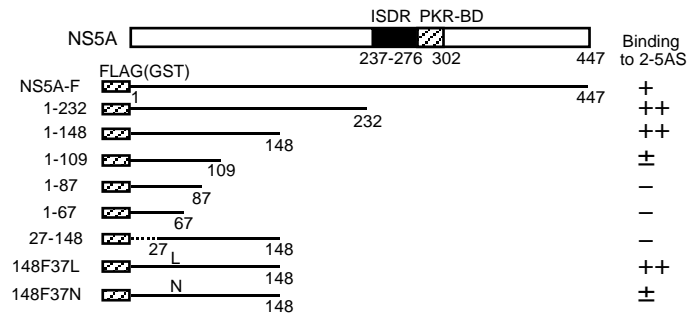
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Table 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence*
NS5A-1	5'-TATAGA <u>AATTC</u> TTCCGGATCCTGGCTAAAG-3'
NS5A-27	5'-TATAGGATCCCTCCTGCCGAAATTGCCG-3'
NS5A-109-R	5'-TATAGA <u>AATTC</u> CT ACGCCCTGGAATAGTT-3'
NS5A-148-R	5'-TATAGA <u>AATTC</u> CT ATTCGGGGGGCCGGGAC-3'
NS5A-F37L	5'-AATTGCCGGGAGTCCCTTTTcTgTCATGCCAACGCGGGTACAAGG-3'
NS5A-F37L-R	5'-CCTTGTACCCGCGTTGGCATGAcaaGAAAGGGACTCCCGGCAATT-3'
NS5A-F37N	5'-AATTGCCGGGAGTCCCTTTTcaacTCATGCCAACGCGGGTACAAGG-3'
NS5A-F37N-R	5'-CCTTGTACCCGCGTTGGCATGAgttGAAAGGGACTCCCGGCAATT-3'
NS5A-F37S	5'-AATTGCCGGGAGTCCCTTTTcccTCATGCCAACGCGGGTACAAGG-3'
NS5A-F37S-R	5'-CCTTGTACCCGCGTTGGCATGAgaGAAAGGGACTCCCGGCAATT-3'
NS5A-F37Y	5'-AATTGCCGGGAGTCCCTTTTctacTCATGCCAACGCGGGTACAAGG-3'
NS5A-F37Y-R	5'-CCTTGTACCCGCGTTGGCATGAgtGAAAGGGACTCCCGGCAATT-3'
2-5AS-1	5'-TATAGGATCCATGGAGCACGGACCCAGG-3'
2-5AS-52	5'-TATAGGATCCACCCAGTGAGGGTCTCC-3'
2-5AS-60-R	5'-TATAGGATCC CT TACACCACCTTGGAGAC-3'
2-5AS-104-R	5'-TATAGGATCC CT ACTTAATTTCTTGAT-3'
2-5AS-144-R	5'-TATAGGATCC CT ACTGATGCAGATGGGG-3'
2-5AS-184	5'-TATAGGATCCGATGGCGAGTTCTCTACC-3'
2-5AS-235-R	5'-TATAGGATCC CT ACTCTAGGGCGTACTG-3'
2-5AS-275-R	5'-TATAGGATCC CT ATGTCCAGTAGATTCTG-3'

* The enzyme recognition sites are underlined. Sequences complementary to a stop codon are shown in boldface letters and those for the site-directed mutagenesis in lowercase letters.

(A) NS5A



(B) 2-5AS

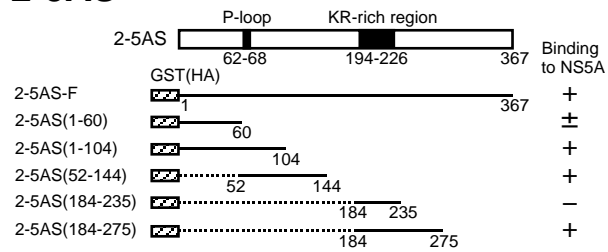


Fig. 1. Taguchi et al.

Fig. 1. Schematic representation of the full-length and various deletion mutants of NS5A and 2-5AS. (A) NS5A. ISDR spans from aa 237 to 276, and the PKR-binding domain (PKR-BD) from aa 237 to 302. The FLAG peptide or GST was fused at the N-terminus of NS5A. The numerals along the constructs indicate aa positions. Results of the binding experiments are shown on the right. (B) 2-5AS. Positions of the P-loop motif and KR-rich region (Yamamoto *et al.*, 2000) are shown. GST or the HA peptide was fused at the N-terminus of 2-5AS. Results of the binding experiments are shown on the right.

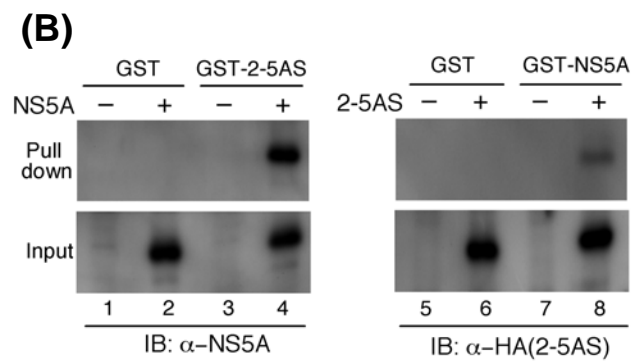
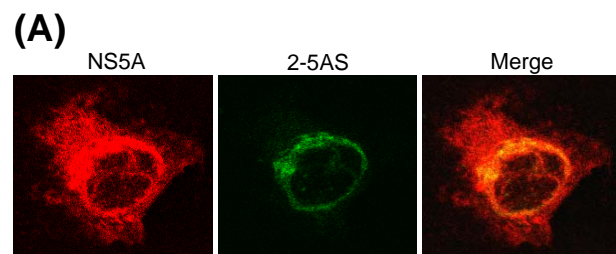


Fig. 2. Taguchi et al.

Fig. 2. NS5A-F and 2-5AS-F colocalise and physically interact with each other in HeLa cells. (A) Confocal laser scanning immunofluorescence microscopy analysis. Cells transiently expressing FLAG-tagged NS5A-F and HA-tagged 2-5AS-F were stained for NS5A-F (left panel) and 2-5AS-F (middle panel). Those two images were merged to show their colocalisation (right panel). (B) Physical interaction between NS5A-F and 2-5AS-F in the cells. Left upper panel: Lysates of cells expressing control GST (lanes 1 and 2) or GST-tagged 2-5AS-F (lanes 3 and 4) in the absence (lanes 1 and 3) or presence of NS5A-F (lanes 2 and 4) were pulled down and probed with anti-NS5A monoclonal antibody. Left lower panel: Comparable amounts of NS5A-F in the lysates were verified. Right upper panel: Lysates of cells expressing control GST (lanes 5 and 6) or GST-tagged NS5A-F (lanes 7 and 8) in the absence (lanes 5 and 7) or presence of HA-tagged 2-5AS-F (lanes 6 and 8) were pulled down and probed with anti-HA monoclonal antibody. Right lower panel: Comparable amounts of 2-5AS-F in the lysates were verified.

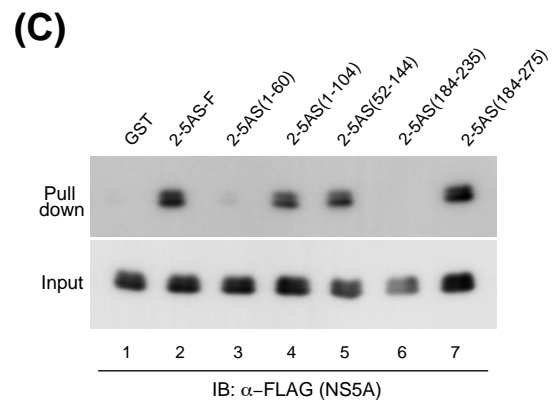
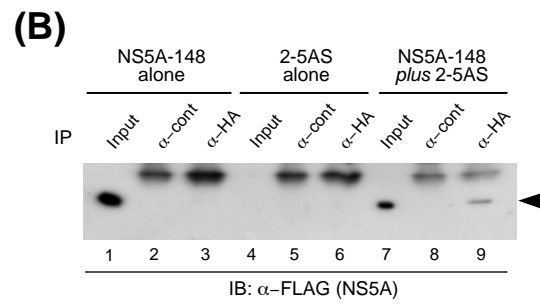
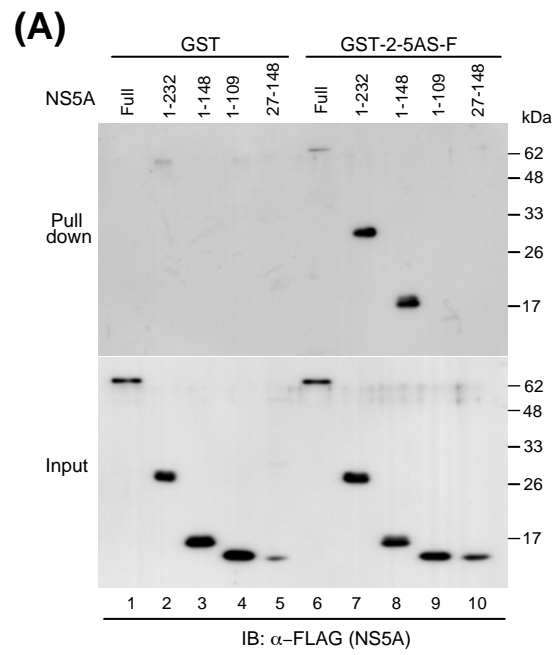


Fig. 3. Taguchi et al.

Fig. 3. An N-terminal region of NS5A (aa 1 to 148) and two separate regions of 2-5AS (aa 52 to 104, and aa 184 to 275) are involved in the interaction. (A) Upper panel: Lysates of HeLa cells expressing control GST (lanes 1 to 5) or GST-tagged 2-5AS-F (lanes 6 to 10) together with FLAG-tagged NS5A-F (lanes 1 and 6) and its deletion mutants (lanes 2 to 5 and 7 to 10) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: Comparable amounts of NS5A in the lysates were verified. (B) Lysates of cells expressing FLAG-tagged NS5A(1-148) alone (lanes 1 to 3), HA-tagged 2-5AS-F alone (lanes 4 to 6) or both (lanes 7 to 9) were either left untreated (input, 5% of total; lanes 1, 4 and 7) or immunoprecipitated with control rabbit IgG (lanes 2, 5 and 8) or anti-HA polyclonal rabbit antiserum (lanes 3, 6 and 9). The immunoprecipitates were probed with anti-FLAG monoclonal antibody. Arrowhead indicates NS5A(1-148). (C) Upper panel: Lysates of cells expressing control GST (lane 1), GST-tagged 2-5AS-F (lane 2) or its deletion mutants (lanes 3 to 7) together with FLAG-tagged NS5A(1-148) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: Comparable amounts of NS5A(1-148) in the lysates were verified.

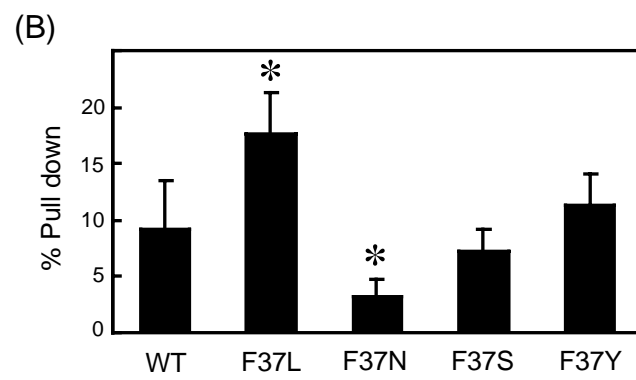
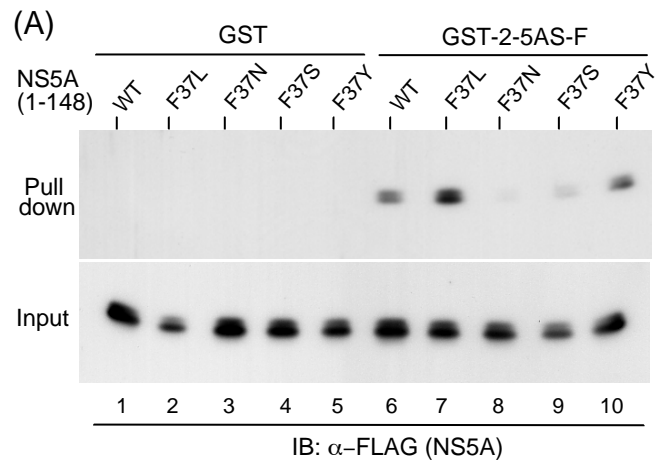


Fig. 4. Taguchi et al.

Fig. 4. Single-point mutations at residue 37 of NS5A influence the interaction with 2-5AS.

(A) Upper panel: Lysates of HeLa cells expressing control GST (lanes 1 to 5) or GST-tagged 2-5AS-F (lanes 6 to 10) together with FLAG-tagged wild-type (WT) NS5A(1-148) (lanes 1 and 6) and its single-point mutants (lanes 2 to 5 and 7 to 10) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: Comparable amounts of NS5A in the lysates were verified. (B) The intensities of the bands for NS5A that had been pulled down were measured and the ratios over total amounts of the input were calculated. Means \pm SD of 4 independent experiments are shown. *, $P < 0.05$, compared with WT.

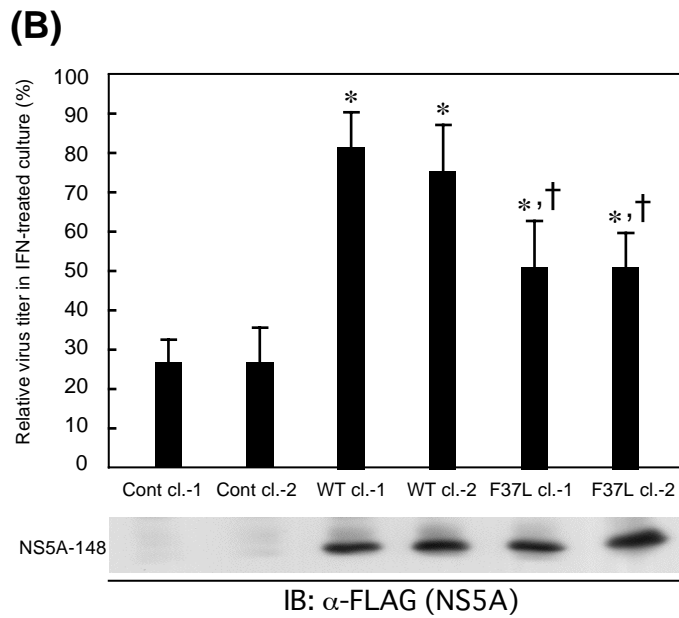
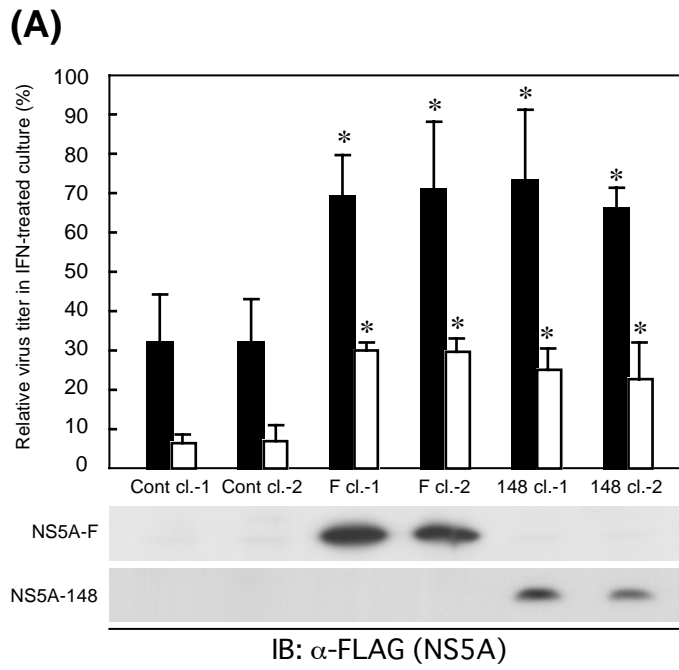


Fig. 5. Taguchi et al.

Fig. 5. NS5A-F and NS5A(1-148) inhibit antiviral activity of IFN in L929 mouse cells. (A) Two clones each of L929 cells stably expressing NS5A-F (F cl.-1 and -2) or NS5A(1-148) (148cl.-1 and -2) and non-expressing control (Cont cl.-1 and -2) were treated with IFN or left untreated for 24 h. The cells were then infected with EMCV (50 p.f.u.) and plaques formed on the monolayer cells were counted. Percent plaque numbers on IFN-treated cells as compared to the untreated controls were calculated. Filled and open columns represent the results obtained with the cells treated with 5 and 25 U of IFN per ml, respectively. Means \pm SD of 4 independent experiments are shown. *, $P < 0.01$, compared with the non-expressing control. Expression levels of NS5A are shown on the bottom. (B) Effect of F37N mutation on the IFN-inhibitory activity of NS5A(1-148). Two clones each of L929 cells stably expressing wild-type (WT) NS5A(1-148) or its single-point mutant NS5A(1-148)F37N and non-expressing control were treated with IFN (5 U/ml) or left untreated, which were then infected with EMCV (50 p.f.u.). Percent plaque numbers on IFN-treated cells as compared to the untreated controls were calculated. Means \pm SD of 4 independent experiments are shown. *, $P < 0.01$, compared with non-expressing control. †, $P < 0.01$, compared with WT. Expression levels of NS5A are shown on the bottom.

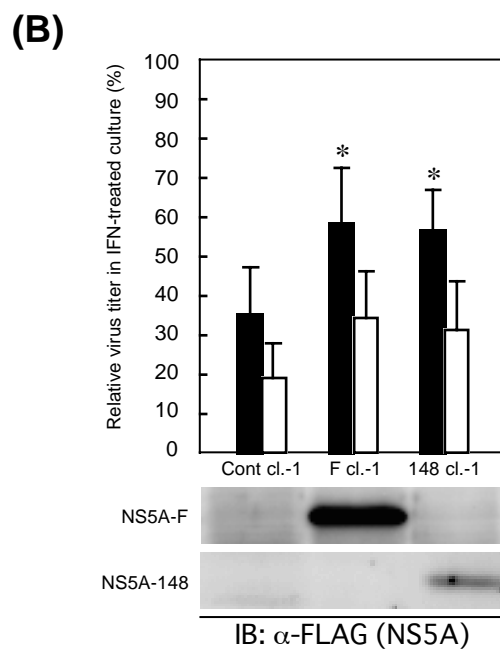
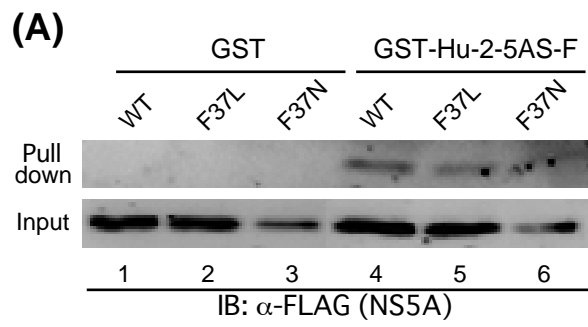
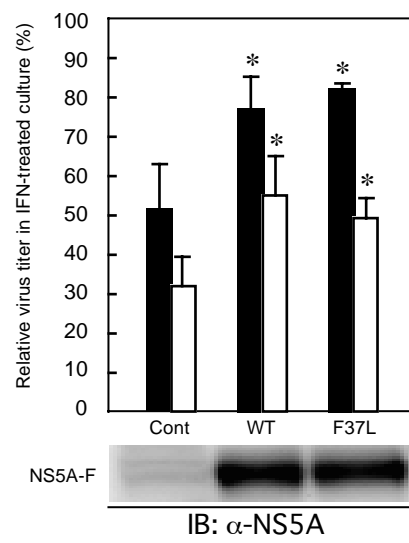


Fig. 6. Taguchi et al.

Fig. 6. NS5A physically interacts with human 2-5AS and inhibits antiviral activity of IFN in Huh-7 human hepatoma cells. (A) Upper panel: Lysates of cells transiently expressing control GST (lanes 1 to 3) or GST-tagged Hu-2-5AS-F (lanes 4 to 6), in the presence of FLAG-tagged wild-type (WT) NS5A(1-148) (lanes 1 and 4) and its single-point mutants (lanes 2, 3, 5 and 6) were pulled down and probed with anti-FLAG monoclonal antibody. Lower panel: Comparable amounts of NS5A in the lysates were verified. (B) Huh-7 cells stably expressing NS5A-F or NS5A(1-148) and the non-expressing control were treated with IFN or left untreated for 24 h. The cells were then infected with EMCV (50 p.f.u.) and plaques formed on the monolayer cells were counted. Percent plaque numbers on IFN-treated cells as compared to the untreated controls were calculated. Filled and open columns represent the results obtained with the cells treated with 5 and 25 U of IFN per ml, respectively. Means \pm SD of 3 independent experiments are shown. *, $P < 0.01$, compared with the non-expressing control. Expression levels of NS5A are shown on the bottom.

(A)



(B)

HCV replicon	No. of G418 ^r colonies
WT*	200~500
F37L	200~500
F37N	0†

Fig. 7. Taguchi et al.

Fig. 7. Analysis using HCV subgenomic RNA replicons. (A) Antiviral activity of IFN is counteracted by HCV replicons. Huh-7 cells harboring an HCV replicon (WT) or its F37L mutant and the parental control cells without HCV replicon (Cont) were treated with IFN or left untreated for 24 h. The cells were then infected with EMCV (50 p.f.u.) and plaques formed on the monolayer cells were counted. Percent plaque numbers on IFN-treated cells as compared to the untreated controls were calculated. Filled and open columns represent the results obtained with the cells treated with 5 and 25 U of IFN per ml, respectively. Means \pm SD of 4 independent experiments are shown. *, $P < 0.01$, compared with the control Huh-7 cells. Expression levels of NS5A are shown on the bottom. (B) F37N mutation of NS5A impairs replication competence of HCV replicon. *, An HCV subgenomic RNA replicon transcribed from pFK5B2884Gly (WT) and mutant HCV replicons possessing the F37L or F37N mutations were transfected into Huh-7 cells and G418-resistant colonies were obtained. †, A few colonies that had arisen, if any, had Phe (WT) at the 37th residue of NS5A.