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Suppression of Interferon-related Promoter Activation by Hepatitis C Virus Proteins Expressed in Cultured Cells

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ABSTRACT

Interferon is important for anti-viral defense of the host. The E2, NS3/4A, and NS5A proteins of hepatitis C virus (HCV) have recently been reported to confront anti-viral action induced by interferon. However, roles of the individual HCV proteins in anti-interferon action are still not well understood. We have isolated an HCV strain, HCV-K, from a patient with acute hepatitis. Nucleotide sequencing of the entire genomic DNA of HCV-K revealed that the isolate belongs to the genotype 1b, which is generally resistant to interferon therapy. In the present study, we expressed individual HCV-K proteins in mammalian cells and investigated effects of the proteins on interferon signal transduction. The results showed that the core, E1, NS4A, and NS4B proteins suppressed activation of interferon stimulation responsive element (ISRE) and gamma activation sequence (GAS) reporters. These results suggest that multiple HCV proteins have a function in suppression of the anti-viral effect by interferon and may indicate a novel role of E1 and NS4B proteins in interferon antagonism.

Key words: *Hepatitis C Virus, Interferon, ISRE, GAS*

It is estimated that hepatitis C virus (HCV) has infected at least 170 million people in the world. About 70% of patients with acute HCV infection progress to chronic HCV infection. Furthermore, 30–40% of them suffer from liver cirrhosis 20–30 years later, and some of them develop hepatocellular carcinoma. No vaccine effective for HCV has been developed so far. Currently, the only approved therapy for HCV is interferon (IFN)-α or pegylated-IFN-α (PEG-IFN-α) in monotherapy or in combination with rebavirin[13)]. However, its effectiveness is limited depending on the viral genotype and initial virus quantity[15,19)]. Although genotypes 2a and 2b are generally susceptible to IFN therapy, genotype 1b, which is prevalent in Japan, is highly resistant to the therapy. The reason for this is not clear.

IFN is a host key player to confront virus infection. Cells sense virus infection through Toll-like receptors or intracellular sensors, RIG-I or mda5, and further activate transcription factors IRF-3 and NF-κB, subsequently activating the IFN-β promoter. Induced IFN-β activates the interferon stimulation responsive element (ISRE) through the IFN-α/β receptor and the Jak-Stat pathway in an autocrine or paracrine fashion. The activation induces production of massive IFN-α through IRF-7 induction by the ISRE activation and results in successive amplified production of anti-viral effectors such as protein kinase R (PKR) and oligoadenyl synthase [reviewed in][4,5,10)].

HCV is a positive single-stranded RNA virus belonging to the family *Flaviviridae*. It contains ca. 9600 nucleotides and encodes an approximately 3000-amino-acid-long polyprotein, which is cleaved into at least 10 proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) through the activity of cellular peptidases as well as viral encoded proteases[2,12)].

Recently, HCV proteins have been shown to antagonize the IFN system. Aizaki et al[1)] demonstrated that expression of the HCV 1b non-struc-

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tural proteins NS3, NS4 and NS5, or NS5A alone in HepG2 cells rendered the cells sensitive to encephalomyocarditis virus (EMCV) infection after IFN-α treatment. The structural proteins core, E1 and E2, however, did not have such antagonizing activity, suggesting that the HCV non-structural proteins, NS4 and NS5, antagonized IFN-induced anti-viral action. On the other hand, Keskinen et al[11] observed that U-2 OS cells inducibly expressing the core protein, the NS4B protein or a polyprotein containing the core, E1 and E2 proteins was partially sensitive to vesicular stomatitis virus (VSV) infection after IFN-α treatment. Thus, the results obtained from the HCV 1b genotype are controversial.

In the present study, we obtained an HCV genomic cDNA of genotype 1b from a patient with acute hepatitis, and we transiently expressed nine out of the ten HCV proteins in HeLa cells and HuH-7 cells from cDNA and examined effects of the respective HCV proteins on IFN-related promoters systematically to investigate mechanisms of the resistance of HCV 1b to IFN therapy.

MATERIALS AND METHODS

Cells and viruses

Human hepatoma-derived HuH-7 cells and human cervical cancer-derived HeLa cells were propagated in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum. African green monkey kidney-derived CV1 cells were grown in MEM supplemented with 10% fetal calf serum.

Plasmid preparation

The HCV-K strain, 1b genotype, was isolated from a patient suffering from acute hepatitis in Hiroshima University Hospital. A full-length genomic cDNA clone of the HCV-K strain was constructed by using RT-PCR and connecting at restriction enzyme sites (DDBJ/EMBL/GenBank accession number AB249644). The DNA fragments encoding individual proteins were amplified by using the HCV-K genomic cDNA as a template with simultaneous addition of the start codon with a Kozak translation start consensus sequence, 5'-ACCATG-3', and a stop codon, 5'-TAG-3', at respective 5' and 3' ends.

For the cDNAs of NS2, NS3, NS4A, NS4B, NS5A, and NS5 proteins, an HA-tag, N'-YPYDVPDYA-C', was attached at the C terminus of each protein. The regions of E1 and E2 were extended toward the N terminus to involve a hydrophobic peptide functioning as a signal sequence. Amino acid numbers of amplified regions were as follows: 1–191 for C, 155–383 for E1, 340–746 for E2, 810–1026 for NS2, 1027–1657 for NS3, 1658–1711 for NS4A, 1712–1972 for NS4B, 1973–2419 for NS5A, and 2420–3010 for NS5B.

The amplified fragments were subcloned into the multicloning site of pKS336, a eukaryotic pol II expression vector possessing the human elongation factor promoter and a blastocidin-*S* acetyl transferase gene[20]. For a control, the cDNA of heat shock protein 70 (Hsp70) 1a with the C-terminal HA tag was also inserted into the vector.

Immunofluorescent staining

CV1 cells were transfected with expression vectors by using FuGENE6 (Roche Diagnostics) and fixed with 0.5% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min at 24 hr after transfection. The cells were then treated with 100 mM glycine in PBS and 0.1% Triton X-100 in PBS and incubated with either an anti-HA monoclonal antibody (262K, Cell Signaling Technology), an anti-core monoclonal antibody (10G5H4, a gift from T. Fujiwara), an anti-NS5A monoclonal antibody (B7, a gift from T. Fujiwara), an anti-E1 monoclonal antibody (ViroStat) or an anti-E2 goat polyclonal serum (Biodesign International). The cells were subsequently incubated with an Alexa 488-conjugated anti-mouse or anti-goat IgG antibody (Molecular Probes) and observed under a fluorescent microscope (TE2000-S, Nikon).

Immunoprecipitation and SDS-PAGE

Subconfluent CV1 cells were transfected with plasmids, metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 min at 24 hr post transfection, and solubilized with a RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Proteins were immunoprecipitated with a specific antibody and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 10% or 15% gel and then visualized using a BAS2000 Bio-imaging Analyzer (Fuji Film) as described previously[21].

Reporter assay

A reporter assay was performed basically as described by Gotoh et al[8]. HuH-7 and HeLa cells in a 24-well plate were transfected with 0.3 μg/well of a firefly luciferase ISRE reporter plasmid, pISRE-Luc (Clontech), 0.03 μg/well of a

ABBREVIATIONS

GAS, gamma activation sequence; IRF, interferon regulatory factor; ISRE, interferon-stimulated responsive element; Jak, Janus kinase; mda5, melanoma differentiation-associated gene 5; NF-κB, nuclear factor κB; RIG-I, retinoic acid-inducible gene I; SeV, Sendai virus; Stat, signal transducer and activator of transcription; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor inducing IFN.

Renilla luciferase-expressing plasmid under the control of thymidine kinase promoter, pRL-TK (Promega), and 0.3 μg/well of a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, cells were incubated with 1000 IU/ml human IFN-α (Mochida Pharmaceutical Co.) for 6 hr and then harvested for measuring luciferase activity. Firefly luciferase reporter activity in the cell extracts was normalized to Renilla luciferase activity or in some cases to protein contents. Fold induction of the ISRE promoter was calculated by dividing the relative luciferase activity of IFN-α-treated cells by that of mock-treated cells. Data represent the mean values of the normalized luciferase activities from triplicate samples. For measurement of GAS promoter activation, pGAS-Luc (Clontech) was used as a reporter plasmid and 5 ng/ml recombinant human IFN-γ (Strathmann Biotec) was used for stimulation.

For IFN-β promoter activation, 0.3 μg/well of pIFΔ (–125) lucter, which has a firefly luciferase gene under control of the human IFN-β promoter (provided by S. Goodbourn[18]), was introduced into cells in a 24-well plate together with 0.03 μg/well of pRL-TK and 0.3 μg/well of a plasmid expressing an HCV protein. After 24 hr, cells were treated with 100 μg/ml of poly I:C for 6 hr and harvested for luciferase assay.

RESULTS

Expression of HCV proteins derived from the HCV-K strain in mammalian cells

We initially examined the expression of HCV-K proteins in mammalian cells. The constructed expression plasmids were introduced into CV1 cells and stained with protein-specific or anti-HA tag antibody, followed by immunofluorescent staining (Fig. 1). The core, E1 and E2 proteins were found mainly in the peri-nuclear region, probably around the endoplasmic reticulum. The E1 and E2 proteins appeared to be delivered to the ER by the attached signal sequence. NS2, NS4B, NS5A, and NS5B were observed mainly in the cytosol with homogenous or patchy patterns. NS3 and NS4A appeared to reside mainly in the nucleus.

Transfected cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine, and proteins were immunoprecipitated with one of the E1-, E2-specific and anti-HA tag antibodies (Fig. 2). The results demonstrated that NS2, NS3, NS4A, NS4B, and NS5B appeared to migrate to the posi-

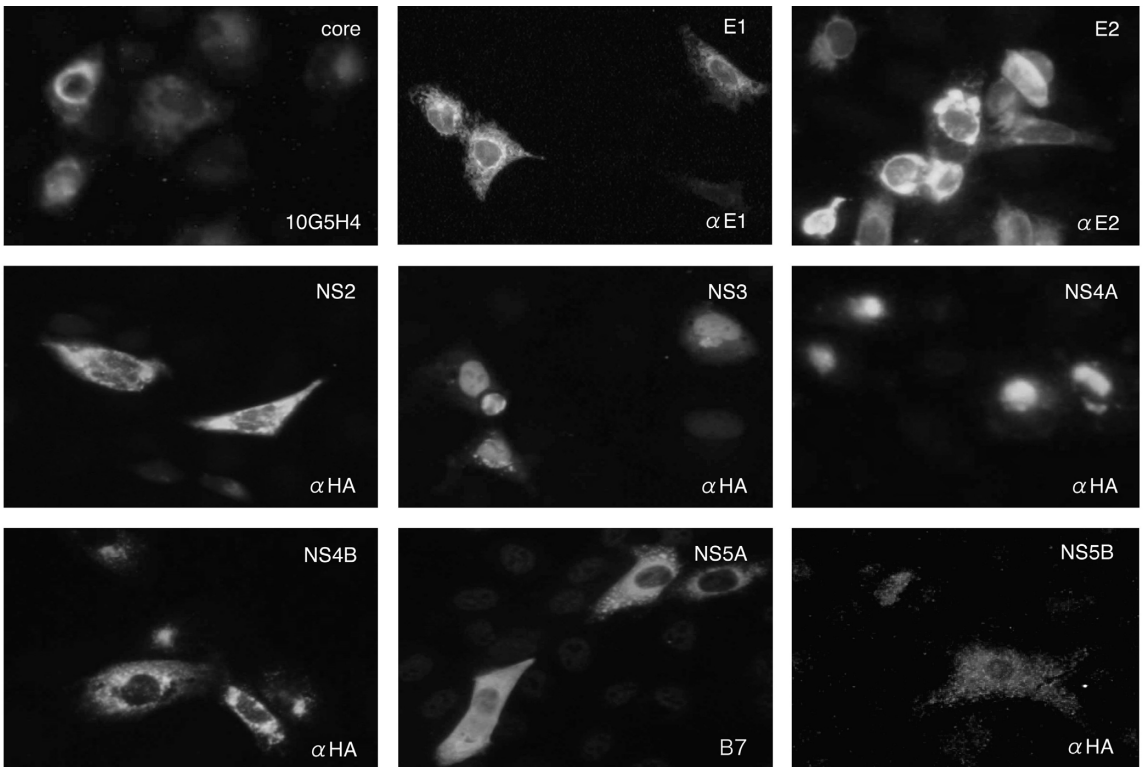

Fig. 1. Immunofluorescent staining of HCV-K proteins.
CV1 cells were transfected with an expression plasmid encoding either core, E1, E2, HA-tagged NS2, HA-tagged NS3, HA-tagged NS4A, HA-tagged NS4B, HA-tagged NS5A, or HA-tagged NS5B protein as indicated in the figure. After 24 hr, the cells were fixed and subjected to immunostaining with anti-HCV core monoclonal antibody (10G5H4), anti-HCV E1 monoclonal antibody (αE1), anti-HCV E2 goat polyclonal antibody (αE2), anti-HCV NS5A monoclonal antibody (B7), and anti-HA monoclonal antibody (αHA) together with an Alexa488-conjugated anti-mouse IgG or anti-goat IgG antibody. Primary antibodies used are also indicated in the figure.

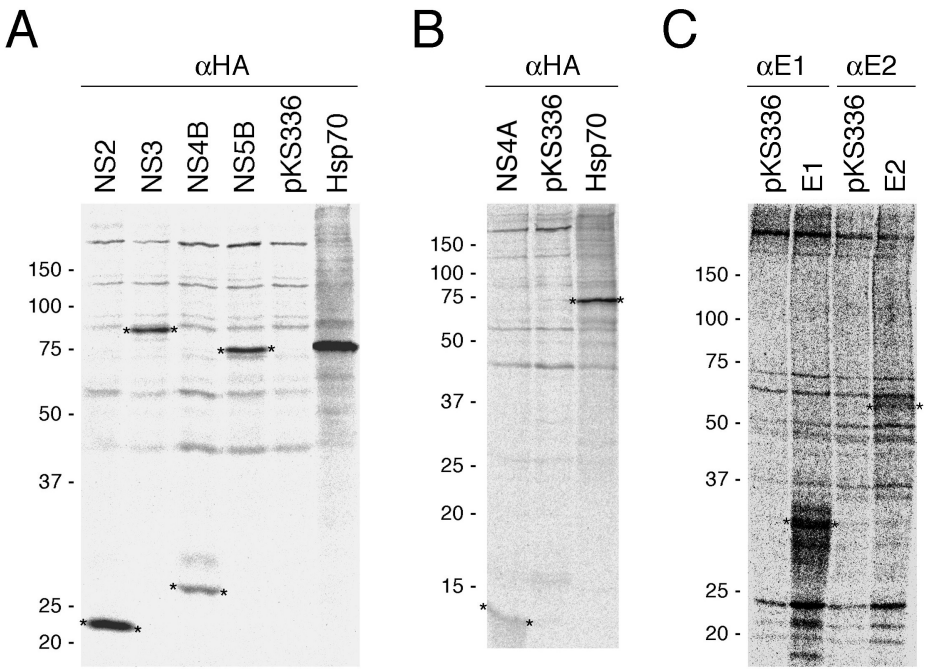

Fig. 2. Detection of HCV-K proteins in SDS-PAGE.
CV1 cells were transfected with plasmids expressing E1, E2, HA-tagged HCV proteins, NS2, NS3, NS4A, NS4B, HA-tagged Hsp70, and an empty vector (pKS336), and they were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min at 24 hr post transfection. The cells were then immunoprecipitated with an anti-HA antibody (αHA), an anti-E1 antibody (αE1) or an anti-E2 antibody (αE2) and analyzed by SDS-PAGE using 10% (A, C) and 15% (B) gels. Positions of molecular size markers are shown in the figure. Asterisks mark the migrating positions of individual expressed proteins.

tions of ca. 20, 80, 10, 30, and 70 kilodaltons, respectively (Figs. 2A, 2B). These are expected sizes from their respective amino acid numbers including an HA tag, 226, 640, 63, 270, and 600, and largely conformed to the reference[12]. The E1 and E2 proteins migrated to the positions of ca. 33 kilodaltons (in two bands) and 60 kilodaltons, respectively (Fig. 2C). These are consistent with the results of previous SDS-PAGE analysis of glycosylated E1 and E2[14,23]. The immunoprecipitated bands of core and NS5A were not clear because of overlapping of the bands with host proteins (data not shown). These results indicate that the HCV proteins, E1, E2, NS2, NS3, NS4A, NS4B, and NS5B, were expressed in mammalian cells and, as far as investigated, their size and antigenicity were authentic. Although the size of core and NS5A was not clear in electrophoresis, these proteins were thought to be expressed in cells based on the results of immunofluorescent experiment.

Effects of the HCV proteins on ISRE activation

We next examined ISRE promoter activation by IFN-α to investigate effects of the HCV proteins on IFN signaling. HuH-7 cells were transfected with pISRE-luc, pRL-TK, and one of the HCV protein expression plasmids, and levels of activation of ISRE-responsive reporter were measured (Fig. 3). SeV C protein, which is known to inhibit IFN

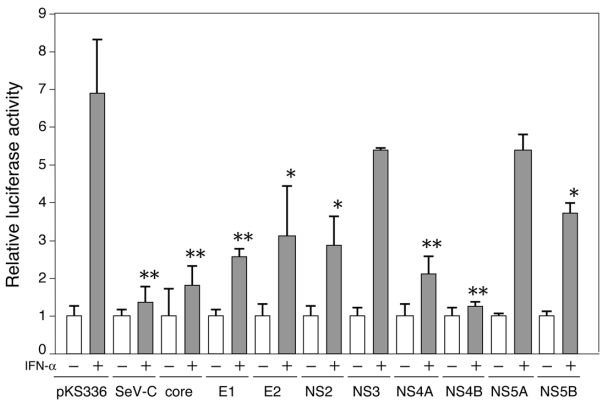

Fig. 3. Effects of HCV-K proteins on ISRE promoter activation by IFN-α.
HuH-7 cells were cotransfected with pISRE-Luc, pRL-TK, and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 1000 IU/ml IFN-α for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity was normalized to Renilla luciferase activity. Fold induction of ISRE promoter was calculated by dividing the relative luciferase activity of IFN-α-treated cells (closed bars) by that of mock-treated cells (open bars). Data represent the mean values of the fold inductions from triplicate samples. Asterisks indicate a significant difference from the IFN-α-treated control pKS336 sample (*$p < 0.05$, **$p < 0.01$, Fisher's PSLD test).

signal transduction[7,9], suppressed ISRE activation by IFN-α treatment. The core, E1, NS4A, and NS4B proteins significantly suppressed ISRE activation. The E2, NS2, and NS5B proteins moderately suppressed the activation, but NS3 and NS5A did not suppress the activation. Similar results were also obtained when HeLa cells were used (data not shown). We also investigated effects of the HCV proteins on IFN-γ signaling (Fig. 4). GAS activation by IFN-γ was also inhibited by all of the HCV proteins except NS3 and NS5A in HeLa cells. These results demonstrate that HCV proteins can disturb IFN-α and IFN-γ signal transduction.

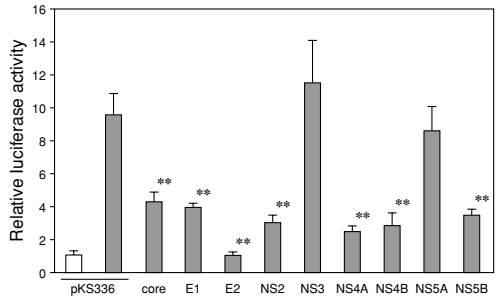

Fig. 4. Effects of HCV-K proteins on GAS promoter activation by IFN-γ.
HeLa cells were cotransfected with pGAS-Luc and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 5 ng/ml IFN-γ for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity in the cell extracts was normalized to protein contents. Data from IFN-γ-treated cells (closed bars) and mock-treated cells (open bars) are shown in the graph. Data represent the mean values of the normalized luciferase activities from 6 samples. Asterisks indicate a significant difference from the IFN-γ-treated control pKS336 samples (**$p < 0.01$, Fisher's PSLD test).

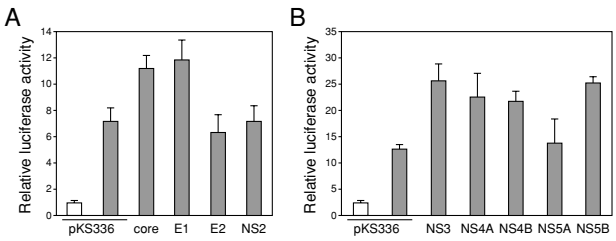

Fig. 5. Effects of HCV-K proteins on IFN-β promoter activation.
HeLa cells were cotransfected with pIFΔ (–125) lucter and a plasmid expressing either SeV-C protein, HCV-K core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A or NS5B protein. After 24 hr, the cells were incubated with 100 μg/ml poly I:C for 6 hr. The cells were then harvested and luciferase activity was measured. Firefly luciferase reporter activity in the cell extracts was normalized to protein contents. Data from poly I:C-treated cells (closed bars) and mock-treated cells (open bars) are shown in the graph. Data represent the mean values of the normalized luciferase activities from 6 samples.

Effects of the HCV proteins on activation of IFN-β promoter

IFN-β promoter activation by poly I:C, which is a trigger of massive IFN-α production, was also examined in the presence of the HCV proteins (Fig. 5). None of the nine proteins investigated significantly suppressed IFN-β promoter activation. Some of the proteins such as core, E1, NS3, NS4A, NS4B and NS5B rather appeared to activate the IFN-β promoter.

DISCUSSION

In the present study, we constructed plasmids expressing individual HCV proteins on the basis of the HCV-K strain, belonging to genotype 1b, which is generally resistant to IFN treatment. We performed reporter assays after introduction of the plasmids into HuH-7 cells or HeLa cells, and we investigated effects of the proteins on IFN-related promoters. There was no difference in the IFN-β promoter activation induced by poly I:C possibly through TLR-3 and TRIF. However, the core, E1, NS4A and NS4B proteins suppressed activation of both of the ISRE and GAS reporters. These results demonstrate that HCV proteins can disturb IFN-α and IFN-γ signal transduction and may indicate that the HCV proteins disturb the factors commonly employed both in the ISRE and GAS activation such as Stat1 and Jak1.

E2 and NS5A proteins have been reported to counter anti-viral activity induced by IFN[6,24]. However, we did not observed suppression of ISRE activation by either NS5A or E2 in a reporter assay. Accordingly, the IFN antagonistic activity of NS5A and E2 proteins does not appear to be attributed to suppression of ISRE activation but to direct inhibition of an IFN effector, PKR. Indeed, it has been reported that the NS5A interferon sensitivity-determining region (ISDR: amino acids 2209–2248), which correlates with sensitivity to IFN treatment[3], directly interacts with PKR and inhibits its anti-viral activity[6,22]. Deletion of the ISDR or mutations in the ISDR abolished binding of NS5A with PKR[6,17,22]. On the other hand, E2 has an amino acid sequence called PKR-eIF2α phosphorylation homology domain (PePHD), which is similar to the target sequences for autophosphorylation of PKR and phosphorylation of eIF2α by PKR, and E2 inhibits PKR function *in vitro*[24].

The present study suggests that some of the HCV 1b proteins have anti-IFN activity probably through suppression of ISRE activity. The fact

that the Jak-Stat pathway has been shown to be suppressed in cultured cells expressing the entire HCV genome [for example][16] suggests that the HCV proteins have anti-IFN activity. Aizaki et al[1] demonstrated that expression of a polypeptide containing NS3, NS4, and NS5 proteins and the NS5A protein of an HCV 1b genotype strain canceled resistance to EMCV infection after IFN treatment. They also demonstrated that a polyprotein containing core, E1, E2, NS2, and NS3 did not have such activity. Anti-viral activity was not observed, however, when VSV was used as a challenge virus instead of encephalomyocarditis virus. On the other hand, Keskinen et al[11] reported that cells expressing the core protein, the NS4B protein, and a polyprotein containing core, E1, and E2 were partially sensitive to VSV infection after IFN-α treatment but that cells expressing the NS3A-NS4A protein and the NS5A protein were not. Differences in IFN-antagonistic activities so far reported in individual HCV proteins may be due to differences in virus strains, cells, and experimental systems. Of the two reports described above, our results are closer to the results presented in the latter, although the results for NS4A and NS5A are different. Disturbance of IFN-related promoters by E1 or NS4B of the HCV-K strain in the present study suggests novel functions of E1 and NS4B against the IFN system.

For the next step of these experiments, stable transformants expressing the HCV-K proteins should be established to clarify effects of the proteins on Jak-Stat signal transduction. Furthermore, it may be interesting to compare anti-IFN activities of HCV proteins in IFN-sensitive and IFN-resistant strains. In order to clarify the anti-interferon activity of HCV proteins more precisely, the use of a full-length genome replicon system or the use of a virus infection system should be considered in the future.

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