ク）および高密度細胞培養システム (BelloCell) で培養を検討した。

３．キメラウイルスの精製

限外濾過、密度勾配遠心、ヘパリンクロマトグラフィーを組み合わせた方法で精製した。ウイルス量は定量的 RT-PCR およびコアタンパク質の EIA にて測定した。

４．キメラウイルス粒子の免疫

紫外線照射にて不活化した部分精製 J6/JFH-1（HCV core 70 pmol 相当）をフロイントの完全アジュバントに加え、エマルジョンを形成させ、腹腔内投与により免疫した。１週間おきに２回フロイントの不完全アジュバントにてエマルジョンを形成させ、同様に腹腔内へ追加免疫した。最初の免疫から 62 日目に採血し、血清を EIA による E2 タンパク質への反応性評価および HCV 中和価の測定に用いた。

５．感染阻害活性の測定

希釈した血清とウイルス液を混合し、１時間 37℃にて培養し、あらかじめ培養した Huh7 に加え、３時間培養した。血清/ウイルス液を除き、洗浄後、新鮮培地を加え４８時間培養した。その後、Isogen を加えて、total RNA の抽出した。total RNA を用いた定量的 RT-PCR により total RNA 1 _g あたりの HCV RNA の copy 数を測定した。

（倫理面への配慮）

本研究計画の実験計画は所属施設に提出されその承認を得ている。取り扱うすべての DNA および病原性微生物に関しては適切な封じ込めレベルの実験施設で取り扱われる。取り扱うすべての DNA に関して組み換え DNA 実験計画を提出し承認を得ている。ヒトの遺伝子解析を行う予定はない。

C. 研究結果

１．ＨＣＶ感染に係わる宿主因子の解析

従来の Huh7 細胞から限界希釈法により 70 クローンの亜細胞株を得た。HCV の感染性を比較すると親株よりも最大 10 倍程度まで感染性の良いクローンや感染性のより低い細胞株、さらに感染性の全くない細胞株が存在することが明らかとなった。次にルシフェラーゼ遺伝子を持つレプリコンによる HCV 遺伝子の複製能を検討すると、複製能も様々な程度の細胞株が存在したが、感染感受性と複製能の間に相関関係は無かった。そこで、HCV の感染性に関与することが報告されている細胞表面マーカーを解析した。CD81, SR-BI, LDLr について検討すると、CD81 の発現の有無が亜細胞株の感染感受性と相関していることが明らかとなった。つまり、CD81 の発現がある細胞株は感染感受性があり、CD81 の発現のない細胞株では感染性が見られなかった。さらにレプリコンによる解析により、CD81 を発現していない細胞は複製能が高かった。そこで、CD81 を発現せず、感染感受性の無い細胞株（Huh7-25）に CD81 発現プラスミドのトランスフェクションにより、CD81 を強制発現すると感染感受性が回復した。さらに CD81 を持続して発現する細胞株を樹立した（Huh7-25-CD81）。この Huh7-25-CD81 はこれまでに報告された Huh7.5.1 などの細胞よりも高い感染感

受性を示し、ウイルス培養に適した細胞株であることが明らかとなった。

２．構造タンパク質が JFH-1 以外の株由来であるキメラウイルスの作製。

遺伝子型１ｂの HCV 株である J1 株、Con1 株、TH 株および遺伝子型 2a の HCV 株である J6CF 株の構造領域遺伝子を JFH-1 株の全長遺伝子に組み換えて、キメラウイルス遺伝子を作製した。全長 RNA を合成して、Huh7-25-CD81 細胞にトランスフェクションして、ウイルス産生を検討した。これらのキメラウイルスの中で、TH/JFH-1 および J6/JFH-12 種類のキメラウイルスは持続的にウイルスが産生され、Huh7 細胞に感染性を示した。

２．キメラウイルス感染細胞の培養

ウイルスを大量に取得するために、３種類の培養系を検討した。まず、Huh7-25-CD81 をマイクロキャリア上に培養する条件を検討した。しかし、至適条件下で Huh7-25-CD81 をマイクロキャリア上で培養し、ＨＣＶを感染させると、細胞増殖が停止して培養液中のウイルス産生量があまり増加しないことが明らかとなった。また、高密度細胞培養システム（BelloCell）にて行ったが、HCV に感染した細胞の増殖は悪く、ウイルス生産量は上がらなかった。これらに対して、単純な平面培養系である多段式培養器（セルスタック）が最もウイルスが得られた。

３．キメラウイルスの精製

J6/JFH1 を限外濾過膜、密度勾配遠心、ヘパリンクロマトグラフィーおよび限外濾過膜による濃縮、バッファー置換の工程にて精製を行った。

４．キメラウイルス粒子免疫マウス血清の HCV 中和価

E2 タンパク質を固相化した EIA にて、正常マウス血清と比較して、J6/JFH-1 粒子で免疫したて得られたマウスの血清は、900 倍希釈で有意に E2 タンパク質に反応した。さらに、HCV 感染系にて評価したところ、免疫血清には 20 倍希釈で 85%の J6/JFH-1-HCV の感染を阻害する活性が認められた。さらに本血清は、20 倍希釈で 65%の TH/JFH-1 の感染を阻害する活性が認められた。この結果は、遺伝子型 2a の HCV を免疫して誘導される抗体中には遺伝子型１ｂの HCV の感染を阻害する抗体が存在することを示している。

D. 考察

この問題を明らかにするために、まず、HCV 感染感受性およびレプリコン複製能の高い細胞の作製を試みた。Huh7 細胞をクローニングすることにより、親細胞株中には感染感受性の異なる亜細胞株が混在していることが明らかとなった。感染感受性を決定する重要な宿主因子は細胞表面の CD81 1 分子であった。また、ウイルス複製能は逆に CD81 の発現の無い亜細胞株で高いものが見られた。本研究で得られた CD81 を強制発現して得られた Huh7-25-CD81 細胞は感染感受性が高く、ウイルス培養に適していると考えられた。最近では肝細胞のタイトジャンクションに発現する Claudin-1 という分子が HCV 感染に重要であることが報告されたことからも、HCV の感染感受性にはさらに多くの宿主因子が関与

していると考えられる。

ウイルス粒子を大量に取得するために、より効率の良い感染細胞培養法を開発する必要がある。種々の方法で感染細胞培養を試みた。感染前と感染後で培養の至適条件が異なることが示唆された。従って、今後は培養装置を含めて感染細胞の至適培養条件を探索することが必要であると考えられた。

特定の HCV 株で免疫して得られた抗体が、異なる株の HCV の感染を阻害するのかを明らかにすることは、HCV ワクチン開発において重要である。遺伝子型 2a の構造タンパク質をもつ J6/JFH-1 を大量調製し、部分精製した本粒子をマウスに免疫して得られた血清が、J6/JFH1 および遺伝子型 1b の構造タンパク質をもつ TH/JFH-1 の感染を阻害することを明らかにした。この結果は、特定の HCV 株を抗原とした場合でも汎中和抗体を誘導することができることを示しておりワクチン開発における重要な知見の一つとなると考えられる。今後、本結果をモノクローナル抗体レベルで解析することを検討している。さらに、本研究で得られたツールは、宿主免疫からの逃避機構における B 細胞エピトープの変異を解析する上でも重要と考えられる。

E. 結論

1. ＨＣＶ感染感受性の高い細胞株（Huh7-25-CD81）を樹立した。本細胞株は JFH-1 レプリコン系において、Huh7.5.1 と同等以上の HCV 産生能を持つ。
2. ウイルス粒子を大量に取得するための感染細胞培養法を検討した。特に持続感染系においては、多段培養装置（セルスタック）が好適であることが判明した。
3. JFH-1 以外の HCV 株の構造タンパク質領域の遺伝子を JFH-1 株の全長遺伝子のその領域と置き換えてキメラウイルス遺伝子を作製することにより、遺伝子型の異なる構造タンパク質を持つキメラウイルスを作製する技術を確立した。
4. 遺伝子型 2a の構造タンパク質をもつ J6/JFH-1 キメラウイルス粒子を抗原として免疫したマウス血清中に、抗原に使用したウイルスだけでなく遺伝子型 1b の構造タンパク質をもつ TH/JFH-1 キメラウイルスの感染を中和する抗体が認められた。

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Ⅲ．研究成果の刊行に関する一覧表

III. 研究成果の刊行に関する一覧表

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石井孝司他	E型肝炎	食品由来感染症と食品微生物			印刷中
石井孝司他	E型肝炎	感染・炎症・免疫	37	58-9	2007
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Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response

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Viral infections activate cellular expression of type I interferons (IFNs). These responses are partly triggered by RIG-I and mediated by Cardif, TBK1, IKKε and IRF-3. This study analysed the mechanisms of dsRNA-induced IFN responses in various cell lines that supported subgenomic hepatitis C virus (HCV) replication. Transfection of dsRNA into Huh7, HeLa and HEK293 cells induced an IFN expression response as shown by IRF-3 dimerization, whilst these responses were abolished in corresponding cell lines that expressed HCV replicons. Similarly, RIG-I-dependent activation of the IFN-stimulated response element (ISRE) was significantly suppressed by cells expressing the HCV replicon and restored in replicon-eliminated cells. Overexpression analyses of individual HCV non-structural proteins revealed that NS4B, as well as NS34A, significantly inhibited RIG-I-triggered ISRE activation. Taken together, HCV replication and protein expression substantially blocked the dsRNA-triggered, RIG-I-mediated IFN expression response and this blockade was partly mediated by HCV NS4B, as well as NS34A. These mechanisms may contribute to the clinical persistence of HCV infection and could constitute a novel antiviral therapeutic target.

INTRODUCTION

Type I interferon (IFN) plays a central role in eliminating virus, not only following clinical therapeutic application but also as a cellular immune response (Samuel, 2001; Taniguchi & Takaoka, 2002). Hepatitis C virus (HCV) infection is characterized by persistence and replication of the virus in the liver, despite an intact host immune system (Alter, 1997). Indeed, even after administration of the currently most potent IFN reagents, as many as half of the patients are refractory to the treatment and fail to eradicate the virus (Fried *et al.*, 2002). These features have led to speculation that HCV escapes from or attenuates the host antiviral response (Katze *et al.*, 2002).

Cellular antiviral responses are primarily mediated by IFN and IFN-stimulated genes (ISGs), including 2,5-oligoadenylate synthetase, dsRNA-dependent protein kinase R (PKR) and MxA proteins, as well as by as yet uncharacterized genes (Itsui *et al.*, 2006; Stark *et al.*, 1998). A study of experimental chimpanzee HCV infection has shown that various cytokines and chemokines are induced in the liver during the course of acute HCV infection and its clearance, and that a considerable proportion of the genes is induced by type I IFN (Bigger *et al.*, 2001).

Control of expression of ISGs is mediated by binding of type I IFNs to their receptors. Following receptor binding, STAT1 and STAT2 are phosphorylated to form ISGF-3, which translocates to the nucleus and binds the IFN-stimulated response element (ISRE), located in the promoter/enhancer region of ISGs, and activates transcription of ISGs (Samuel,

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2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-*β* gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-*β* induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-*β* (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFN*β*-Fluc was constructed by cloning the human IFN-*β* promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum at 37 °C with 5 % CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7, HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1 × 10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1 × 10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12 % Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I : C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I : C), whilst overexpression of full-length RIG-I increased poly(I : C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I : C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

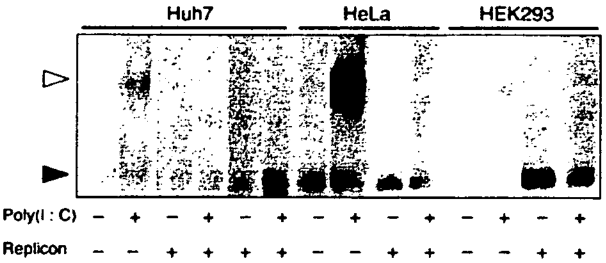

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I : C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

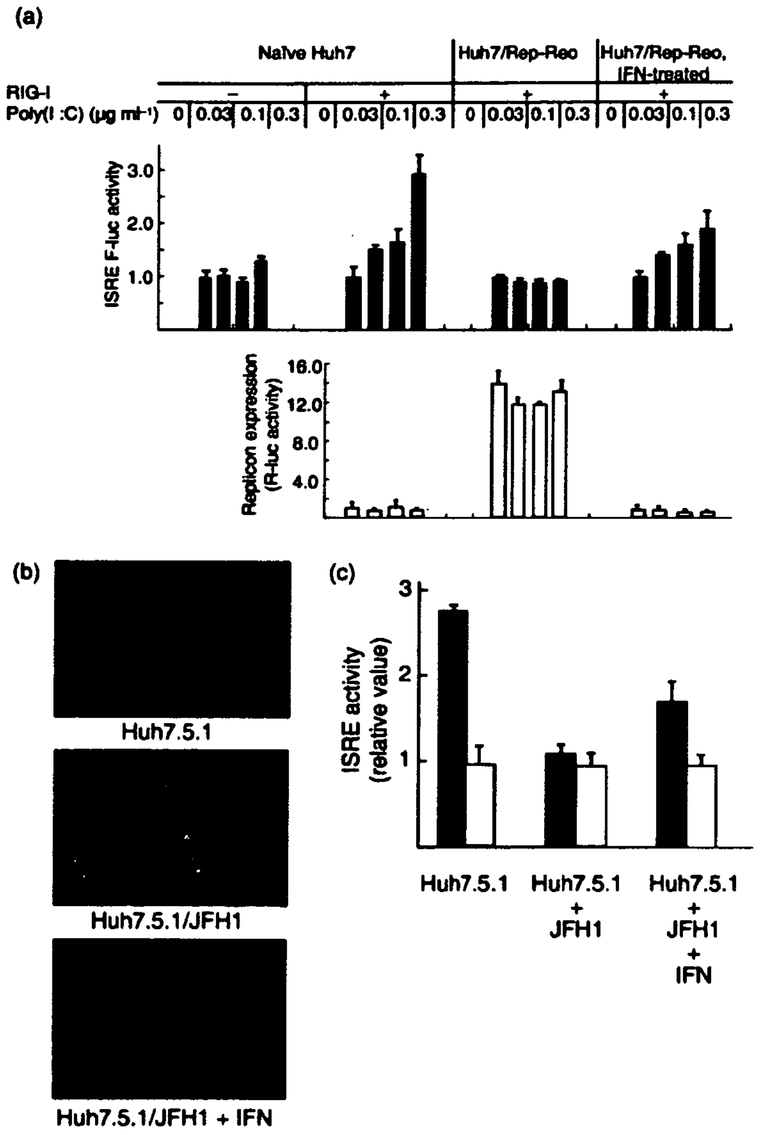

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I : C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I : C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

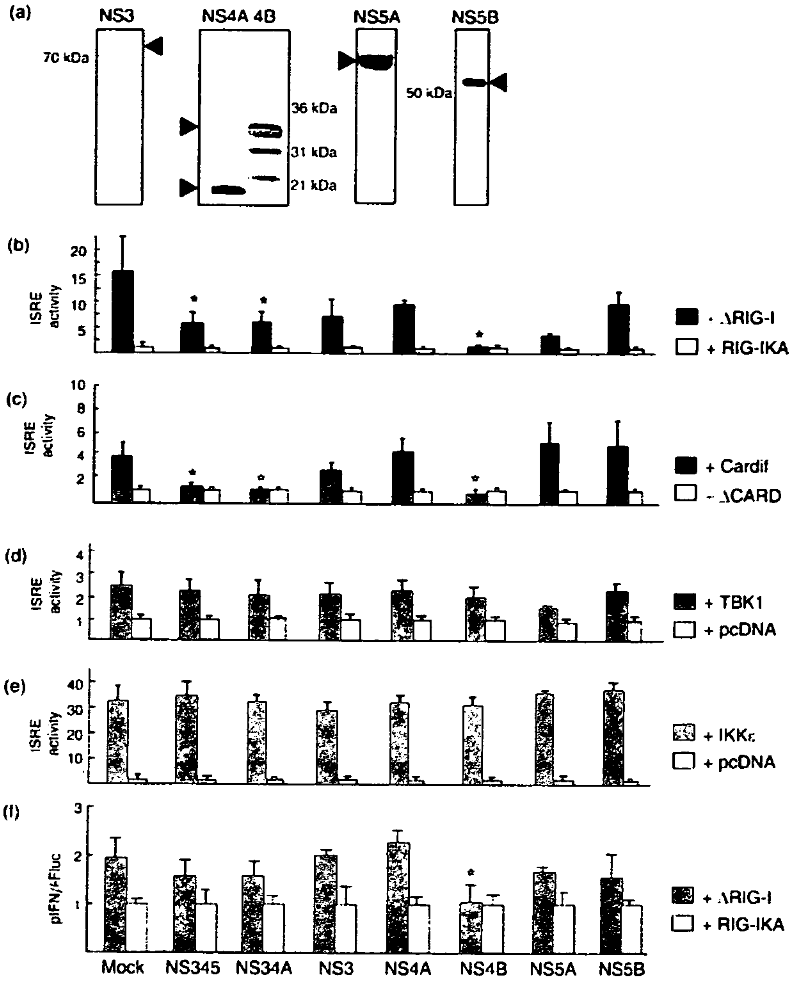

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

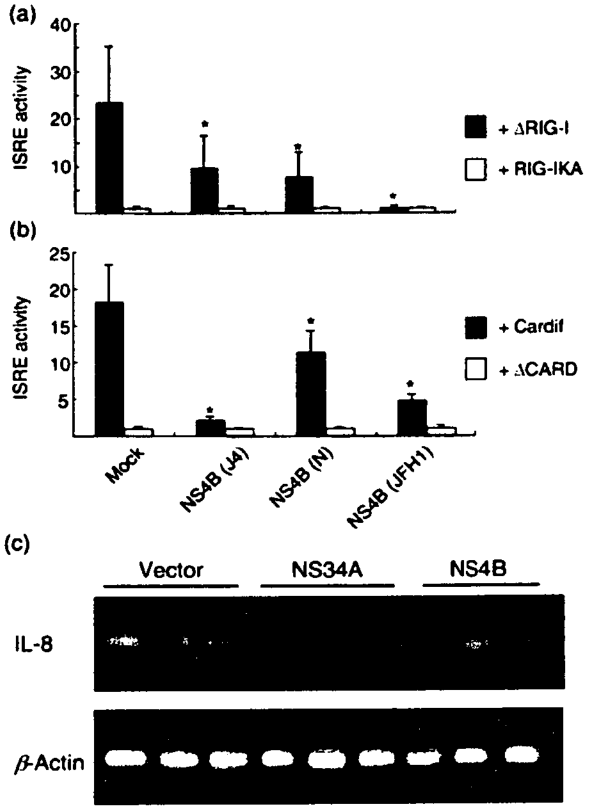

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

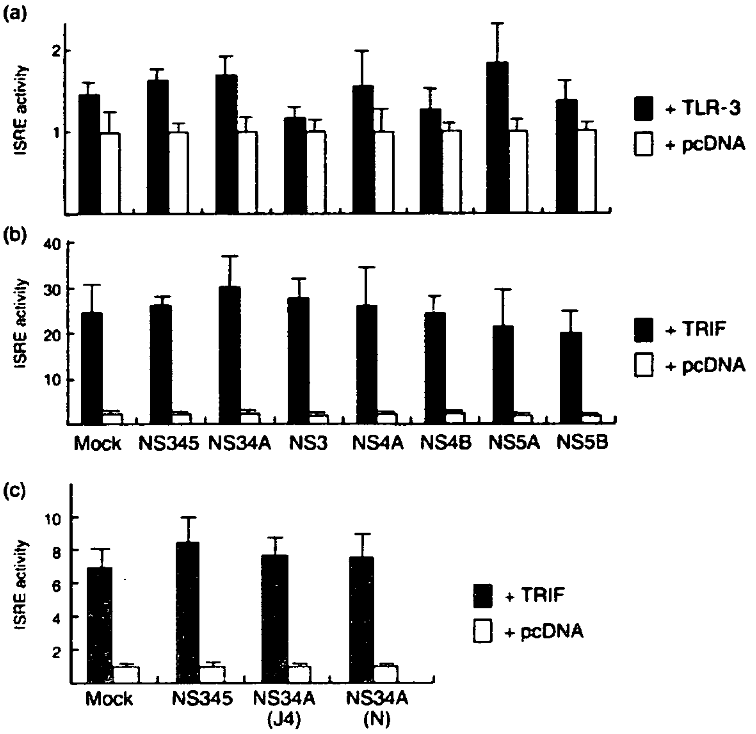

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pISRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

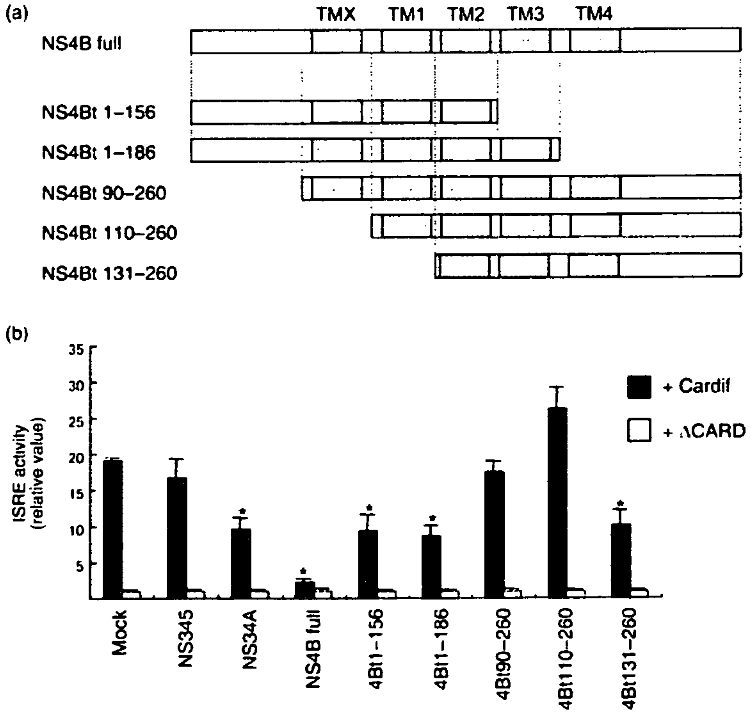

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means ± SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

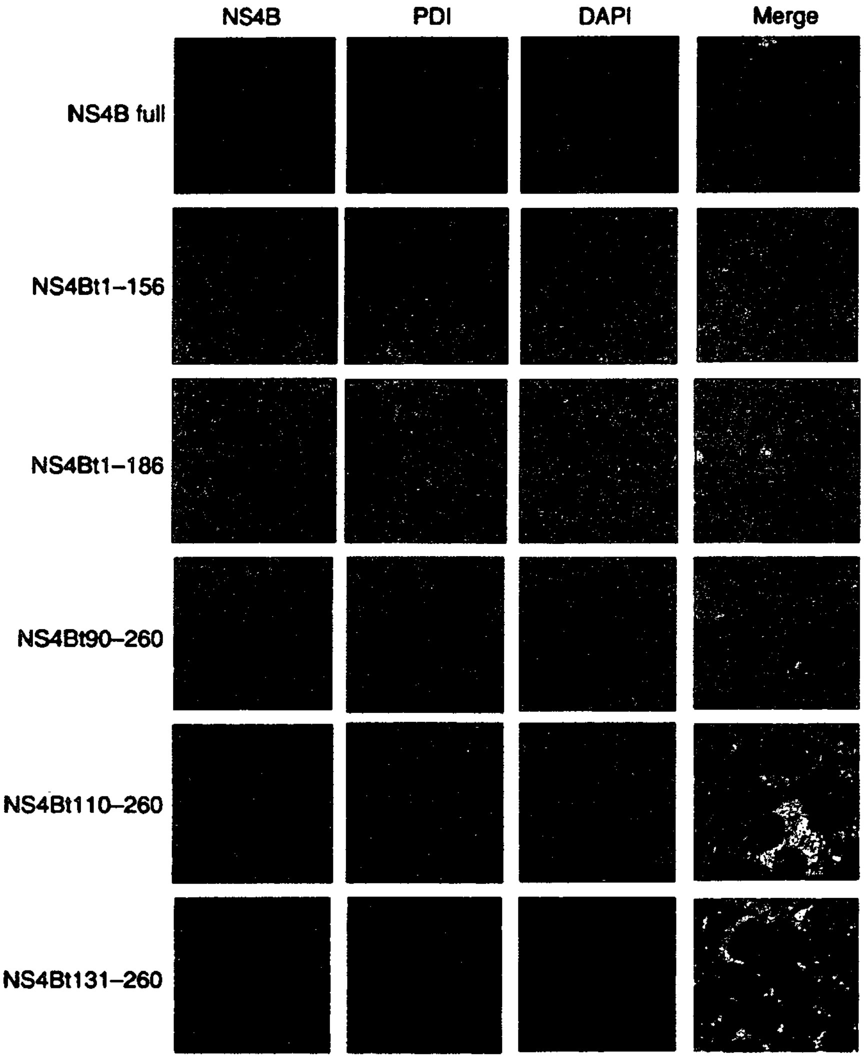

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).

NS4B has been reported to induce an unfolded protein response or ER stress (Zheng *et al.*, 2005). Accumulation of unfolded or misfolded proteins in the ER is detected by three ER sensor proteins, ATF6, IRE-1 and PKR-like ER kinase (PERK), and triggers the unfolded protein response as a stress response and induces expression of molecular chaperon proteins, global shut-off of protein translation and apoptotic cell death (Mori, 2000). Therefore, it may be possible that transgenic overexpression of NS4B induces ER stress and suppresses overall protein synthesis, including that of IFNs. In our experiments, however, NS4B suppressed the ISRE-mediated IFN gene activation but did not suppress non-specific protein expression, as demonstrated by *Renilla* luciferase activity in the control plasmid driven by the herpes simplex thymidine kinase promoter. In addition, the growth and viability of cells that overexpressed NS4B did not differ from untransfected cells or from those transfected with the other HCV proteins. IL-8 overproduction induced by ER stress was not observed in our NS4B-overexpressing cells (Fig. 4c). These findings indicated that the inhibitory effect of NS4B is specific to the IFN induction pathway and is not a non-specific effect through ER stress.

In conclusion, we have shown that dsRNA-induced IFN expression was suppressed by NS4B. These virus–host interactions probably contribute to HCV persistence and to the pathogenesis of HCV-associated liver disease.

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