

	1	10	20	30	40	50	60	70
NN	SMSYSWTGALITPCAAEESKLPINPLSNLLRHNNMVIATTSRSAGLRQKKVTFDRLQVLDDBHYRDVLKE							
Con1	---T-----T---A-----L-----S-----							
O	---T-----A-----SQ-----							
JFH1	---SP--E-----Y--K--C--K--SQ-A-----T---A--DS---D							
	80	90	100	110	120	130	140	
NN	MKAKASTVKAKLLSIEEACKLTPPHSAKSKYGYGAKDVRNLSRAVNHIRSVWEDLLETETPIDTTIMA							
Con1	-----V-----R--F-----K-----K-----							
O	-----V-----R--F-----K-----H--K-----V--							
JFH1	I-LA--K-S-R--TL---Q-----R---F--E--S--G-----K--K---PQ--P---							
	150	160	170	180	190	200	210	
NN	KSEVFCVQPEKGGRRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQYSPKQRFVFLVNTWKS							
Con1	-N-----G-----A--A							
O	-N-----G-----A--A							
JFH1	-N---D-A--K-----Y-----ITQK-----A-----A---Y-LKA-AE							
	220	230	240	250	260	270	280	
NN	KKCPMGFSYDTRCFDSTVTENDIRVEESIYQCCDLAPEAKLAIKSLTERLYVGGPLTNSKGQNCGYRRCR							
Con1	---A-----RQ--R-----I-----							
O	---T---A-----RQ--R-----I-----							
JFH1	--D-----R--T---A-S-PE--RT--H-----MF-----T-----							
	290	300	310	320	330	340	350	
NN	ASGVLTTSCGNTLTCLKASAAKRAKLDCTMLVNGDDLTVVICESAGTQEDAAASLRVFTTEAMTRYSAPP							
Con1	-----A-----C-----E--A-----							
O	-----C-----							
JFH1	-----M--I--V--L--K--GIVAP---C-----S--Q--E--ERN--A-----							
	360	370	380	390	400	410	420	
NN	GDPPQPEYDLELITSCSSNVSVAHDSAGKRVYYLTRDPTTPLARAAWETARHTPVNSWLGNIIMYAPTLW							
Con1	---K-----							
O	-----							
JFH1	---R-----LGPR-R-R-----V--S-I-----Q---I--							
	430	440	450	460	470	480	490	
NN	ARMILMTTHFFSILLAEQLEKALDCQIYGACYSIEPLDLPQIIERLHGLSAFSLHSYSPGEINRVASCLR							
Con1	-----Q-----							
O	-----T-----Q-----							
JFH1	V--V-----MV-DT-DQN-NFEM--SV--VN---A-----D--M-T--HH-LT---A--							
	500	510	520	530	540	550	560	
NN	KLGVPPLRVWRHRARSVRAKLLSQGGRANTCGKYLFWAVKTKLKLTPIPAASRLDLGWFVAGYSGGDI							
Con1	-----R-----R-----Q--S-----							
O	-----R-----							
JFH1	--A-----KS--A--S-I-R--K--V--R-----L-E-RL---S--TV-AG---							
	570	580	590	591				
NN	YHLSRARPRWFMWCLLLSVGVGIYLLPNR							
Con1	-----							
O	-----							
JFH1	F--V-----SLLFG---F---LF---A-							

FIG. 8. Amino acid sequence alignment of NS5B encoded by HCV strains NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid numbers. Conserved residues are shown by dashes. The region spanning 521 to 591 aa, which is involved in the interaction with CyPB, is boxed.

recombinant GST-CyPB. The addition of recombinant GST-CyPB increased the binding of genotype 1b NS5B to poly(U) RNA (Fig. 7B and C). However, this augmentation of RNA binding was not observed with NS5B from the JFH1 strain (Fig. 7B and C). From the above results, it is suggested that the RNA binding of JFH1 NS5B is free from regulation by CyPB.

DISCUSSION

Until now, we and another group have utilized subgenomic replicons carrying genotype 1b NN and HCV-N strains to

demonstrate that CsA suppresses HCV genome replication (22, 29). This study reveals that CsA is effective on full-genome replicons to almost the same extent. In addition, other available genotype 1b replicons carrying the Con1 and O strains also have a high sensitivity to CsA, consistent with our proposal that HCV genotype 1b is highly sensitive to CsA. However, a fulminant-type genotype 2a replicon, JFH1, was less responsive to CsA, although a high dose of CsA suppressed the replication of this strain.

CyPB interacts with genotype 1b NS5B to stimulate its RNA

binding activity. In contrast, CyPB binds JFH1 NS5B but does not regulate the function of JFH1 NS5B. This is consistent with a previous speculation that genotype 1b and JFH1 replicons utilize the same cellular factors in distinct manners (21). The NS5B sequence of NN strain has 95.0, 95.9, and 70.4% homology to that of Con1, O, and JFH1, respectively (Fig. 8). The region spanning amino acids (aa) 521 to 591 of NS5B, which is involved in the interaction with CyPB (31), is highly conserved among genotype 1b strains NN, Con1, and O while that of JFH1 has 21 substituted residues in this region. The proline at 540 aa, which is important for CyPB binding (31), is conserved but the adjacent residues such as isoleucine at 539 aa and alanine at 541 aa are replaced by leucine and glutamic acid, respectively, in JFH1. Through molecular interactions, CyPB seems to make the conformation of NS5B of genotype 1b strains but not JFH1 suitable for RNA binding (31). The diverse regulation system of NS5B by CyPB among strains may be due to differences in either the sequence or the entire conformation of NS5B. Further study is important for elucidating the regulation mechanism of RNA binding activity of NS5B by CyPB.

Thus, replication in JFH1 replicon is independent of CyPB. Interestingly, human immunodeficiency virus type 1 (HIV-1) strains also have a diversity of CyP dependence on viral proliferation (3, 33). CyPA plays an important role in the life cycle of HIV-1. The interaction of the HIV-1 capsid protein with CyPA that resides within the target cells of infection is critical for HIV-1 replication (7, 24). In peripheral blood mononuclear cells or Jurkat T cells, CsA suppresses the proliferation of HIV-1 group main (M) strain (3). However, certain strains of group outlier (O), such as MVP5180 and MVP9435, are resistant to CsA (3, 33), suggesting the different dependency of the replication on CyPA. Authors have suggested that MVP5180 and MVP9435 clones adapt to replicate independently of CyPA and that this adaptation provides a significant replication advantage for the virus *in vivo* (3). In vesicular stomatitis virus (VSV) strains, a role for CyPA in virus replication also has been reported (2). CyPA is required for the infection of the VSV-NJ strain but not the VSV-IND strain. These authors proposed that during evolutionary divergence from the ancestral lineages that initially were dependent on CyPA for replication, VSV-IND may have adapted to reduce its dependency on CyPA (2). In the case of HCV, a fulminant type genotype 2a replicon (JFH1) replicates independently of CyPB. It has previously been reported that JFH1 has a much higher competency of replication in the cells than other strains (13). The adaptation to independence from CyPB may contribute to the high capacity of replication of JFH1.

Although the JFH1 replicon is less sensitive to CsA, high concentrations of CsA still suppress replication of the JFH1 replicon. Moreover, the introduction of the siRNA designed to recognize several CyP subtypes [si-CyP(broad)] moderately diminishes HCV RNA in the JFH1 replicon. We suspect that a CyP family member other than CyPB is involved in HCV genome replication. Further analysis is needed on the role of other CyP subtypes.

As there a replicon system for a fulminant-type genotype 1b replicon or chronic-type genotype 2a replicon does not yet exist, we cannot conclude whether chronic-type genotype 2a replicons or fulminant-type replicons are less sensitive to CsA

or not. However, there is a clinical report describing cotreatment of patients with chronic hepatitis C with IFN and CsA that resulted in a higher sustained virological rate than with treatment of IFN alone (11). In this report, increase in the sustained virological rate was prominent with patients carrying genotype 1 HCV (51.7% versus 21.9%), while it was relatively weak in patients carrying genotype 2 HCV (66.7% versus 58.3%) (11). Thus, genotype may affect the sensitivity of HCV replication to CsA. However, we cannot exclude the possibility that the diminished sensitivity to CsA is a characteristic only of the fulminant-type genotype 2a strain.

Our results suggest that sensitivity to CsA and replication dependency to CyPB is different among HCV strains. This finding is an important insight into the diversity of the mechanism of HCV genome replication and its sensitivity to antiviral agents.

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NS3 protein of *Hepatitis C virus* associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner

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The N-terminal 198 residues of NS3 (NS3-N) of *Hepatitis C virus* (HCV) subtype 1b obtained from 29 patients, as well as full-length NS3 (NS3-Full), were analysed for their subcellular localization, interaction with the tumour suppressor p53 and serine protease activity in the presence and absence of the viral cofactor NS4A. Based on the subcellular-localization patterns in the absence of NS4A, NS3-N sequences were classified into three groups, with each group exhibiting either dot-like, diffuse or a mixed type of localization. Chimeric NS3-Full sequences, each consisting of an individual NS3-N and a shared C-terminal sequence, showed the same localization patterns as those of the respective NS3-N. Site-directed mutagenesis experiments revealed that a single or a few amino acid substitutions at a particular position(s) of NS3-N altered the localization pattern. Interestingly, NS3 of the dot-like type, either NS3-N or NS3-Full, interacted with p53 more strongly than that of the diffuse type, in both the presence and the absence of NS4A. Moreover, NS3-N of the dot-like type suppressed *trans*-activating activity of p53 more strongly than that of the diffuse type. Serine protease activity did not differ significantly between the two types of NS3. In HCV RNA replicon-harboring cells, physical interaction between NS3 and p53 was observed consistently and p53-mediated transcriptional activation was suppressed significantly compared with HCV RNA-negative control cells. Our results collectively suggest the possibility that NS3 plays an important role in the hepatocarcinogenesis of HCV by interacting differentially with p53 in an NS3 sequence-dependent manner.

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INTRODUCTION

Chronic, persistent infection with *Hepatitis C virus* (HCV) often leads to liver cirrhosis and hepatocellular carcinoma (HCC) (Saito *et al.*, 1990). However, the exact mechanisms of HCV-associated pathogenesis and carcinogenesis are largely unknown.

HCV possesses a single-stranded, positive-sense RNA genome of 9.6 kb, which encodes a polyprotein of approximately 3000 aa. The polyprotein is processed into at least 10 structural and non-structural (NS) viral proteins by cellular and viral proteases (Reed & Rice, 2000). One of the viral proteases, the NS3 serine protease, has become a research focus, as it is indispensable for virus replication and, therefore, would be a good target for antiviral drugs. The serine

protease is encoded in the N-terminal portion of NS3 and is responsible for cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions. NS4A, a cofactor for NS3, stabilizes it to augment its serine protease activity, being virtually essential for complete cleavage of the HCV polyprotein (Reed & Rice, 2000). The C-terminal portion of NS3 possesses the NTPase/helicase activity (Kim *et al.*, 1995), which is essential for viral RNA replication.

In addition to its key role in the life cycle of HCV, possible involvement of NS3 in viral persistence and hepatocarcinogenesis has been studied. For example, NS3 was reported to transform NIH3T3 (Sakamuro *et al.*, 1995) and rat fibroblast (Zemel *et al.*, 2001) cells. We also demonstrated that NIH3T3 cells constitutively expressing C-terminally truncated NS3 (aa 1–433) were more resistant to actinomycin D-induced apoptosis than control cells (Fujita *et al.*, 1996). It was also reported that NS3 could block transforming growth factor- β /Smad3-mediated apoptosis (Cheng *et al.*,

Supplementary figures showing subcellular-localization patterns and a sequence alignment are available in JGV Online.

2004). Moreover, the NS3–4A complex was shown to suppress beta interferon (IFN- β) induction by inhibiting retinoic acid-inducible gene I-mediated activation of IFN regulatory factor 3, counteracting innate immune responses to help establish persistent HCV infection (Foy *et al.*, 2003, 2005; Breiman *et al.*, 2005).

The tumour-suppressor protein p53 functions principally to control cell-cycle arrest and apoptosis upon various cellular stresses, ensuring completion of DNA repair and the integrity of the genome (Levine, 1997). It has been documented that oncogenic viral proteins, such as papillomavirus E6 (Münger & Howley, 2002; Longworth & Laimins, 2004), adenovirus E1B 55K (Martin & Berk, 1998), simian virus 40 large T antigen (Sheppard *et al.*, 1999) and hepatitis B virus X protein (Truant *et al.*, 1995), inhibit p53-mediated apoptosis via interacting with p53. In the case of HCV, NS5A (Lan *et al.*, 2002) and core protein (Kao *et al.*, 2004) were reported to suppress p53-dependent apoptosis. Our previous studies showed that NS3 colocalized with p53 in the nucleus (Ishido *et al.*, 1997; Muramatsu *et al.*, 1997) and that they formed a complex through an N-terminal portion of NS3 (aa 29–174) and a C-terminal portion of p53 (Ishido & Hotta, 1998). In a clinical setting, we found a strong correlation between HCC and predicted secondary structure of an N-terminal portion of NS3 (Ogata *et al.*, 2003). These observations prompted us to investigate the possible correlation between NS3 sequence diversity and p53 interaction. We report here that subcellular localization of NS3 and its interaction with p53 vary with different NS3 sequences.

METHODS

Plasmid construction. cDNA fragments encoding the N-terminal 198 residues of NS3 (NS3-N; aa 1–198) of HCV subtype 1b (HCV-1b) isolates were described previously (Ogata *et al.*, 2002, 2003). *Bam*HI and *Hind*III recognition sites were introduced by PCR into the 5' and 3' ends of the cDNAs, respectively. The cDNAs were digested with *Bam*HI and *Hind*III and subcloned into pcDNA3.1/*Myc*-His(–)C (Invitrogen). A single point mutation(s) was introduced into some plasmids by using a QuikChange site-directed mutagenesis kit (Stratagene). Expression plasmids for Myc-tagged full-length NS3 (NS3-Full) of different HCV isolates, MKC1a, M-H05-5, M-45, M-H17-2 and M-42, were reported elsewhere (Hidajat *et al.*, 2005). To express NS3–4A *in cis*, the corresponding region was amplified from pTMns2-5B/810-2721 (Muramatsu *et al.*, 1997) and subcloned into pcDNA3.1/*Myc*-His(–)C to generate pcDNA3.1/MKC1a/4A. Expression plasmids for chimeric NS3-Full flanked with NS4A were constructed, in which the N-terminal 355 residues were derived from M-H05-5 or M-H17-2, whereas the C-terminal 330 residues were derived from MKC1a/4A. They were designated pcDNA3.1/M-H05-5/4A and pcDNA3.1/M-H17-2/4A. The NS3 sequences were subcloned also into pSG5 (Stratagene).

An *Eco*RI fragment encoding full-length NS4A was obtained from pBSns4A (Muramatsu *et al.*, 1997) and subcloned into pcDNA3.1/*Myc*-His(–)C and pSG5. Myc-tagged NS4A was amplified from pFK5B/2884Gly (a kind gift from Dr R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) and subcloned into pEF1/*Myc*-His (Invitrogen). An expression plasmid for Myc-tagged NS4B was reported elsewhere (Tanaka *et al.*, 2006). To express a polyprotein

consisting of full-length NS5A and C-terminally truncated NS5B (NS5A/5BAC; aa 1973–2720 of the entire HCV polyprotein), the corresponding region was amplified from pTMns2-5B/810-2721 (Muramatsu *et al.*, 1997) and subcloned into pTM1 (Moss *et al.*, 1990).

An *Xho*I fragment encoding full-length wild-type p53 was obtained from pBSp53/1-393 (Ishido & Hotta, 1998) and subcloned into pcDNA3.1/*Myc*-His(–)C. pSG5/p53 (Florese *et al.*, 2002) was also used.

All of the plasmid constructs were verified for the correct sequence by DNA sequencing.

Cell culture and protein expression. Huh-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For protein expression, cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) (Fuerst *et al.*, 1986). After 1 h, the cells were transfected with the expression plasmids by using Lipofectin reagent (Invitrogen). After cultivation overnight, the proteins expressed in the cells were analysed by co-immunoprecipitation, immunoblot and immunofluorescence techniques, as described below. For the luciferase reporter assay, Huh-7 cells were transfected with plasmids by using Eugene 6 transfection reagent (Roche) and cultivated for 24 h before analysis.

Huh-7 cells stably harbouring an HCV subgenomic RNA replicon were prepared as described previously (Taguchi *et al.*, 2004; Hidajat *et al.*, 2005), using pFK5B/2884Gly (Lohmann *et al.*, 2001). Cured Huh-7 cells were prepared by treating the HCV replicon-harbouring cells with IFN- α (1000 IU ml^{–1}) for 1 month (Hidajat *et al.*, 2005). Full-length HCV RNA-harbouring Huh-7 cells, designated O, and IFN-cured cells, designated Oc, were described previously (Ikeda *et al.*, 2005).

Indirect immunofluorescence. Cells expressing Myc-tagged NS3 were fixed with methanol at –20 °C for 20 min and incubated with an anti-Myc mouse mAb (9E10; Santa Cruz Biotech) for 1 h at room temperature. In some experiments, an anti-NS3 mouse mAb (4A-3; a kind gift from Dr I. Fuke, Research Foundation for Microbial Diseases, Osaka University, Kagawa, Japan) was used to detect NS3-Full. An anti-haemagglutinin (HA) mouse mAb (HA.11; Covance Inc.) served as a control IgG. After being washed with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MBL) and observed under a laser-scanning confocal microscope (LSM510 version 3.0; Carl Zeiss).

Immunoprecipitation and immunoblotting. Cells expressing NS3 (Myc-tagged or untagged) and p53 were lysed in a stringent RIPA buffer containing 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate and protease inhibitor cocktail (Roche) for 30 min on ice. The cell lysates were centrifuged and the supernatants were cleared by mixing with 0.25 μ g normal rabbit IgG (Santa Cruz Biotech) and 15 μ l protein A-Sepharose beads (Amersham Biosciences) at 4 °C for 30 min on a rotator to reduce non-specific precipitation. The cleared lysates were incubated with anti-p53 rabbit polyclonal antibody (FL-393; Santa Cruz Biotech) at 4 °C for 1 h and subsequently with 15 μ l protein A-Sepharose beads for another 1 h. The beads were washed six times with RIPA buffer and the immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting (see below). To analyse the interaction of NS3 expressed in the context of HCV RNA replication with p53, the HCV subgenomic or full-length RNA replicon-harbouring cells were lysed in a mild RIPA buffer without 0.1% SDS and 0.1% sodium deoxycholate. The lysates were subjected to immunoprecipitation analysis in the same way as described above, except that the beads were washed with PBS instead of RIPA buffer. Anti-FLAG rabbit polyclonal antibody (Sigma) served as a control.

Immunoblot analysis was performed as described previously (Hidajat *et al.*, 2005). Mouse mAbs against Myc (9E10), NS3, NS4A (S4-13; a kind gift from Dr I. Fuke) and p53 (Ab-1; Calbiochem) were used as primary antibodies and peroxidase-labelled goat anti-mouse IgG (MBL) as a secondary antibody. The protein bands were visualized by an enhanced chemiluminescence method (ECL; Amersham Biosciences) and the intensity of the bands was quantified by using NIH Image 1.61.

Luciferase reporter assay. p53-Luc (Stratagene), which contains the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element plus an inducible *cis*-enhancer element, containing 14 repeats of the p53-binding sequence (TGCCTGGACTTGCCTGG), was used as a reporter plasmid. pRL-SV40 (Promega), which expresses *Renilla* luciferase, was used as a control plasmid to check transfection efficiency. Huh-7 cells prepared in a 24-well tissue-culture plate were transfected transiently with p53-Luc (10 ng), pRL-SV40 (1 ng), pSG5/p53 (5 ng) and pSG5/NS3-N or pSG5/NS3-Full (250 ng) in the absence or presence of pSG5/NS4A (75 ng). After 24 h, the cells were harvested and a luciferase assay was performed by using the Dual-Luciferase Reporter Assay system (Promega), as described previously (Kadoya *et al.*, 2005). Firefly and *Renilla* luciferase activities were measured by using a Luminescencer-JNR AB-2100 (Atto). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

NS3 serine protease activity. HeLa cells transiently coexpressing NS5A/5BΔC and Myc-tagged NS3 were lysed in gel-loading buffer containing 50 mM Tris/HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The lysates were separated by SDS-PAGE and analysed by immunoblotting using anti-NS5A (8926; a kind gift from Dr I. Fuke) and anti-Myc antibodies (9E10). Intensity of the bands corresponding to the cleaved NS5A and the uncleaved NS5A/5BΔC was measured. Arbitrary units of serine protease activity of each NS3 were calculated by the following formula: protease activity (arbitrary units) = NS5A/(NS5A/5BΔC + NS5A).

RESULTS

NS3-N sequences of different HCV-1b isolates exhibit distinct subcellular-localization patterns in a sequence-dependent manner

We first examined the subcellular localization of NS3-N in HeLa cells. As shown in Fig. 1(a), we noticed three distinct patterns of NS3-N localization: (i) dot-like staining in both the cytoplasm and the nucleus, (ii) diffuse staining predominantly in the cytoplasm and (iii) a mixed pattern of the former two. Of the 29 HCV-1b isolates tested, 15 (52%) exhibited exclusively the dot-like staining, nine (31%) the diffuse staining and the remaining five (17%) the mixed pattern. The subcellular-localization patterns of four NS3-N sequences each from the dot-like, diffuse and mixed staining groups are also shown in Supplementary Fig. S1 (available in JGV Online). Similar results were obtained when NS3-N sequences were expressed in Huh-7 cells (data not shown), suggesting that the distinct localization patterns among different NS3 sequences are not restricted to a particular cell line.

In order to see which amino acid residue(s) affected the subcellular localization of NS3, we determined the sequences of all 29 isolates. Some of the sequences showing the typical

localization patterns, along with a standard sequence, are shown in Fig. 1(b). For more information, the sequences of all 29 isolates are shown in Supplementary Fig. S2 (available in JGV Online). We did not find any common amino acid residue(s) that was/were associated with a particular localization pattern. We noticed, however, that a substitution at position 17 or 18 (Ile to Val) was observed with some NS3 sequences of the dot-like pattern, but not with any NS3 sequences of the other localization patterns. Also, a substitution(s) at positions 150–153 (Val to Ala, Ile to Val) appeared to be more frequent in NS3 sequences of the dot-like pattern. To examine the possible importance of those substitutions, we introduced a point mutation(s) to some NS3-N of the dot-like pattern (Fig. 1c). Introduction of two point mutations at positions 18 and 153 into NS3-N of isolate H05-5 did not alter the localization pattern. However, introduction of an additional two point mutations at positions 80 and 122 altered the localization pattern significantly, with the majority of the cells exhibiting the typical diffuse staining. Similarly, introduction of two mutations at positions 17 and 86, but not of either one alone, into NS3-N of isolate 45 altered the localization pattern from dot-like to diffuse staining. As for isolate 63, a single point mutation at position 150 alone was enough to change the localization pattern of NS3-N. These results suggest that residues at positions 17 or 18, 80–86 and/or 150–153 play an important role in determining the localization pattern of some, but not all, NS3 sequences.

NS3-N binds to p53 and inhibits its *trans*-activating activity in an NS3 sequence-dependent manner

We previously reported that a region near the N terminus of NS3 (aa 29–174) was involved in complex formation with p53 (Ishido & Hotta, 1998). In this study, we examined whether interaction between NS3-N and p53 differs with different NS3-N sequences. We selected two NS3-N sequences each from the dot-like (H05-5 and 45) and diffuse (H17-2 and 42) staining groups. Co-immunoprecipitation analysis demonstrated that NS3-N of isolate H05-5 interacted with p53 most strongly, followed by that of isolate 45, both in the absence (Fig. 2a) and the presence (Fig. 2b) of NS4A. On the other hand, NS3-N of the diffuse-staining group interacted only weakly with p53. The specificity of the interaction between NS3-N and p53 was confirmed by a control experiment, in which neither NS4A nor NS4B bound to p53 under the same experimental conditions (Fig. 2c, left and centre panels). The specificity of the NS3–p53 interaction was also secured by another control experiment using an irrelevant (anti-FLAG) antibody (Fig. 2c, right panel).

Next, we examined the possible effect of NS3-N on p53 function. The plasmid p53-Luc harbours 14 copies of p53-responsive elements and a minimum promoter upstream of a luciferase gene, and is used to monitor p53-dependent transcriptional activity. Interestingly, NS3-N of H05-5 and that of isolate 45 inhibited p53-dependent transcription of the luciferase gene strongly and moderately, respectively

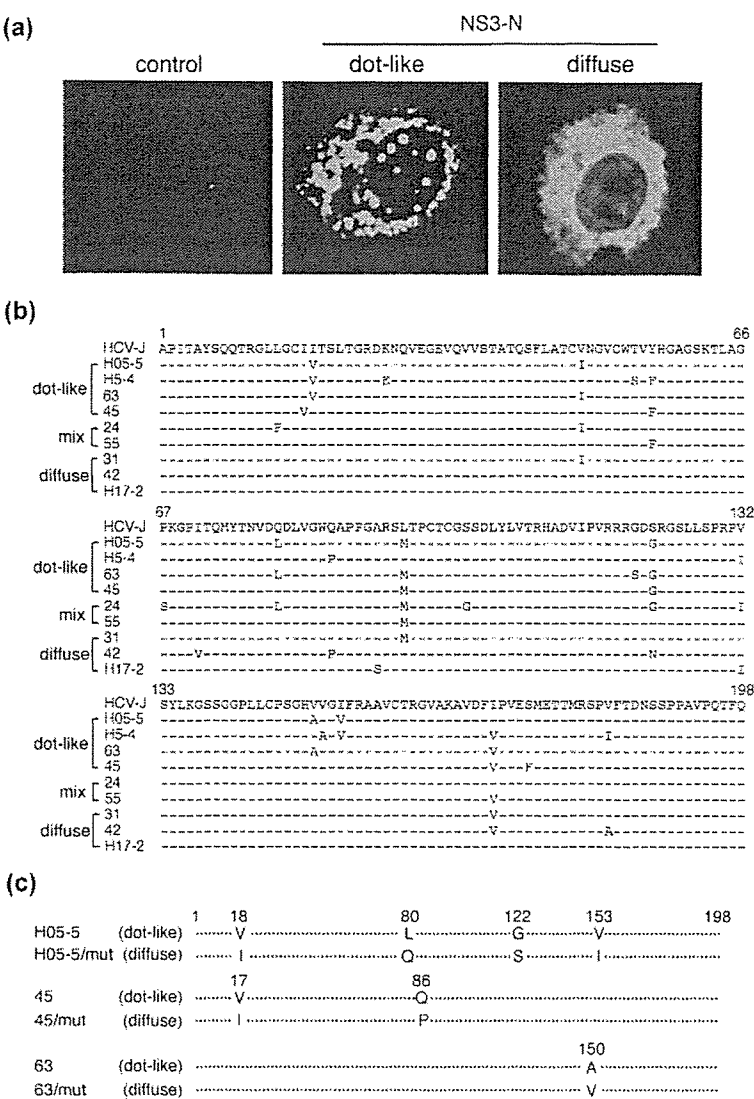


Fig. 1. Distinct subcellular-localization patterns of NS3-N of different HCV-1b isolates and the alignment of their sequences. (a) NS3-N was expressed in HeLa cells using a vaccinia virus-T7 hybrid expression method. Typical immunofluorescence images of the dot-like (middle panel) and diffuse (right panel) localization patterns of NS3-N of isolates H05-5 and H17-2, respectively, are shown. As a control, cells expressing NS3-N of H05-5 (left panel) were stained with an irrelevant (anti-HA) antibody. (b) Sequence alignment of representative sequences of each of the staining groups along with a standard sequence of the HCV-J strain (top). Dashes indicate residues identical to those of HCV-J. The numbers along the sequence indicate amino acid positions. (c) Identification of residues that alter the localization patterns of NS3-N of the isolates H05-5, 45 and 63. Substituted residues at the indicated positions are shown.

(Fig. 2d). On the other hand, no inhibition was observed with NS3-N of isolate 42 and even a slight increase in p53-dependent transcription was observed with NS3-N of H17-2.

NS3 forms a stable complex with its cofactor NS4A, which may counteract the NS3-mediated inhibitory action of p53-dependent transcription. In fact, we observed that inhibition of the p53-dependent transcription by NS3-N of the H05-5 isolate was alleviated to some extent, but not completely, by coexpression of NS4A (Fig. 2e).

To further test the possibility that the alteration in the localization pattern of NS3-N affects its interaction with p53, we compared NS3-N of H05-5 with its point mutant H05-5/mut (Fig. 1c) in terms of their p53-binding abilities and inhibitory effects on p53-dependent transcription. The result obtained demonstrated that NS3-N of H05-5/mut, which showed diffuse localization, had weaker p53-binding capacity (Fig. 3a) and exerted weaker inhibition on p53-dependent

transcription (Fig. 3b) compared with NS3-N of the parental H05-5, showing the dot-like localization. Similar results were obtained with isolates 45 and 63 and their point mutants (data not shown). Our results thus suggest that NS3-N of the dot-like localization pattern interacts with p53 more strongly and inhibits p53-mediated transcriptional activation more efficiently than that of the diffuse localization.

NS3-Full sequences exhibit the same subcellular-localization patterns as those of NS3-N sequences derived from the same isolates and interact differentially with NS4A and p53 in an NS3 sequence-dependent manner

As shown above, NS3-N exhibited a distinct subcellular-localization pattern in a sequence-dependent manner when expressed alone (see Fig. 1). Moreover, we have reported that NS3, either NS3-N or NS3-Full, enters the nucleus when

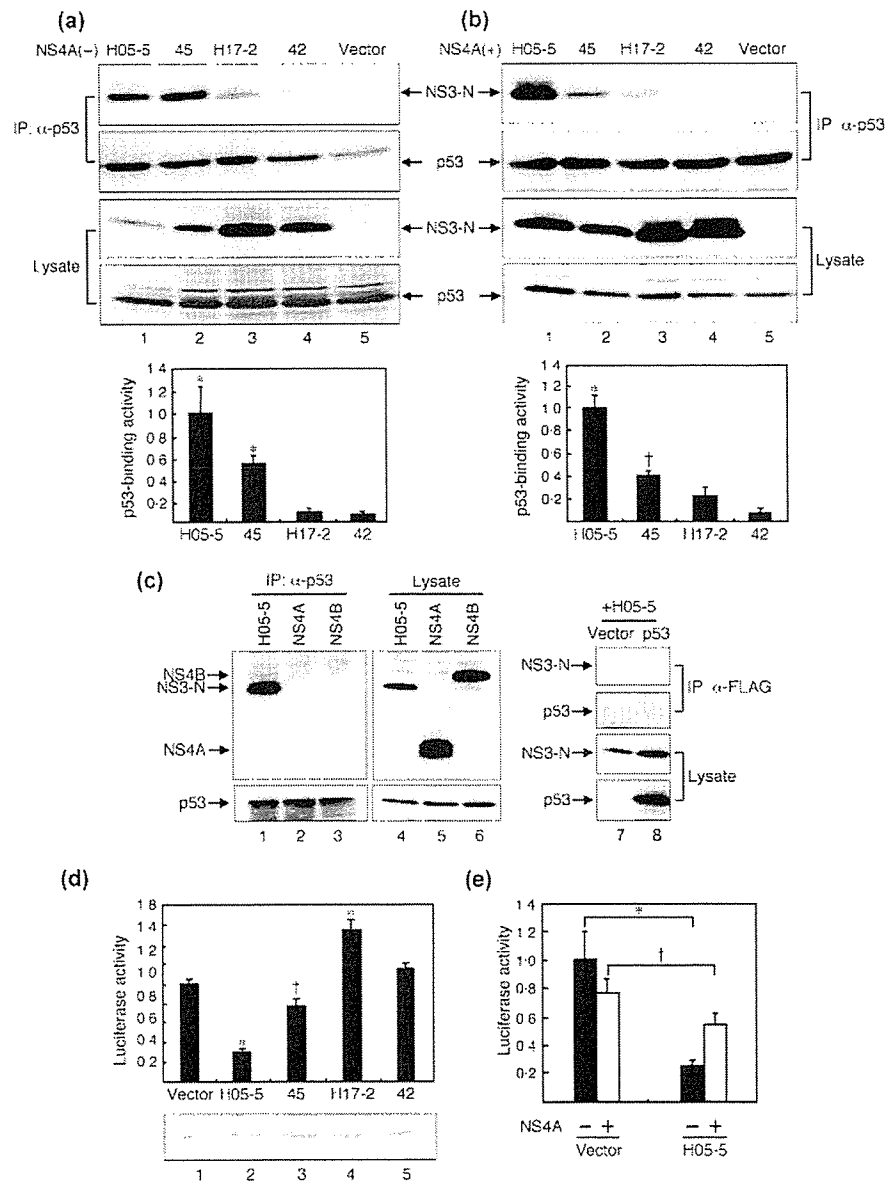


Fig. 2. Physical and functional interactions between NS3-N and p53 in an NS3 sequence-dependent manner. NS3-N and p53 were coexpressed in the absence (a) and presence (b) of NS4A. Cells that did not express NS3-N served as a control. Cell lysates were immunoprecipitated by using an anti-p53 antibody and probed by immunoblotting using an anti-Myc antibody to detect NS3-N (top row). Efficient immunoprecipitation of p53 was verified (second row). Lysates were probed directly (without being immunoprecipitated with anti-p53 antibody) with anti-Myc and anti-p53 antibodies, respectively, to verify comparable expression levels of NS3-N (third row) and p53 (bottom row). The intensity of the bands for NS3-N co-immunoprecipitated with p53 was quantified and normalized to the expression levels of NS3-N in the lysates. Filled columns and bars represent mean \pm SD obtained from three independent experiments. The p53-binding intensity of NS3-N of the isolate H05-5 was expressed as 1.0. * $P < 0.01$; † $P < 0.05$, compared with isolate 42. (c) Cells expressing Myc-tagged H05-5, NS4A or NS4B together with p53 were analysed by immunoprecipitation using an anti-p53 antibody (left). Lysates were probed directly with anti-Myc and anti-p53 antibodies, respectively (middle). Cells expressing Myc-tagged H05-5 with or without p53 were analysed by immunoprecipitation using an irrelevant (anti-FLAG) antibody (right). (d) Inhibition of p53-dependent transcription by NS3-N in an NS3 sequence-dependent manner. pSG5-based NS3-N expression plasmids were each co-transfected with pSG5/p53, p53-Luc and pRL-SV40 in Huh-7 cells and cultivated for 24 h. Firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. The luciferase activity in the control cells without NS3-N expression was expressed arbitrarily as 1.0. Results are shown as mean \pm SD from three independent experiments. * $P < 0.01$; † $P < 0.05$, compared with the control. Expression levels of NS3-N in the cells are shown at the bottom. (e) Inhibition of p53-dependent transcription by NS3-N of H05-5 in the absence (filled bars) and presence (open bars) of NS4A. Results are shown as mean \pm SD from three independent experiments. * $P < 0.01$; † $P < 0.05$, compared with the control.

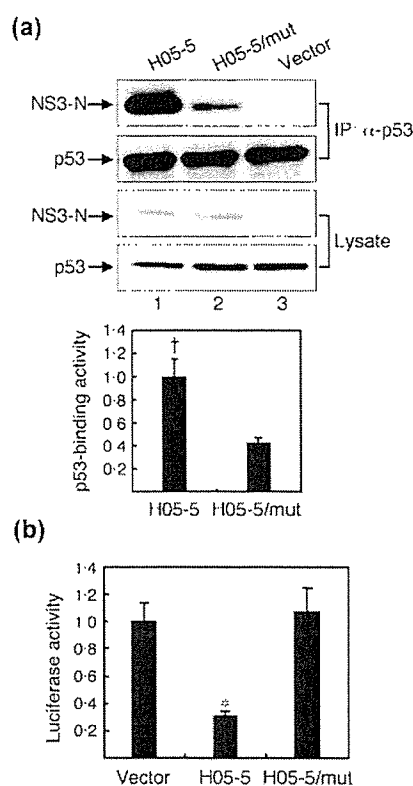


Fig. 3. Comparison between NS3-N of the isolate H05-5 and its point mutant H05-5/mut in their capacity to interact with p53. (a) Physical interaction with p53 was analysed as described in the legend to Fig. 2(a). Filled columns and bars represent mean \pm SD obtained from three independent experiments. The p53-binding intensity of NS3-N of H05-5 was expressed as 1.0. $\dagger P < 0.05$. (b) Functional interaction with p53 was analysed as described in the legend to Fig. 2(c). Results are shown as mean \pm SD from three independent experiments. $* P < 0.01$ compared with H05-5/mut.

coexpressed with p53 and that the p53-mediated nuclear localization of NS3 is inhibited by NS4A in an NS3 sequence-dependent manner (Muramatsu *et al.*, 1997). Therefore, we examined the subcellular-localization patterns of NS3-Full of different sequences, both when expressed alone and when coexpressed with p53 and/or NS4A. The NS3-Full sequences tested differ from each other only in the N-terminal 180 residues that are derived from the clinical isolates, with the C-terminal 451 residues being shared among all the strains tested (Fig. 4a; Hidajat *et al.*, 2005). When expressed alone, NS3-Full of all four strains exhibited the same subcellular-localization patterns as those of NS3-N of the same strains (Fig. 4b; data not shown for M-45 and M-42). When coexpressed with NS4A, NS3-Full was localized in the cytoplasm, especially in perinuclear regions, regardless of the strain tested. Interestingly, when p53 was additionally coexpressed with NS4A, NS3-Full of the dot-like type (M-H05-5 and M-45) showed an increased tendency to accumulate in the nucleus together with p53 (Fig. 4b), with

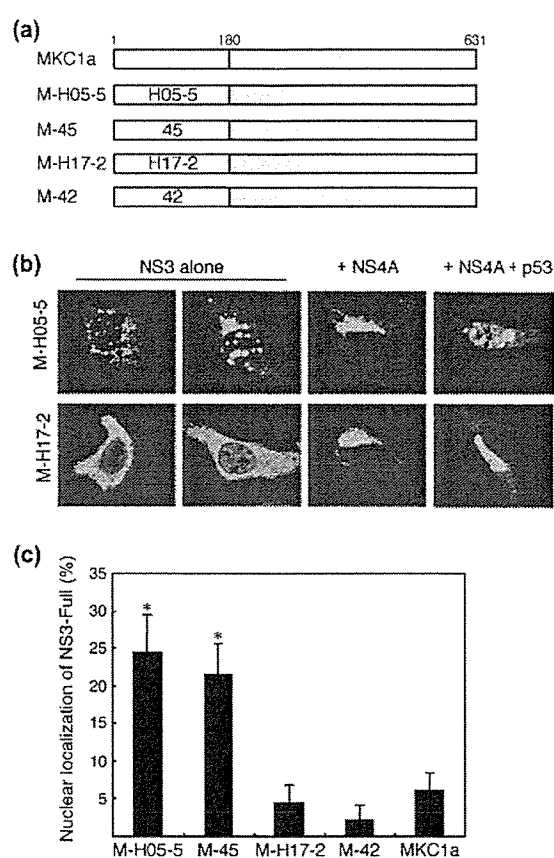


Fig. 4. Subcellular localization of NS3-Full in the presence and absence of NS4A and p53. (a) Schematic representation of NS3-Full of different sequences. The N-terminal 180 residues are derived from clinical isolates (H05-5, H17-2, 42 and 45) and the C-terminal 451 residues from the parental MKC1a strain. (b) Subcellular localization of M-H05-5 (upper) and M-H17-2 (lower) when expressed alone, when coexpressed with NS4A and when coexpressed with NS4A and p53. The cells were stained with either anti-NS3 (left panels) or anti-Myc antibody (left-middle, right-middle and right panels). (c) Percentage of cells with nuclear accumulation of NS3-Full when coexpressed with NS4A and p53. $* P < 0.01$ compared with M-42.

nearly 25 % of the cells exhibiting nuclear localization of NS3 (Fig. 4c). Concomitant expression of NS4A in the cytoplasm of the same cells was confirmed by double-staining immunofluorescence analysis (data not shown), the result being consistent with our previous observation (Ishido *et al.*, 1997). On the other hand, NS3-Full of the diffuse type (M-H17-2, M-42 and MKC1a) was localized almost exclusively in the cytoplasm together with NS4A. Similar results were obtained when NS3-N sequences of different isolates were coexpressed with p53 and/or NS4A (data not shown).

We then tested complex formation between NS3-Full of the five different sequences and p53. The results demonstrated clearly that NS3-Full of the dot-like type (M-H05-5 and

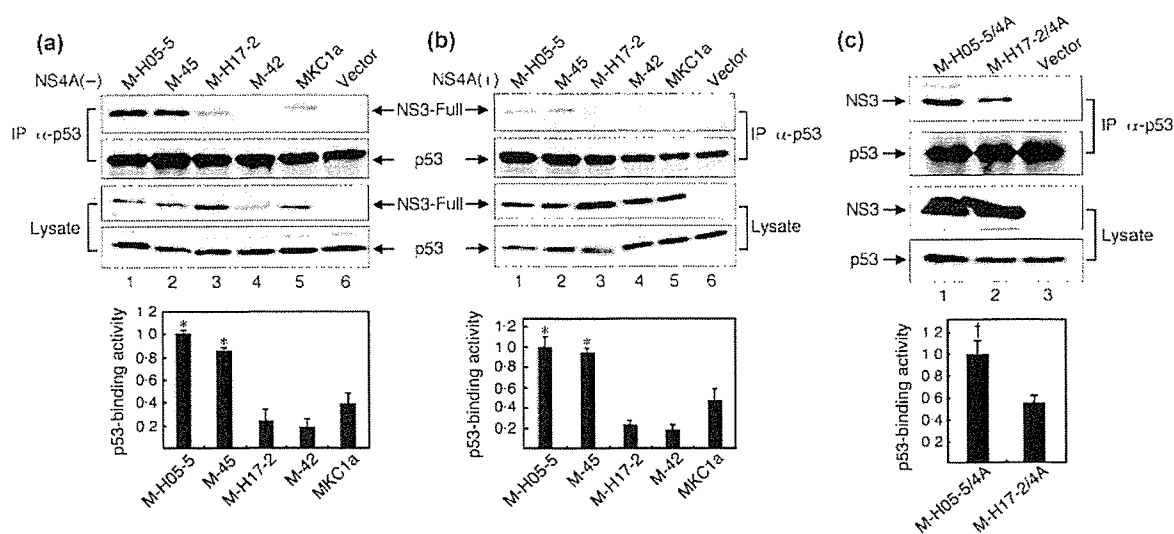


Fig. 5. Physical interaction between NS3-Full and p53 in an NS3 sequence-dependent manner. NS3-Full of different strains in the absence (a) and presence of NS4A coexpression *in trans* (b) were analysed as described in the legend to Fig. 2(a). Filled columns and bars represent mean \pm SD obtained from three independent experiments. The p53-binding intensity of M-H05-5 was expressed as 1.0. * P < 0.01 compared with M-42. (c) M-H05-5/4A and M-H17-2/4A (NS3-4A coexpression *in cis*) were analysed as described in the legend to Fig. 2(a), except that anti-NS3 antibody was used instead of anti-Myc antibody. Filled columns and bars represent mean \pm SD obtained from three independent experiments. The p53-binding intensity of M-H05-5/4A was expressed as 1.0. † P < 0.05 compared with M-H17-2/4A.

M-45) interacted with p53 more strongly than that of the diffuse type (M-H17-2, M-42 and MKC1a), both in the absence and presence of NS4A (Fig. 5a, b). In this connection, it should be noted that the interaction between NS3-Full and p53 was weaker in the presence of NS4A than in its absence. We also examined the interaction of NS3 with p53 when full-length NS3-4A was expressed *in cis*, where NS3-4A complex formation occurs more efficiently than *in trans*. The results demonstrated that M-H05-5/4A interacted with p53 more strongly than did M-H17-2/4A (Fig. 5c), again suggesting NS3 sequence-dependent interaction with p53.

NS3 binds to p53 and inhibits its *trans*-activating activity in HCV RNA replicon-harboring cells

In order to determine whether NS3 expressed in the context of HCV replication interacted with p53, we used Huh-7 cells harbouring an HCV subgenomic RNA replicon and examined physical and functional interactions between NS3 and p53. Co-immunoprecipitation analysis revealed that NS3 interacted physically with p53 in HCV subgenomic RNA replicon-harboring cells, albeit with much lower efficiency than in the plasmid-based expression system (Fig. 6a). We also used the full-length HCV RNA replicon, whose NS3 is detected more strongly than that of the subgenomic RNA replicon by the anti-NS3 mAb used in this study. The result demonstrated that NS3 expressed in the context of HCV RNA replication interacted efficiently with p53, irrespective

of whether p53 was expressed ectopically or endogenously (Fig. 6b). The specificity of the interaction between NS3 and p53 was confirmed by the lack of interaction between NS4A and p53 in HCV subgenomic RNA-harboring cells (Fig. 6c). Next, we compared *trans*-activating activity of p53 between HCV RNA replicon-harboring cells and the HCV-negative controls (parental and cured Huh-7 cells). We observed that p53-dependent transcription was suppressed significantly in cells harbouring an HCV RNA replicon, either subgenomic or full-length, compared with the parental and cured Huh-7 cells (Fig. 6d, e). These results suggest collectively that NS3 expressed in the context of HCV replication inhibits p53 function.

Serine protease activity of NS3-Full in the absence and presence of NS4A

The N-terminal portion of NS3 possesses a serine protease activity that can cleave the NS5A/5B junction even in the absence of NS4A (Lin *et al.*, 1994). By using NS5A/5BΔC as a substrate, we compared the serine protease activities of NS3-Full of different subcellular-localization patterns. A tendency was noted that, in the absence of NS4A, NS3-Full of the dot-like type showed slightly weaker protease activity than that of the diffuse type (Fig. 7). This difference might be attributable, at least partly, to the fact that NS5A/5BΔC was localized diffusely in the cytoplasm (Kim *et al.*, 1999; Mottola *et al.*, 2002; data not shown) and, therefore, could be recognized more easily by NS3 of the same localization pattern than by NS3 of the other type. In the presence of

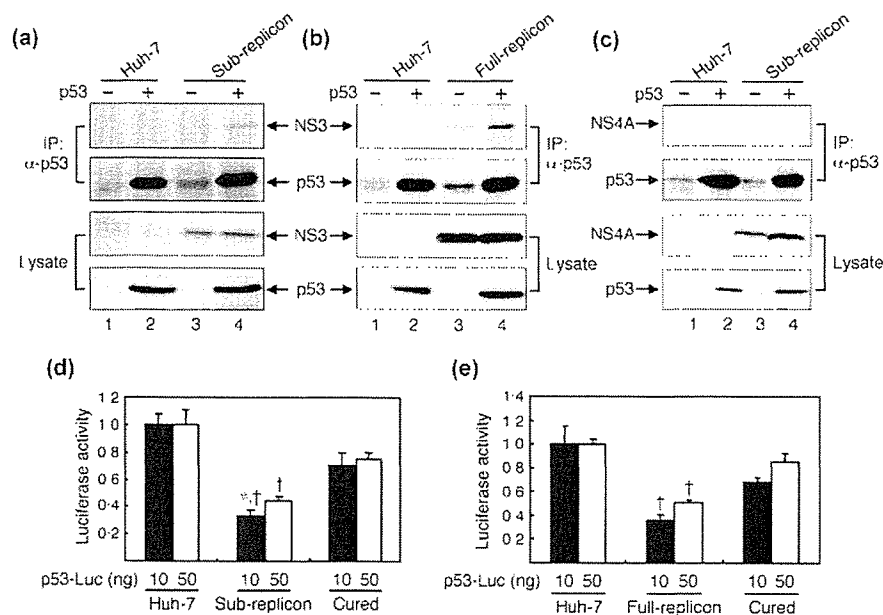


Fig. 6. Physical and functional interactions between NS3 and p53 in HCV RNA replicon-harboring cells. p53 was expressed ectopically in Huh-7 cells harbouring either HCV subgenomic (a) or full-length (b) RNA replicon and the parental Huh-7 cells by using a vaccinia virus–T7 hybrid expression method. Cell lysates were analysed as described in Methods. (c) The lack of interaction between NS4A and p53 in HCV subgenomic RNA replicon-harboring Huh-7 cells was confirmed. Inhibition of p53-dependent transcription in Huh-7 cells harbouring either HCV subgenomic (d) or full-length (e) RNA replicon. Cells were co-transfected with p53-Luc (10 and 50 ng, filled and open bars, respectively), pSG5/p53 (10 ng) and pRL-SV40 (1 ng) as an internal control. After 24 h, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. Luciferase activities in the parental Huh-7 cells were expressed arbitrarily as 1.0. Cured cells also served as a control. Results are shown as mean \pm SD from three independent experiments. * $P < 0.01$; † $P < 0.05$, compared with the parental and cured Huh-7 cells.

NS4A, on the other hand, all of the NS3-Full sequences, which accumulated at a perinuclear region of the cytoplasm (see Fig. 4b), exhibited an enhanced and comparable degree of serine protease activity among the five strains (Fig. 7). Similar results were obtained when Huh-7 cells were used instead of HeLa cells (data not shown).

DISCUSSION

In the present study we demonstrated that, when expressed alone, NS3 of HCV-1b isolates, either NS3-N or NS3-Full, exhibited distinct subcellular-localization patterns, i.e. (i) dot-like staining both in the cytoplasm and the nucleus, (ii) diffuse staining predominantly in the cytoplasm and (iii) a mixed type, in a sequence-dependent manner (Figs 1 and 4). Although no significant correlation has been observed so far between the localization patterns of NS3 and the HCC status of the patients, it was interesting to find that NS3-N and NS3-Full of the dot-like staining pattern interacted with p53 more strongly than that of the diffuse-staining pattern (Figs 2a, 3a and 5a). Similar results were obtained when NS3 was coexpressed with NS4A (Figs 2b, 5b and 5c). We also observed that both NS3-N and NS3-Full of the dot-like

staining pattern, but not those of the diffuse pattern, were more prone to colocalize with p53 in the nucleus even in the presence of NS4A (Fig. 4). Luciferase reporter analysis demonstrated that NS3-N of the dot-like type, but not that of the diffuse type, suppressed p53-dependent transcriptional activation significantly (Figs 2d and 3b).

When cells are exposed to a variety of stresses, p53 is induced to accumulate in the nucleus, where it functions as a transcription factor for cell-cycle regulators such as p21 (Levine, 1997). Our present results demonstrated that NS3-N of isolate H05-5 inhibited p53-dependent transcription of a reporter gene strongly (Figs 2d and 3b). We need to assess two possible mechanisms for the NS3-N-mediated p53 inhibition: NS3 might inhibit either p53 expression or p53 function itself. Our results showed that p53 expression levels were not altered significantly by NS3-N, irrespective of the localization patterns (Fig. 2a, b, bottom). Similar results that neither p53 mRNA nor protein levels were downregulated by NS3 were reported by Kwun *et al.* (2001). Overexpression of p53 was even observed in hepatocytes of some, if not all, HCV-infected patients (Loguercio *et al.*, 2003). It is likely, therefore, that NS3-N inhibits p53 function by interacting with it physically.

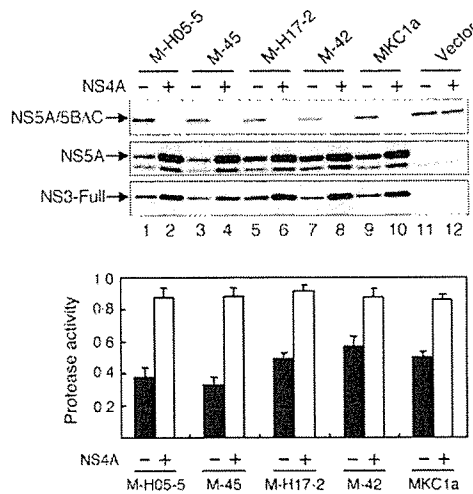


Fig. 7. Serine protease activity of NS3-Full in the absence and presence of NS4A. NS3-Full of different strains and NS5A/5BΔC were coexpressed in HeLa cells without (lanes with odd numbers) or with (lanes with even numbers) NS4A using a vaccinia virus-T7 hybrid expression method. Cell lysates were subjected to immunoblotting using anti-NS5A or anti-Myc antibody to detect NS5A/5BΔC (top), NS5A (middle) and NS3 (bottom). The intensity of the bands was quantified and arbitrary units of serine protease activity were calculated as described in Methods. Serine protease activities of NS3-Full in the absence (filled bars) and presence (open bars) of NS4A obtained from three independent experiments are shown.

We previously reported that a region of p53 near the C terminus (aa 301–360) was involved in complex formation with NS3 (Ishido & Hotta, 1998). This region includes the p53 oligomerization domain (aa 324–355) (Levine, 1997). It is known that the p53 tetramer binds to the p53-response element on promoter sequences most efficiently and, therefore, is most effective in *trans*-activation of its target genes (McLure & Lee, 1998; Weinberg *et al.*, 2004). Recently, it was reported that proteins of the S100 family disrupted p53 tetramerization via binding to its tetramerization domain (Fernandez-Fernandez *et al.*, 2005). Therefore, it is reasonable to assume that interaction of NS3-N with p53 interferes with its tetramer formation and DNA binding, thereby inhibiting p53-dependent transcriptional activation. It was also reported that a C-terminal portion of p53 (aa 364–393) negatively regulated its DNA-binding capacity (Müller-Tiemann *et al.*, 1998) and that the 14-3-3 proteins could associate with this region to counteract the negative regulation, which resulted in increased DNA binding of p53 (Waterman *et al.*, 1998). It is tempting to speculate that, by binding to a nearby region of p53, NS3-N may impair the association of 14-3-3 proteins with p53, which results in comparably decreased DNA binding of p53. Moreover, p53 is subject to post-translational modifications, including phosphorylation and acetylation, that affect p53 function (Appella & Anderson, 2001). Further study is needed to

determine whether such p53 modification status is affected, either directly or indirectly, by NS3-N.

Consistent with the results obtained from transient-expression experiments, physical interaction between NS3 and p53 was also observed in Huh-7 cells harbouring either an HCV subgenomic or full-length RNA replicon, albeit to a smaller extent than in the transient-expression system (Fig. 6). It should be noted that NS3 expressed by the full-length RNA replicon is detected more strongly by the anti-NS3 antibody used in this study than that of the subgenomic RNA replicon. In HCV RNA replicon-harbouring cells, the HCV non-structural proteins are incorporated into the HCV RNA replication complex and, therefore, it is conceivable that only a minor fraction of NS3 is available for the interaction with p53. Nevertheless, p53-mediated transcriptional activation was suppressed significantly in HCV RNA replicon-harbouring cells compared with the controls (Fig. 6d, e). We must consider the possibility that not only NS3, but also other HCV proteins, are involved in the observed p53 inhibition. In fact, interaction between NS5A and p53 has been reported (Lan *et al.*, 2002; Qadri *et al.*, 2002).

In conclusion, our present results have demonstrated that NS3 of HCV-1b can be divided into three groups based on the subcellular-localization patterns and that NS3 of the dot-like localization pattern interacts with, and inhibits the function of, the tumour suppressor p53 more strongly than that of the diffuse type. The observed difference may account, at least partly, for a different degree of the oncogenic capacity of different HCV-1b isolates.

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Hepatitis C virus NS5B delays cell cycle progression by inducing interferon- β via Toll-like receptor 3 signaling pathway without replicating viral genomes

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Abstract

To clarify the pathogenesis of hepatitis C virus (HCV), we have studied the effects of HCV proteins using human hepatocytes. Here, we found that HCV NS5B, an RNA-dependent RNA polymerase, delayed cell cycle progression through the S phase in PH5CH8 immortalized human hepatocyte cells. Since treatment with anti-interferon (IFN)- β neutralizing antibody restored the cell cycle delay, IFN- β was deemed responsible for the cell cycle delay in NS5B-expressing PH5CH8 cells. The induction of IFN- β and the cell cycle delay were overridden by the down-regulation of Toll-like receptor 3 (TLR3) through RNA interference in NS5B-expressing PH5CH8 cells. Moreover, the NS5B full form was required for the cell cycle delay, the induction of IFN- β , and the activation of the IFN- β signaling pathway. Our findings revealed that NS5B induced IFN- β through the TLR3 signaling pathway in immortalized human hepatocytes even without replicating viral genomes.
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Keywords: Hepatitis C virus; NS5B; Interferon- β ; TLR3; Hepatocyte cells

Introduction

Since more than 170 million individuals are estimated to be infected with hepatitis C virus (HCV) worldwide, this disease is a global health problem (Thomas, 2000). HCV belongs to the family Flaviviridae, whose positive-stranded RNA genome encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins in the following order: NH₂-core-envelope 1-envelope 2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Kato, 2001; Kato et al., 1990). These viral proteins are not only involved in viral replication but also may affect a variety of cellular functions (Bartenschlager and Lohmann, 2000; Kato, 2001). Although persistent infection

with HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Colombo, 1996; Kato, 2001), the molecular mechanisms leading to liver cell dysplasia and HCC remain elusive.

It has been thought that unregulated cell cycle progression may be a cause of malignant transformation of normal cells. On the other hand, inhibition of cell cycle progression through the S phase may cause replication error during DNA replication, which induces genomic instability and malignant transformation. Therefore, it is important to clarify the effect of HCV proteins on cell cycle progression in order to understand the molecular mechanism underlying the pathogenesis of HCV, including the development of HCC. A number of previous reports suggested that four HCV proteins—the core, NS3, NS4B, and NS5A—are involved in modulating cell cycle progression (Arima et al., 2001; Kato, 2001; Ray and Ray, 2001; Reed and Rice, 2000). For instance, the core protein promotes cell proliferation through the Ras/Raf signaling pathway and the anti-apoptotic function (Mar-

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usawa et al., 1999; Tsuchihara et al., 1999). However, the core has been described to both enhance and repress the function of p21^{Waf1/Cip1/Sdi1}, a Cdk inhibitor (Dubourdeau et al., 2002; Jung et al., 2001; Lu et al., 1999; Ray et al., 1998). Recently, Scholle et al. found no significant cell cycle delay in human hepatoma HuH-7-based HCV RNA-replicating cells that were autonomously replicating genome-length HCV RNA, in comparison with cured cells of the same line from which HCV RNA had been eliminated by treatment with interferon (IFN)- α (Scholle et al., 2004). Hence, the effects of cell cycle regulation by HCV proteins are still controversial. Cancerous cell lines, such as the human hepatoma HuH-7 cell line (Hsu et al., 1993), which harbors a mutant *p53* gene, may not be suitable for addressing the effects of HCV proteins on cell cycle progression.

The PH5CH8 cell line was established by immortalization using the SV40 large-T antigen from non-neoplastic liver tissue of an HCV-related HCC patient (Ikeda et al., 1998; Noguchi and Hirohashi, 1996). PH5CH8 cells possess wild-type *p53* and *Rb* tumor suppressor genes. In nude mice, these cells reveal a non-malignant phenotype upon colony formation and tumorigenicity (Noguchi and Hirohashi, 1996), although the SV40 large-T antigen would partially repress the function of *p53*. Therefore, the PH5CH8 cell line is considered to be more relevant for studying the role of HCV proteins during hepatocarcinogenesis. We have previously reported that the HCV core protein activates the IFN-inducible 2'-5'-oligoadenylate synthetase gene in PH5CH8 cells (Naganuma et al., 2000). Recently, we demonstrated that the core protein's activation of this gene was mediated through the IFN-stimulated response element (ISRE) (Danskø et al., 2003). Furthermore, we found that the core protein promoted microsatellite instability in PH5CH8 cells (Naganuma et al., 2004). In fact, microsatellite instability was detected in approximately 20% of the tumor tissues from HCC patients examined, whereas no microsatellite instability was detected in normal liver tissues from the same patients (Dore et al., 2001; Kondo et al., 2000). In order to clarify the effect of HCV proteins on cell cycle progression in PH5CH8 cells, we examined cell cycle progression after the cells were released from the G1/S boundary in PH5CH8 cells expressing HCV proteins. We found that NS5B delays cell cycle progression by inducing IFN- β through the activation of the Toll-like receptor 3 (TLR3) signaling pathway without replicating viral genomes.

Results

HCV NS5B causes the delay of S phase progression

In a previous study of virus–host interactions, we examined whether or not HCV proteins affect cell cycle progression in PH5CH8 cells that stably expressed core or NS proteins. PH5CH8 cells were infected with retrovirus pCXbsr as a negative control (Ctr) or pCXbsr encoding either an HCV structural protein (HA-core) or NS protein (NS3, HA-NS4B, HA-NS5A, HA-NS5B, or NS5B), and we obtained

PH5CH8 cells stably expressing each HCV protein. The expression of each HCV protein was confirmed by Western blot analysis (Fig. 1A). Then, the HCV protein-expressing cells were synchronized at the G1/S boundary, and cell cycle progression (from the S phase to the G2-M phase, then turning back to the G1 phase) was analyzed after the cells were released from synchronization. This cell cycle analysis revealed no significant differences in cell cycle progression between cells (PH/Ctr) infected with a control pCXbsr retrovirus and cells expressing core, NS3, NS4B, or NS5A (Fig. 1B). Unlike the PH/Ctr cells, the apparent delay of S phase progression was found in cells (PH/NS5B) expressing NS5B, regardless of the presence of the HA tag (Fig. 1B). To exclude the possibility that pCXbsr-derived retrovirus proteins synergistically affect the cell cycle together with NS5B, the retrovirus pCX4bsr vector (Akagi et al., 2003), which eliminates the production of any fusion proteins resulting from initiation at upstream AUG codons within the *gag* region of the vector, was used for the cell cycle analysis. As a result, the delay of S phase progression was found again in PH5CH8 cells expressing NS5B (Figs. 1A and C), suggesting that the retrovirus proteins are not involved in the delay of S phase progression. BrdUrd incorporation analysis was also carried out using PH/Ctr and PH/NS5B cells (Fig. 1D). In the PH/Ctr cells, DNA synthesis began early in the S phase (4 h after release). In the late S phase (8 h), more than 61% of the cells indicated final DNA synthesis. Thereafter, the cells either finished DNA replication in the G2-M phase (12 h) or returned to the G1 phase. In contrast, we found that DNA replication in most PH/NS5B cells predominantly remained in the early or middle S phase (8 h), and 49% of the cells were prolonged in the late S phase (12 h). To quantitatively evaluate this delay in S phase progression, the cells that had finished DNA replication were accumulated during the G2 phase by treatment with Nocodazole (Noc), which inhibits the progression of the G2 to M phases, after the cells were released from the G1/S transition. Whereas 77% of PH/Ctr cells reached the G2-M phase, only 37% of PH/NS5B cells did so (Fig. 1D). This level of decrease in cell numbers in the G2-M phase was not observed in PH5CH8 cells expressing core, NS3, NS4B, or NS5A (Fig. 1E), suggesting that NS5B specifically causes the delay of S phase progression in PH5CH8 cells. We further observed that the growth rate of PH/NS5B cells was significantly decreased relative to PH/Ctr cells (Fig. 1F), although the cell cycle distribution in asynchronous PH/NS5B cells was almost the same as that in asynchronous PH/Ctr cells (Fig. 1D). These results indicated that NS5B might delay the cell cycle progression of PH5CH8 cells in the S phase.

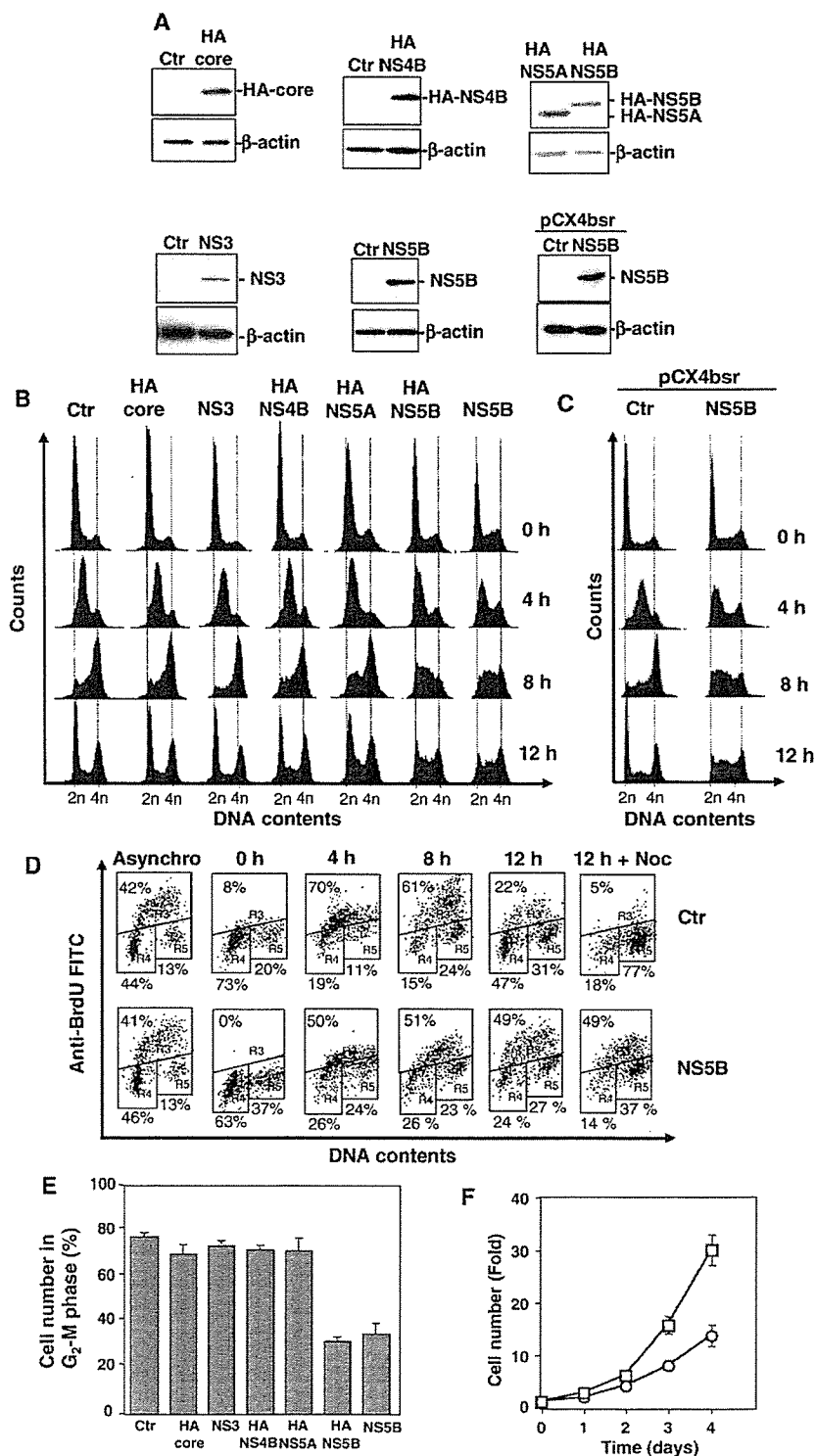
Cell cycle delay by NS5B is also found in other immortalized human hepatocytes

To clarify whether or not the delay of S phase progression by NS5B occurs in other human cell lines, we prepared three cell lines (Fig. 2A) that stably express NS5B—non-neoplastic human hepatocyte NKNT-3 (Kobayashi et al., 2000), hepatoma

HuH-7, and cervical carcinoma HeLa—and subjected them to the cell cycle analysis described above. The results revealed that S phase progression was delayed in NKNT-3, but not in HuH-7 or HeLa cells (Fig. 2B). This indicates that the delay of the cell cycle by NS5B expression is not limited to PH5CH8 cells.

IFN- β mediates the delay of S phase progression by NS5B

Since it has been reported previously that IFN- β induced the delay of S phase progression in human cultured cells (Vannucchi et al., 2000), we speculated that IFN- β was



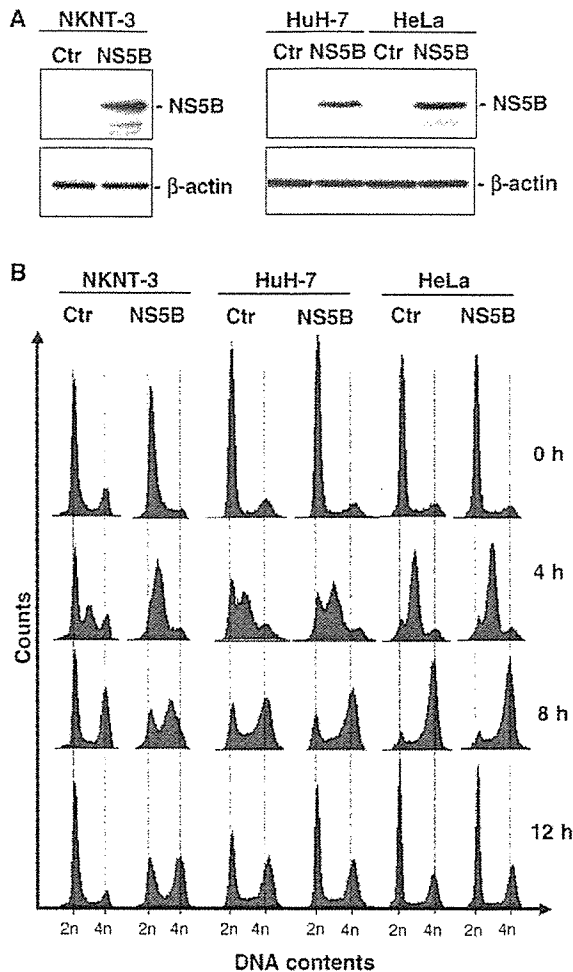


Fig. 2. NS5B delays S phase progression in another immortalized human hepatocytes. (A) Western blot analysis of NKNT-3, HuH-7, and HeLa cells infected with pCXbsr retrovirus encoding NS5B. The pCXbsr retrovirus was used as a control infection (Ctr). Anti-NS5B and anti- β -actin antibodies were used for the immunoblotting analysis. (B) Cell cycle analysis of NKNT-3, HuH-7, and HeLa cells expressing NS5B (NS5B series) were synchronized, and cell cycle progression was analyzed as indicated in Fig. 1B. NKNT-3, HuH-7, and HeLa cells that were infected with the pCXbsr retrovirus were also analyzed as a control (Ctr series).

induced by NS5B. To evaluate this hypothesis, we examined whether or not PH/NS5B and NKNT-3 cells expressing NS5B (NK/NS5B) induce the expression of IFN- β . RT-PCR analysis

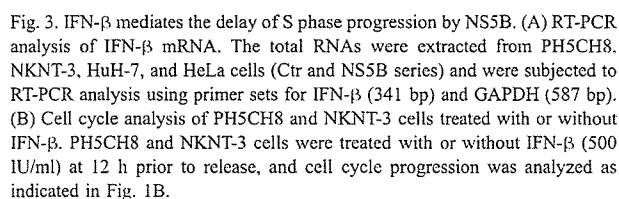
clearly indicated that they did, and, at the same time, we found that HuH-7 and HeLa cells did not, despite their expression of NS5B (Fig. 3A). We next examined whether or not S phase progression is delayed in PH5CH8 and NKNT-3 cells treated with IFN- β prior to release from the G1/S boundary. As we expected, the S phase progression was stalled in PH5CH8 and NKNT-3 cells treated with IFN- β (Fig. 3B). We also observed that IFN- γ did not possess this activity of IFN- β (data not shown). These results suggest that the induction of IFN- β is implicated in the cell cycle delay in two immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3.

To confirm the effect of IFN- β on cell cycle delay, we further examined whether or not treatment with anti-IFN- β neutralizing antibody can restore the cell cycle delay in PH/NS5B cells. The results showed that this treatment canceled the delay of S phase progression in PH/NS5B cells (Fig. 4A). BrdUrd incorporation analysis also showed that the proportion of cells reaching the G2-M phase was increased by the treatment with anti-IFN- β antibody in PH/NS5B cells (Fig. 4B). These observations indicated that the expression of IFN- β mediated cell cycle delay during the S phase in PH/NS5B cells and suggested that the expression of NS5B induced IFN- β in PH5CH8 and NKNT-3 cells even without replication of the viral genome.

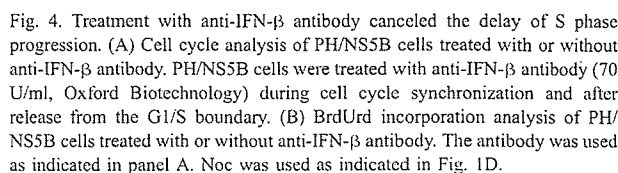
Activation of TLR3 signaling pathway by NS5B

Since IFN- β is known as a major cytokine induced by the activation of the TLR3 and TLR4 signaling pathways (Takeda et al., 2003), we next focused on which TLR pathway was activated for the production of IFN- β in PH/NS5B cells. To answer this question, TLR3- and TLR4-specific siRNAs were used to knock down TLR3 and TLR4 expression in PH/NS5B cells. TLR3 and TLR4 mRNAs were drastically decreased in PH/NS5B cells transfected with TLR3 and TLR4 siRNAs, respectively, but not in PH/NS5B cells transfected with GL2 siRNA (Elbashir et al., 2001) used as a control (Fig. 5A). This result indicates that the siRNAs used specifically contribute well to the degradation of TLR3 and TLR4 mRNAs. In this condition, IFN- β mRNA was significantly decreased in only PH/NS5B cells transfected with TLR3 siRNA (Fig. 5A), indicating that IFN- β expression in PH/NS5B cells is mediated through the TLR3 signaling pathway. The growth rate of PH/NS5B cells transfected with TLR3 siRNA was also accelerated, although TLR4 siRNA showed a rather lethal effect (Fig. 5B).

Fig. 1. HCV NS5B causes the delay of S phase progression. (A) Expression of HCV proteins in human cells introduced by retrovirus-mediated gene transfer. Western blot analysis of PH5CH8 cells infected with pCXbsr retroviruses encoding HCV proteins (HA-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B) or pCX4bsr retrovirus encoding NS5B. pCXbsr or pCX4bsr retrovirus was used as a control infection (Ctr). Anti-HA (3F10, Roche), anti-NS3 (Novacastra), anti-NS5B, and anti- β -actin (Sigma) antibodies were used for the immunoblotting analysis. (B) Cell cycle analysis of PH5CH8 cells expressing HCV proteins. PH5CH8 cells that expressed HA-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, or NS5B were synchronized at G1/S boundary, and then cell cycle progression was monitored by flow cytometry after the release of the cells into the S phase at the indicated times. PH/Ctr cells that were infected with pCXbsr retrovirus were also analyzed as a control. (C) Cell cycle analysis of PH5CH8 cells infected with pCX4bsr retrovirus encoding NS5B. The cell cycle analysis was performed as described in panel B. (D) BrdUrd incorporation analysis of PH/Ctr and PH/NS5B cells. Cell cycle distribution of dot-plots of BrdUrd fluorescence versus DNA contents was analyzed in asynchronous or synchronized PH/Ctr and PH/NS5B cells. To measure the cells reaching the G2-M phase at 12 h after release, the cells were accumulated by Noc treatment. (E) Analysis of the cells reaching the G2-M phase. The percentage of cells at that phase was assessed by Noc treatment as indicated in panel D. The data are means \pm SD of values from three independent experiments. (F) Growth curve of PH/Ctr and PH/NS5B cells. PH/Ctr (squares) or PH/NS5B (circles) were plated onto 6-well plates (3×10^4 cells per well), and the kinetics of cell proliferation during 4 days in culture were determined by trypan blue treatment. The data indicate average values \pm SD from three independent experiments.



To obtain further evidence that the induction of IFN- β by NS5B is mediated through TLR3, we prepared human embryonic kidney (HEK) 293 cells stably expressing TLR3 derived from PH5CH8 cells since it has been reported that ectopic expression of TLR3 can reconstruct the TLR3 signaling pathway in HEK293 cells (Alexopoulou et al., 2001). First, HEK293 cells were infected with retrovirus pCXbsr encoding NS5B or pCXbsr as a negative control (Ctr), yielding HEK293 cells (HEK/NS5B) stably expressing NS5B and control HEK293 cells (HEK/Ctr). Next, HEK/NS5B and HEK/Ctr cells were infected with



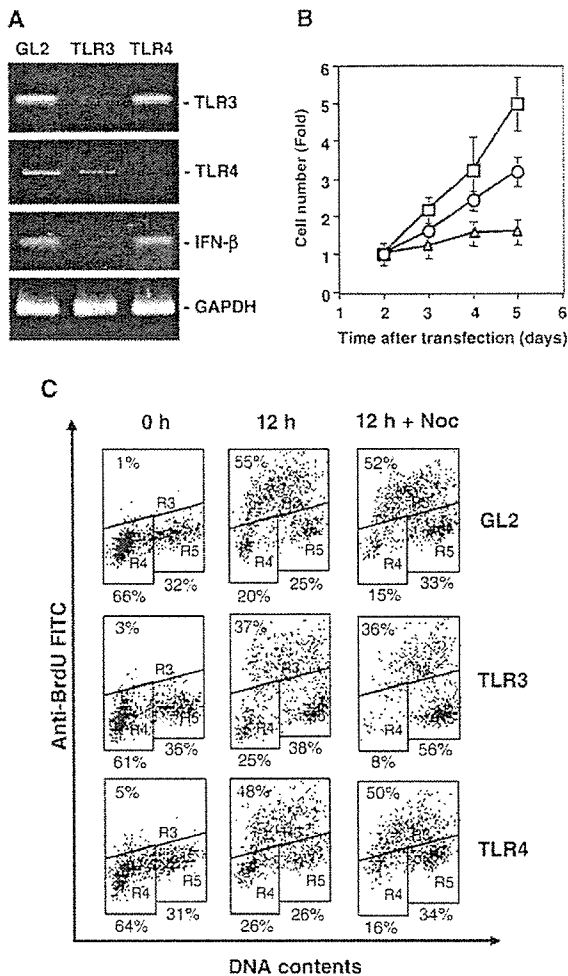


Fig. 5. Activation of IFN- β gene by NS5B is mediated through the TLR3 signaling pathway. (A) Down-regulation of IFN- β mRNA by transfection of TLR3 siRNA. PH/NS5B cells were transfected with dsRNA duplexes targeting TLR3, TLR4, or luciferase GL2. After 3 days, the expression levels of TLR3, TLR4, IFN- β , and GAPDH mRNAs were examined by RT-PCR. (B) Growth curve of PH/NS5B cells transfected with siRNAs. After 2 days of transfection, the proliferation kinetics of PH/NS5B cells transfected with GL2 (circles), TLR3 (squares), and TLR4 (triangles) siRNAs were analyzed as indicated in Fig. 1F. (C) BrdUrd incorporation analysis of PH/NS5B cells transfected with GL2, TLR3, and TLR4 siRNAs. After 2 days of transfection, the cells were synchronized, and cell cycle progression was analyzed as indicated in Fig. 1D.

retrovirus pCXpur encoding TLR3 or pCXpur as a negative control, yielding cells stably expressing TLR3 and control HEK293 cells. The expression of NS5B or TLR3 was confirmed by Western blot analysis (Fig. 6A). We then performed a dual-luciferase reporter assay using an IFN- β gene promoter. The results revealed that the luciferase activity was enhanced in only the HEK293 cells stably expressing both NS5B and TLR3 (Fig. 6B). This suggests that TLR3 mediates NS5B's induction of IFN- β . However, since the enhancement of luciferase activity was approximately two-fold, we failed to detect the enhancement of the mRNA expression levels for IFN- β and one of its target genes, ISG56 (data not shown). To accurately assess the

enhancement, high expression levels of NS5B and TLR3 in HEK293 cells will be needed.

The RIG-I-mediated signaling pathway is not implicated in the induction of IFN- β in PH/NS5B cells

Recently, RIG-I, a cellular DExD/H box helicase, was found to be a double-stranded RNA (dsRNA) binding protein that functions independently of TLR3 to induce IFN- β in response to viral infection (Yoneyama et al., 2004). Since another recent study showed that both the TLR3- and RIG-I-mediated signaling pathways are functional in PH5CH8 cells (Li et al., 2005a, 2005b), we examined whether or not the RIG-I-mediated signaling pathway is involved in NS5B's induction of IFN- β . First, PH/NS5B and PH/Ctr cells were infected with retrovirus pCXpur encoding myc-tagged RIG-IC, a dominant negative inhibitor of RIG-I harboring only the helicase domain but not the two N-terminal CARD domains (Yoneyama et al., 2004), or pCXpur as a negative control. Cells that stably expressed myc-tagged RIG-IC were thus obtained. The expression of myc-

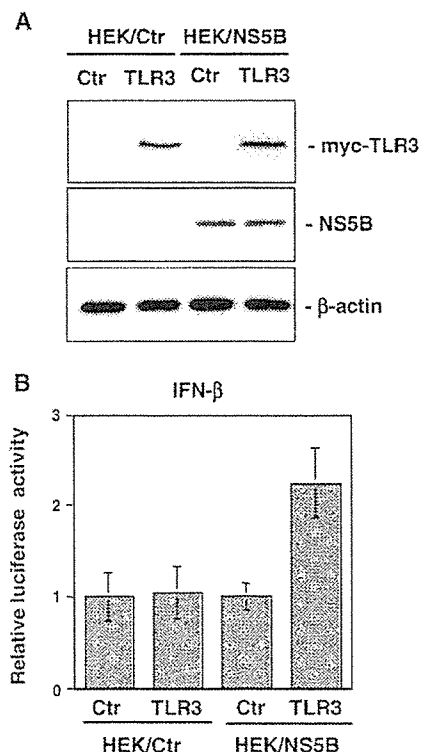


Fig. 6. Ectopic expression of TLR3 enhances the IFN- β gene promoter in only the HEK293 cells stably expressing NS5B. (A) Expression of TLR3 and NS5B in HEK293 cells introduced by retrovirus-mediated gene transfer. Western blot analysis of HEK/Ctr or HEK/NS5B cells infected with pCXpur retrovirus encoding myc-tagged TLR3 was performed. The pCXpur retrovirus was used as a control infection. Anti-myc, anti-NS5B, and anti- β -actin antibodies were used for the immunoblotting analysis. (B) Dual luciferase reporter assay of the IFN- β gene promoter. The cells shown in panel A were transfected with pIFN- β (-125)-Luc, and the dual luciferase assay was performed as described previously (Dansako et al., 2003). Data are means \pm SD from three independent triplicate experiments.