Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus

Florese, Ruth H
Nagano-Fujii, Motoko
Iwanaga, Yasuhiro
Hidajat, Rachmat
Hotta, Hak

(Citation)
Virus Research,90(1-2):119-131

(Issue Date)
2002-12

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000062

Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus

Ruth H. Florese [a], Motoko Nagano-Fujii [a], Yasuhiro Iwanaga [a], Rachmat Hidajat [a], Hak Hotta [a,b,*]

[a] Department of Microbiology, and [b] International Center for Medical Research, Kobe University Graduate School of Medicine. 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

* Corresponding author.

Phone: +81-78-382-5500. Fax: +81-78-382-5519. E-mail address: hotta@kobe-u.ac.jp.

Abstract

Possible inhibitory effects of hepatitis C virus (HCV) proteins on cellular protein synthesis were analyzed using transient expression system. The core protein, the nonstructural protein 4A (NS4A) and NS4B, but not NS3, NS5A or NS5B, inhibited p21/Waf1 expression post-transcriptionally. Further analysis revealed that the inhibition by NS4A and NS4B was mediated at least partly, if not entirely, at the translation level. NS4A-mediated translational inhibition was counteracted to some extent by NS3 co-expressed either in *trans* or *cis*. Co-expression of NS4A and NS4B exerted an additive effect on the translational inhibition. The N-terminal two-thirds of NS4A (amino acids 1-40) was shown to be involved in the translational inhibition. We also tested possible inhibitory effects of NS4A and NS4B on synthesis of other cellular proteins in parallel with p21/Waf1. NS4A and NS4B inhibited p21/Waf1 most strongly, followed by RNase L, p53, a C-terminally truncated form of CREB-RP and 2'-5' oligoadenylate synthetase. p21/Waf1, RNase L and p53 are known to have the PEST (proline-glutamic acid-serine-threonine) motif with relatively high scores in their sequences and considered to be sensitive to intracellular degradation. Taken together, our results suggest that NS4A and NS4B each mediate translational inhibition and, probably, increased degradation of certain cellular proteins.

Key words: Hepatitis C virus; NS4A; NS4B; Translational inhibition; Degradation; Endoplasmic reticulum stress.

1. Introduction

Hepatitis C virus (HCV), a member of Genus *Hepacivirus*, Family *Flaviviridae*, is recognized as the major causative agent of parenterally transmitted and community acquired non-A, non-B hepatitis (Choo et al., 1989). Majority of the patients infected with HCV contract persistent infection that often leads to chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Shimotohno, 1995; Houghton, 1996; Seeff, 1997; Gordon et al., 1998; Farci et al., 2000). An estimated population of 170 million people throughout the world are infected with HCV, with average prevalence being 2-3% (Lauer & Walker, 2001).

The HCV genome is a positive-sense, single-stranded linear RNA molecule of ~9.6 kb in length and consists of a 5' non-coding region, a long open reading frame (ORF) and a 3' non-coding region. The large ORF encodes a polyprotein of about 3, 000 amino acids (aa), which is proteolytically processed into at least 10 structural and nonstructural proteins in the order NH_2-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Grakoui et al., 1993; Mizushima et al., 1994; Hijikata et al., 1996; Houghton et al., 1996). The viral genome exhibits significant genetic heterogeneity, based on which HCV isolates are now classified into 6 distinct but related clades (genotypes) and more than 60 subtypes (Apichartpiyakul et al., 1994; Mellor et al., 1995; Doi et al., 1996; Soetjipto et al., 1996; Robertson et al., 1998).

NS4A exhibits versatile functions (Reed and Rice, 2000). This ~7 kDa protein is localized in the endoplasmic reticulum (ER) (Kim et al., 1999; Wölk et al., 2000) and has several hydrophobic residues in its N-terminal and central segments. It forms a stable complex with the NS3 proteinase through binding to its N-terminal sequence (Bartenschlager

et al., 1995; Failla et al., 1995; Satoh et al., 1995). It has been shown that NS4A enhances the enzymatic activity of the NS3 serine proteinase to cleave the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions of the viral polyprotein (Bartenschlager et al., 1995; Failla et al., 1995; Tanji et al., 1995). NS4A was also shown to inhibit the other HCV-encoded proteinase, NS2-NS3 autocatalyzing proteinase (Darke et al., 1999). It is not known whether NS4A exerts the inhibitory effect through binding to NS3, or through binding to NS2, as reported previously (Flajolet et al., 2000).

NS4B is a hydrophobic 27 kDa protein, which also is localized in ER (Hügle et al., 2001). In pestivirus, a related member of the *Flaviviridae* family, NS4B was found to be involved in viral cytophatogenecity (Qu et al., 2001). NS4B of HCV, in the context of NS4B/NS5A polyprotein, forms a complex with NS4A (Lin et al., 1997). Also, NS3, NS4A and NS4B were reported to modulate hyperphosphorylation of NS5A (Koch et al., 1999; Neddermann et al., 1999) and they form a complex with NS5B, the RNA-dependent RNA polymerase (Ishido et al., 1998). All these interactions suggest that NS4A and NS4B, together with NS3, NS5B and other HCV proteins, form a multisubunit machinery essential for viral replication.

Apart from their roles in virus replication, not much is known about the possible effects of NS4A and NS4B on cellular functions. NS4B was shown to enhance Ha-*ras* oncogene-mediated malignant transformation of NIH3T3 cells (Park et al., 2000). Other investigators recently reported that NS4A and NS4B suppressed translation *in vivo* and inhibited cell proliferation (Kato et al., 2002). We have been independently studying the possible effects of NS4A and NS4B on host cell functions. In the present paper, we report that NS4A and NS4B, but not NS3, NS5A or NS5B, post-transcriptionally inhibited synthesis

of p21/Waf1 and some other cellular proteins. Effects of the other HCV proteins on the inhibitory activities of NS4A and NS4B are also discussed.

2. Materials and methods

2.1. Expression plasmids

The entire NS4A region of HCV-1bJ cDNA (Kaneko et al., 1994; Tanji et al., 1995) was amplified by PCR using a set of primers, J4A-1-S and J4A-2-AS (Table 1). Likewise, a cDNA fragment encoding the full-length NS4B of HCV-1b (M094AJ strain) (Song et al., 1999) was amplified by PCR using the primers, NS4B-1-S and NS4B-2-AS. The amplified fragments were each subcloned into the unique *Eco*RI site of pSG5 expression vector (Stratagene) to generate pSG5-NS4A-F and pSG5-NS4B, respectively. The pSG5 vector is designed to express a protein of interest under the regulation of the simian virus 40 early promoter or the T7 promoter. cDNA fragments encoding various deletion mutants of NS4A were also amplified using appropriate combinations of the following primers; J4A-1-S, J4A-40-AS, J4A-18-S and J4A-2-AS, and subcloned into pSG5 to generate pSG5-NS4A(1-40), pSG5-NS4A(18-40), and pSG5-NS4A(18-54) (Fig. 1).

The entire NS3 region flanked with NS4A was amplified from two different cDNA clones, HCV-1bJ and M094AJ, by using a set of primers, J3-1-S and J4A-2-AS. These fragments were each subcloned into pSG5 to generate pSG5-NS3/4A(1bJ) and pSG5-NS3/4A(94AJ), respectively (Fig. 1). pSG5-NS3ΔC was used to express a C-terminally deleted mutant of NS3 (Fujita et al., 1996; Muramatsu et al., 1997; Ishido et al., 1997, 1998; Ishido & Hotta, 1998).

The entire coding sequence excluding the 3 amino terminal residues of mouse 2'-5'

oligoadenylate synthetase (2'-5'-OAS) (Ichii et al., 1986), the entire coding sequence of human RNase L (Zhou et. al, 1993) tagged with the HA epitope of influenza virus, and a C–terminally deleted mutant of CREB-RP (Min et al., 1995) tagged with a short peptide FLAG were each subcloned to pSG5 to generate pSG5-2'-5'-OAS, pSG5-RNaseL and pSG5-CREB-RP(N) (N stands for nuclear form), respectively. Described elsewhere were pSG5-Core (Wang et al., 2000), pSG5-NS3, pSG5-NS5A, pSG5-NS5B (Ishido et al., 1997, 1998; Ishido and Hotta, 1998; Muramatsu et al., 1997), pSG5-p21 and pSG5-p53 (Wang et al., 2000; Ishido et al., 1997; Ishido and Hotta, 1998; Muramatsu et al., 1997).

A plasmid harboring chloramphenicol acetyltransferase (CAT) reporter gene under the regulation of human p21/Waf1 promoter, pWWP-CAT, was reported previously (el-Diery et al., 1995).

2.2. Cell culture and transfection

NIH3T3 and Ltk$^-$ mouse fibroblast cells, and HuH-7 human hepatoma cells were cultivated in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% calf serum. The cells were transfected with the expression plasmids using FuGENE^{TM}-6 (Roche Diagnostics), according to the manufacturer's protocol. In co-transfection experiments, total amounts of transfected DNA were adjusted equal by adding the vector plasmid in each set of experiments to assure equal transfection efficiency.

2.3. Immunoblot analysis

Cells were lysed with gel loading buffer containing 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 10% glycerol, and incubated at 95°C for 10 min. Lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically blotted onto a polyvinylidene difluoride

filter (PVDF; Biorad). The filters were blocked with phosphate-buffered saline (PBS) containing 5% skim milk and reacted with appropriate mouse monoclonal antibody (mAb) or human serum directed against proteins of interest. Antibodies used were mouse mAb against p21/Waf1 (C187, Santa Cruz Biotech Inc., Santa Cruz, CA; Waf1/Ab4, Calbiochem), p53 (Ab-1, Oncogene), FLAG (M2; Sigma) and HA (16B12, BabCO), and human serum strongly reacting with NS4B. Filters were washed with PBS containing 0.5% Tween 20 and reacted with peroxidase-labeled antibody against either mouse or human IgG. The protein bands in the filters were visualized using enhanced chemiluminescence method (ECL, Amersham-Pharmacia Biotech).

2.4. CAT assay

Cells were harvested 48 h post transfection and disrupted by sonication (140 W, 2 min) in 250 mM Tris-HCl (pH 7.8). Cell extracts were collected by centrifugation at 10,000 rpm for 5 min and assayed for CAT activity, as described previously (Hotta et al., 1992). Briefly, 20 μl of cell extract was mixed with 0.05 μCi of ^{14}C-chloramphenicol, 0.5 mM acetyl coenzyme A and 250 mM Tris-HCl (pH 7.8) and incubated at 37˚C for 5 h. The reaction mixture was extracted with ethyl acetate and loaded onto a silica gel for thin layer chromatography. Acetylated and non-acetylated forms of chloramphenicol were visualized by autoradiography and radioactivity was measured using BAS 2000 analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2.5. Semi-quantitative reverse transcription (RT)-PCR analysis

Total cellular RNA was extracted using TRIZOL LS (Life Technologies, Gaithesburg, MD) and treated with 10 units of RNase-free DNase I (Takara Shuzo, Co., Ltd., Kyoto, Japan) at 37˚C for 30 min to remove plasmid DNA possibly contaminating the samples. The

RNA was then reverse transcribed using Rous associated virus 2 reverse transcriptase (Takara Shuzo) and random hexamer primer (Takara Shuzo) for 10 min at room temperature followed by incubation at 42˚C for 1 h. The resultant cDNA was used for PCR over a range of cycles each consisting of 1 min at 95˚C, 1 min at 53˚C, and 1.5 min at 72˚C. Specific primers used to amplify CAT and p21/Waf1 mRNA are shown in Table 1. As an internal control, mRNA for a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), was amplified using specific primers in parallel experiments. The primers used are also shown in Table 1.

2.6. Northern blot analysis

Total cellular RNA extracted as above was subjected to 1% formaldehyde-agarose gel electrophoresis and blotted onto a nylon filter. The filters were hybridized with DIG-labeled p21/Waf1 cDNA probe (PCR DIG Probe Synthesis Kit, Boehringer Mannheim) in DIG Easy Hyb buffer (Boehringer Mannheim) at 50ºC overnight. The filters were then washed under a stringent condition as suggested by the manufacturer's protocol and an RNA band that hybridized with the probe was visualized using DIG luminescent ready-to-use detection kit (Boehringer Mannheim).

2.7. In vitro translation assay

The pSG5 vector, pSG5-NS4A-F, pSG5-NS4B, pSG5-NS5B and pSG5-p21 were linearized with *Xba*I and *in vitro* transcription was performed using Ribomax RNA Production System-T7 (Promega). The transcription reaction was carried out in the presence of ^{7}mGpppG (New England Biolabs) to synthesize capped RNA. The reaction mixtures were digested with RNase-free DNase I to remove the template DNA and the synthesized mRNA was purified using the RNEasy Mini Kit (Qiagen). The purified RNA was then used for *in*

vitro translation. A constant amount of p21/Waf1 RNA (1 μg) was mixed with increasing amounts of RNA for NS4A, NS4B or NS5B (as control) plus rabbit reticulocyte lysates and translation reaction was performed according to the manufacturer's protocol. The translated products were analyzed by immunoblotting, as described above.

3. Results

3.1. NS4A and NS4B each inhibit synthesis of p21/Waf1 and CAT proteins

p21/Waf1 was transiently expressed with or without HCV proteins in HuH-7 cells, and amounts of the proteins were analyzed by immunoblotting. The amount of p21/Waf1 protein was smaller in cells co-expressing each of Core, NS4A and NS4B than in the cells expressing p21/Waf1 alone (Fig. 2A), suggesting that Core, NS4A and NS4B each inhibited p21/Waf1 synthesis. On the other hand, such inhibition was not observed with NS3, NS5A or NS5B. The inhibitory effects of Core and NS4A were nearly at the same level, and stronger than that of NS4B (Fig. 2B). The inhibition of p21/Waf1 synthesis by Core, NS4A and NS4B was reproducibly observed in separate experiments using HuH-7, NIH3T3 and Ltk^- cells (data not shown). Moreover, inhibition of protein synthesis by Core, NS4A and NS4B was consistently observed using CAT assay (data not shown). We previously reported the inhibition of p21/Waf1 synthesis by Core (Yoshida et al., 2001). In the following analyses, therefore, we focused on NS4A and NS4B.

3.2. NS4A and NS4B each inhibit gene expression post-transcriptionally

To examine the possible mechanism involved in the NS4A- and NS4B-mediated inhibition, we first determined transcription levels. Cells were co-transfected with pWWP-CAT and pSG5-NS4A-F, or pWWP-CAT and pSG5 vector, as in the case of CAT

assay, and steady-state CAT mRNA levels were measured by semi-quantitative RT-PCR analysis. The result showed that amounts of CAT mRNA did not differ between NS4A-expressing cells and the control (Fig. 3A). An equal amount of total cellular RNA in each sample was verified by RT-PCR for G3PDH.

Similar results were obtained with NS4B. In this experiment, cells were co-transfected with (i) pSG5-p21 and pSG5-NS4B, (ii) pSG5-p21 and pSG5 vector or (iii) pSG5 vector alone, and steady-state p21/Waf1 mRNA levels were measured. RT-PCR analysis revealed no difference in p21/Waf1 mRNA levels between NS4B-expressing cells and the control (Fig. 3B). It should be noted that plain PCR without RT did not amplify the target sequence, demonstrating the specificity of our RT-RCR procedure for detection of p21/Waf1 mRNA. Northern blot analysis confirmed that there was no significant inhibition by NS4B of p21/Waf1 mRNA levels (Fig. 3C). These results strongly suggest that NS4A and NS4B each inhibited p21/Waf1 gene expression post-transcriptionally.

3.3. NS4A and NS4B each inhibit in vitro translation of capped p21/Waf1 RNA

We then performed *in vitro* translation assay using capped p21/Waf1 RNA. Immunoblot analysis of the translated products demonstrated significant inhibition of p21/Waf1 RNA translation by NS4A and NS4B (Fig. 4A and B). With NS4A, nearly a maximum level of inhibition was achieved even at 1:1 ratio of RNA. On the other hand, a dose-dependent inhibition was observed with NS4B over the same RNA ratios tested. The control protein, NS5B, showed no detectable effect on p21/Waf1 RNA translation in parallel experiments. It should be noted that increasing amounts of NS4A (ratios 1:1, 1:4 and 1:7) and NS4B (ratios 1:4 and 1:7) were verified by immunoblot analysis using the respective antibodies (data not shown). These results suggest that NS4A and NS4B inhibit protein

synthesis at the translation level.

3.4. Effect of other HCV proteins on translational inhibition by NS4A and NS4B

It has been well documented that NS4A forms a stable complex with NS3 (Bartenschlager, et al., 1995; Failla, et al., 1995; Satoh et al., 1996). To determine whether or not NS3 influences NS4A-mediated translational inhibition, various forms of NS3 were expressed with NS4A, either in *trans* (NS3ΔC plus NS4A-F) or *cis* (NS3/4A). The inhibitory effect of NS4A-F was significantly negated when co-expressed with NS3ΔC in *trans* (Fig. 5A). NS3/4A of the HCV-1bJ strain [NS3/4A(1bJ)] was shown to exhibit the serine proteinase activity that cleaved the polyprotein into NS3 and NS4A whereas NS3/4A of the M094AJ strain [NS3/4A(94AJ)] was enzymatically inactive and remained uncleaved (data not shown). When expressed in *cis* in the form of NS3/4A(1bJ), the inhibitory effect of NS4A was further negated. However, the uncleaved polyprotein mutant, NS3/4A(94AJ), still showed a considerable level of inhibition. These results suggest that interaction with NS3, either in *cis* or *trans,* significantly negated the NS4A-mediated inhibition of protein synthesis.

NS4B in the context of NS4B/NS5A polyprotein was reported to form a complex with NS4A (Lin et al., 1997), and together with other HCV proteins, they form RNA polymerase complex. To see the possible influences on the inhibitory effect of NS4B, the other HCV proteins, such as Core, NS3, NS4A, NS5A and NS5B, were expressed in *trans* and amounts of p21/Waf1 protein were analyzed by immunoblotting. As shown in Fig. 5B, both Core and NS4A showed additive effects on NS4B-mediated translational inhibition. This was not the case with NS3, NS5A or NS5B; these latter proteins did not show a significant influence on the inhibitory action of NS4B. Under this experimental condition, NS4B expression was not

significantly affected by co-expression of the other HCV proteins, except that NS3 enhanced NS4B expression to some extent (data not shown).

3.5. Involvement of N-terminal sequence of NS4A in the translational inhibition

We then analyzed as to which portion(s) of NS4A was involved in the translational inhibition using CAT assay. The result revealed that NS4A(1-40), but not NS4A(18-54) or NS4A(18-40), brought about nearly the same degree of inhibition compared with NS4A-F (Fig. 6). Immunofluorescence analysis revealed that the expression levels of NS4A(1-40), NS4A(18-54) and NS4A-F were basically the same (data not shown). On the other hand, NS4A(18-40) consistently showed a lower expression level, due possibly to instability of the protein. The results suggested that an N-terminal 40 residues of NS4A were sufficient, with the C-terminal 14 residues being unnecessary, to mediate the inhibitory effect.

3.6. NS4A and NS4B preferentially inhibit synthesis of certain cellular proteins

We also analyzed the possible inhibitory effects of NS4A and NS4B on synthesis of the other cellular proteins, such as p53, CREB-RP(N), RNase L and 2'-5'-OAS. p21/Waf1 was included in parallel experiments. Results obtained with NS4A revealed more than 95% inhibition of p21/Waf1 protein synthesis, followed by RNase L (76%), p53 (67%), CREB-RP(N) (58%), and 2'-5'-OAS (~40%) (Fig. 7A, B). Similarly, NS4B inhibited p21/Waf1 protein synthesis most strongly, with the reduction being 95%, followed by RNase L (65%), p53 (30%) and CREB-RP(N) (30%) (Fig. 7C, D). Again, 2'-5'-OAS was least affected, showing no reduction upon co-expression with NS4B under this experimental condition. The amount of NS4B was practically the same between cells expressing NS4B alone and those co-expressing NS4B and each of p21/Waf1, p53, CREB-RP(N), RNase L and 2'-5'-OAS (data not shown).

4. Discussion

We previously reported post-transcriptional inhibition of p21/Waf1 expression by Core using vaccinia virus T7 hybrid expression system (Yoshida et al., 2001). In the present study, we demonstrated, using a system without vaccinia virus, that NS4A and NS4B, but not NS3, NS5A or NS5B, also inhibited p21/Waf1 expression (Fig. 2). We looked into the possible mechanism(s) involved in the NS4A- and NS4B-mediated inhibition. There was no significant difference in steady-state p21/Waf1 mRNA levels, as demonstrated by RT-PCR and Northern blot analyses (Fig. 3). *In vitro* translation assay revealed inhibited synthesis of p21/Waf1 protein when co-translated with NS4A or NS4B RNA (Fig. 4). Taken together, the present results suggest that NS4A and NS4B inhibit p21/Waf1 expression at the translation level. It should be noted that, under this experimental condition, nearly a maximum level of inhibition was achieved by NS4A at 1:1 ratio of RNA. Considering the size of RNA and its translated product for NS4A (~7 kDa) and NS4B (27 kDa), and considering the result showing a stronger effect of NS4A than that of NS4B (Fig. 2), it would be natural that NS4B at 1:7 excess ratio was required to achieve a maximum degree of inhibition while NS4A did so at ratios of 1:1 to 1:4.

In the case of NS4A, the inhibition was mediated by an N-terminal 40 residues (aa 1-40), but not by a C-terminal 37 residues (aa 18-54) (Fig. 6). It has been reported that NS4A forms a stable complex with NS3 (Bartenschlager et al., 1995; Failla et al., 1995; Satoh et al., 1995; Kim et al., 1996; Lin et al., 1997) and that the central hydrophobic region of NS4A (aa 18-40 or more specifically, aa 22-34) is implicated to be essential for the complex formation with NS3 (Lin et al., 1995; Tanji et al., 1995; Shimizu et al., 1996) and with NS4B

(Lin et al., 1997). Our present results imply the possibility that the minimum NS3-binding domain alone is not sufficient for inhibition of protein synthesis. We also analyzed the possible influence of NS3 on NS4A-mediated inhibition of protein synthesis. When co-expressed with NS3 either in *trans* or *cis*, the inhibitory effect of NS4A-F was significantly, if not completely, negated (Fig. 5A). However, a mutant NS3/NS4A polyprotein that was incapable of cleaving the NS3/NS4A junction still inhibited protein synthesis to a considerable extent. It is reasonable to assume that different conformation of the N-terminal region of NS4A before and after the cleavage accounts for the observed functional difference.

Overexpression of a protein, in general, results in accumulation of unfolded/misfolded proteins in ER, causing ER stress. This ER stress mediates activation of certain enzymes, such as double stranded RNA-activated protein kinase (PKR), PKR-like ER kinase (PERK) (Harding et al., 1999, 2000; Mori, 2000; Bertolotti et al., 2001) and ER transmembrane kinase/ribonuclease (Ire1) (Iwawaki et al., 2001). Activated PKR/PERK phosphorylates eukaryotic initiation factor 2α (eIF2α) to inhibit protein synthesis while activated Ire1 mediates translational inhibition through cleavage of 28S ribosomal RNA. In addition, two other distinct signaling pathways have evolved to relieve the cells of the burden of misfolded proteins. One is the unfolded protein response that consists of transcriptional induction of genes encoding ER-resident molecular chaperones and folding enzymes, such as GRP78, GRP94 and protein disulfide isomerase (Harding et al., 1999; Mori, 2000; Patil et al., 2001). Through this mechanism, ER is equipped with increased capacity to properly fold newly synthesized proteins. The other one is the ER-associated degradation (ERAD) system that degrades misfolded proteins. ERAD requires Hrd1p/Der3p, an ER-resident,

membrane-anchored ubiquitin ligase that has an intrinsic preference for misfolded proteins (Travers et al., 2000; Bays et al., 2001). It is thus likely that not only inhibition of protein synthesis but also increased degradation of newly synthesized proteins take place in cells overexpressing NS4A and NS4B.

We observed that, in cells expressing NS4A or NS4B, p21/Waf1 was inhibited most preferentially, followed by RNase L, p53 and CREB-RP(N) (Fig. 7). 2'-5'-OAS was least affected by NS4A or NS4B. In this connection, the presence of the PEST motif in each protein may be another factor to consider. The PEST motif, which stands for proline (P), glutamic acid (E), serine (S) and threonine (T), targets the protein for rapid degradation (Wolfe, 1993). As shown in Table 2, RNase L has the PEST motif of the highest value (5.88), followed by p21/Waf1 (5.11), p53 (4.71) and CREB-RP(N) (1.06). It should be noted that the PEST motif of p21/Waf1 represents 11.6% (19 of 164 residues) of the entire sequence while that of RNase L 2.4% (18 of 741 residues). No PEST motif exists in 2'-5'-OAS.

Viruses employ certain mechanisms by which to inhibit translation of the host cell in order for viral mRNA to be translated preferentially (Knipe, 1996). In the case of HCV, Core was reported to inhibit translation of capped, but not uncapped, RNA although the precise mechanism of the inhibition remains to be elucidated (Mamiya and Worman, 1999). Since HCV RNA translation occurs via an internal ribosome entry site at the 5' end of the viral RNA and does not depend on the cap structure (Houghton, 1996), the inhibition by Core appears to be aimed at the host-cell translation process. Likewise, but probably through a different mechanism, NS4A and NS4B inhibit synthesis of certain cellular proteins preferentially to help establish better environment for virus replication and persistence.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), and a research grant from Research Foundation of Viral Hepatitis, Japan. This work was also part of a collaborative research project between Southeast Asian countries and Japan conducted by JSPS.

References

Apichartpiyakul, C., Chittivudikarn, C., Miyajima, H., Homma, M., Hotta, H., 1994. Analysis of hepatitis C virus isolates among healthy blood donors and drug addicts in Chiang Mai, Thailand. J. Clin. Microbiol. 32, 2276-2279.

Bartenschlager, R., Lohmann, V., Wilkinson, T., Koch, J.O., 1995. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J. Virol. 69, 7519-7528.

Bays, N.W, Gardner, R.G, Seelig, L.P, Joazeiro, C.A, Hampton, R.Y, 2001. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat. Cell Biol. 3, 25-29.

Bertolotti, A., Zhang, Y., Hendershot, L.M, Harding, H.P, Ron, D., 2000. Dynamic Interaction of BiP and ER stress transducers in the unfolded-protein response. Nat. Cell Biol. 2, 326-332.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359-362.

Darke, P.L., Jacobs, A.R., Waxman, L., Kuo, L.C., 1999. Inhibition of hepatitis C virus NS2/3 processing by NS4A peptides: Implications for control of viral processing. J. Biol. Chem. 274, 34511-34514.

Doi, H., Apichartpiyakul, C., Ohba, K.I, Mizokami, M., Hotta, H., 1996. Hepatitis C virus (HCV) subtype prevalence in Chiang Mai, Thailand and identification of novel subtypes of HCV major type 6. J. Clin. Microbiol. 34, 569-574.

el-Deiry, W.S., Tokino, T., Waldman, T. Oliner, J.D., Velculescu, V.E., Burrell, M., Hill, D.E., Healy, E., Rees, J.L., Hamilton, S.R., 1995. Topological control of $p21^{WAF1/CIP1}$ expression in normal and neoplastic tissues. Cancer Res. 55, 2910-291.

Failla., C., Tomei, L., De Francesco, R., 1995. An amino terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. J. Virol. 69, 1769-1777.

Farci, P., Shimoda, A., Coiana, A., Diaz, G., Peddis, G., Melpolder, J.C, Strazzera, A., Chien, D.Y, Munoz, S.J, Balestrieri, A., Purcell, R.H, Alter, H. J, 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 288, 339-342.

Flajolet, M., Rotondo, G., Daviet, L., Bergametti, F., Inchauspe, G., Tiollais, P., Transy, C., Legrain, P., 2000. A genomic approach of hepatitis C virus generates a protein interaction map. Gene 242, 369-379.

Fujita, T., Muramatsu, S., Itoh, M., Hotta, H., 1996. Suppression of actinomycin D-induced apoptosis by the NS3 protein of hepatitis C virus. Biochem. Biophys. Res. Commun. 229, 825-831.

Gordon, S.C., Bayati, N., Silverman, A.L., 1998. Clinical outcome of hepatitis C as a function of mode of transmission. Hepatology 28, 562-567.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67, 1385-1395.

Harding, H.P, Zhang, Y., Ron, D., 1999. Protein translation and folding are coupled by an endoplasmic reticulum-resident kinase. Nature 397, 271-274.

Harding, H.P, Zhang, Y., Bertolotti, A., Zeng, H., Ron, D., 2000. Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol.

Cell 5, 897-904.

Hijikata, M. Kato, N., Ootsuyama, Y., Nakagawa, M., Shimotohno, K., 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. USA 88, 5547-5551.

Hotta, H., Miyamoto, H., Hara, I., Takahashi, N., Homma, M., 1992. Genomic structure of the ME491/CD63 antigen gene and functional analysis of the 5'-flanking regulatory sequences. Biochem. Biophys. Res. Commun. 185, 436-442.

Houghton, M., 1996. Hepatitis C viruses. In: B.N. Fields, D.M. Knipe, and P.M. Howley (Eds), Virology, 3rd Edn., Lippincott-Raven, Philadelphia, pp 1035-1058.

Hügle, T., Fehrmann, F., Bieck, E., Kohara, M., Krausslich, H.G., Rice, C.M, Blum, H.E, Moradpour, D., 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. Virology 284, 70-81.

Ichii, Y., Fukunaga, R., Shiojiri, S., Sokawa, Y., 1986. Mouse 2-5A synthetase cDNA: nucleotide sequence and comparison to human 2-5A synthetase. Nucleic Acids Res. 14, 10117.

Ishido, S., Muramatsu, S., Fujita, T., Iwanaga, Y., Tong, W.Y., Katayama, Y., Itoh, M., Hotta, H., 1997. Wild-type, but not mutant–type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus. Biochem. Biophys. Res. Commun. 230, 431-436.

Ishido, S., Fujita, T., Hotta, H., 1998. Complex formation of NS5B with NS3 and NS4A proteins of hepatitis C virus. Biochem. Biophys. Res. Commun. 244, 35-40.

Ishido, S., Hotta, H., 1998. Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. FEBS Lett. 438, 258-262.

Iwawaki, T., Hosoda, A., Okuda, T., Kamigori, Y., Nomura-Furuwatari, C., Kimata, Y.,

Tsuru, A., Kohno, K., 2001. Translational control by the ER transmembrane kinase/ribonuclease IRE1 under ER stress. Nat. Cell Biol. 3, 158-164.

Kaneko, T., Tanji, Y., Satoh, S., Hijikata, M., Asabe, S., Kimura, K., Shimotohno, K., 1994. Production of two phosphoproteins from the NS5A region of the hepatitis C genome. Biochem. Biophys. Res. Commun. 205, 320-326.

Kato, J., Kato, N., Yoshida, H., Ono-Nita, S.K., Shiratori, Y., Omata, M., 2002. Hepatitis C virus NS4A and NS4B proteins suppress translation in vivo. J. Med. Virol. 66,187-199.

Kim, J.L., Morgestern, K.A., Lin, C., Fox, T., Dwyer, M.D., Landro, J.A., Chambers, S.P., Markland, W., Lepre, C.A., O' Malley, E. T., Harbeson, S.L., Rice, C.M., Murcko, M.A., Caron, P.R., Thomson, J.A., 1996. Crystal structure of hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell 87, 343-355.

Kim, J.E., Song, W.K., Chung, K.M., Back, S.H., Jang, S.K., 1999. Subcellular localization of hepatitis C viral proteins in mammalian cells. Arch. Virol. 144, 329-343.

Knipe, D.M., 1996. Virus-host cell interactions. In: B.N. Fields, D.M. Knipe, and P.M. Howley (Eds.), Virology, 3rd Edn., Lippincott-Raven, Philadelphia, pp 273-299.

Koch, J.O., Bartenschlager, R., 1999. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A and NS4B. J. Virol. 73, 7138-7146.

Lauer, G.M., Walker, B.D., 2001. Hepatitis C virus infection. N. Engl. J. Med. 345, 41-52.

Lin, C., Thomson, J.A., Rice, C.M., 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex *in vivo* and i*n vitro*. J. Virol. 69, 4373-4380.

Lin, C., Wu, J.W., Hsiao, K., Su, M.S., 1997. The Hepatitis C virus NS4A protein:

interactions with the NS4B and NS5A proteins. J. Virol. 71, 6465-6471.

Mamiya, N., Worman, H.J., 1999. Hepatitis C virus core protein binds to a DEAD box RNA helicase. J. Biol. Chem. 274, 15751-15756.

Mellor, J., Holmes, E.C., Jarvis, L.M., Yap, P.L. Simmonds, P., The International HCV Collaborative Study Group, 1995. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. J. Gen. Virol, 76, 2493-2507.

Min, J., Shukla, H., Kozono, H., Bronson, S.K, Weissman, S.M, Chaplin, D.D, 1995. A novel CREB family gene telomeric of HLA-DRA in the HLA complex. Genomics 30, 149-156.

Mizushima, H., Hijikata, M., Asabe, S., Hirota, M., Kimura, K., Shimotohno, K., 1994. Two hepatitis C virus glycoprotein E2 products with different C termini. J. Virol. 68, 6215-6222.

Mori, K., 2000. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101, 451-454.

Muramatsu, S., Ishido, S., Fujita, T., Itoh, M., Hotta, H., 1997. Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting the localization. J. Virol. 71, 4954-4961.

Neddermann, P., Clementi, A., De Francesco, R., 1999. Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B and NS5A encoded on the same polyprotein. J. Virol. 73, 9984-9991.

Park, J.S., Yang, J.M., Min, M.K., 2000. Hepatitis C virus nonstructural protein 4B transforms NIH3T3 cells in cooperation with the Ha-*ras* oncogene. Biochem. Biophys.

Res. Commun. 267, 581-587.

Patil, C., Walter, P., 2001. Intracellular signalling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr. Opin. Cell Biol. 13, 349-356.

Qu, L., McMullan, L.K, Rice, C.M, 2001. Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. J. Virol. 75, 10651-10662.

Reed, K.E., Rice, C.M., 2000. Overview of hepatitis C virus genome structure, polyprotein processing and protein properties. Curr. Top. Microbiol. Immunol. 242, 55-84.

Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shin-i, T., Simmonds, P., Smith, D., Stuyver, L., Weiner, A., 1998. Classification, nomenclature and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Arch. Virol. 143, 2493-2503.

Satoh, S., Tanji, Y., Hijikata, M., Kimura, K., Shimotohno, K., 1995. The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4A. J. Virol. 69, 4255-4260.

Seeff, L., 1997. Natural history of hepatitis C. Hepatology 26, 21S-28S.

Shimizu, Y., Yamaji, K., Masuho, Y., Yokota, T., Inoue, H., Sudo, K., Satoh, S., Shimotohno, K., 1996. Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. J. Virol. 70, 27-132.

Shimotohno, K., 1995. Hepatitis C virus as a causative agent of hepatocellular carcinoma. Intervirology 38, 162-169.

Soetjipto, Handajani, R., Lusida, M.I., Darmadi, S., Adi, P., Soemarto, Ishido, S., Katayama, Y., Hotta, H., 1996. Differential prevalence of hepatitis C virus subtypes in healthy blood donors, patients on maintenance hemodialysis, and patients with hepatocellular carcinoma in Surabaya, Indonesia. J. Clin. Microbiol. 34, 2875-2880.

Song, J., Fujii, M., Wang, F., Itoh, M., Hotta, H., 1999. The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. J. Gen. Virol. 80, 879-886.

Tanji, Y., Kaneko, T., Satoh, S., Shimotohno, K., 1995. Phosphorylation of hepatitis C virus- encoded nonstructural protein NS5A. J. Virol. 69, 3980-3986.

Travers, K.J, Patil, C.K, Wodicka, L., Lockhart, D.J, Weissman, J.S., Walter, P., 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101, 249-258.

Wang, F., Yoshida, I., Takamatsu, M., Ishido, S., Fujita, T., Oka, K., Hotta, H., 2000. Complex formation between hepatitis C virus Core protein and p21Waf1/Cip1/Sdi1. Biochem. Biophys. Res. Commun. 273, 479-484.

Wolfe, S., 1993. Molecular and Cellular Biology, pp. 720-721. Wadsworth, Inc. Belmont, California

Wölk, B., Sansonno, D., Krausslich, H.G., Dammacco, F., Rice, C.M., Blum, H.E., Moradpour, D., 2000. Subcellular localization, stability and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline regulated cell lines. J. Virol. 74, 2293-2304.

Yoshida, I., Oka, K., Hidajat, R., Fujii-Nagano, M., Ishido, S., Hotta, H., 2001. Inhibition of p21/Waf1/Cip1/Sdi1 expression by hepatitis C virus core protein. Microbiol. Immunol. 45, 689-697.

Zhou, A., Hassel, B.A., Silverman, R.H., 1993. Expression cloning of 2-5A dependent RNAse: a uniquely regulated mediator of interferon action. Cell 72, 753-765.

Figure Legends

Fig. 1. Schematic representation of the HCV genome and expression plasmids used in this study. Plasmids for full-length NS4A, its deletion mutants, full-length NS4B, NS3ΔC and two forms of the NS3/NS4A polyprotein are depicted. NS3/4A(1bJ) and NS3/4A(94AJ) indicate NS3 proteinase-active and -inactive polyproteins, respectively. Numbers along with the HCV genome and the expression plasmids indicate aa positions.

Fig. 2. Inhibition of p21/Waf1 expression by HCV proteins. (A) HuH-7 cells were transiently transfected with pSG-p21 and each of the expression plasmids for HCV proteins at 1:1 ratio of transfected DNA. The cells were analyzed by immunoblotting. (B) Relative intensity of the bands in (A) was measured. Mean values ± SD obtained from 3 independent experiments are shown. *, $P<0.001$, compared with control (p21 alone).

Fig. 3. Evidence for the lack of inhibition by NS4A and NS4B at the transcription level. (A) Ltk^- cells were co-transfected with pWWP-CAT and pSG5-NS4A-F (1:15 ratio), or pWWP-CAT and pSG5 as a control. Total cellular RNA was analyzed by semi-quantitative RT-PCR for CAT and G3PDH mRNA levels. Gel images of amplicons after 25, 30 and 35 cycles are shown. (B) NIH3T3 cells were co-transfected with (i) pSG-p21 and pSG-NS4B (1:2 ratio), (ii) pSG-p21 and pSG5 or (iii) pSG5 alone. Total cellular RNA was analyzed by semi-quantitative RT-PCR for p21/Waf1 and G3PDH mRNA levels. Gel images of amplicons after 20, 25 and 30 cycles are shown. (C) The cell lysates prepared as in (B) were subjected to Northern blot analysis using DIG-labeled cDNA probe

for p21/Waf1 mRNA. The 18S ribosomal RNA stained with ethidium bromide is shown to verify equal loading of total cellular RNA in each lane.

Fig. 4. Inhibition of *in vitro* translation by NS4A and NS4B. (A) Capped RNA for p21/Waf1, NS4A and NS5B was synthesized *in vitro* using T7 RNA polymerase and linearized respective plasmids as templates. A constant amount of p21/Waf1 RNA (1 μg) was subjected to *in vitro* translation in the presence of increasing amounts of RNA for NS4A-F (1, 4 and 7 μg) or NS5B (4 and 7 μg). The translated products were analyzed by immunoblotting. Relative intensity of the p21/Waf1 band for cells without NS4A expression (control) was expressed as 100%. Mean values ± SD from 4 independent experiments are shown. *, $P<0.005$, compared with control. (B) The same analysis as above was performed with NS4B using increasing amounts of RNA for NS4B (4 and 7 μg). Relative intensity of the p21/Waf1 band for cells without NS4B expression (control) was expressed as 100%. Mean values ± SD from 3 independent experiments are shown. *, $P<0.005$, compared with control.

Fig. 5. Effects of co-expressed HCV proteins on translational inhibition by NS4A and NS4B. (A) Ltk^- cells were transfected with 1 μg of pWWP-CAT with or without 10 μg of pSG5-NS4A-F in the absence or presence of pSG5-NS3ΔC, pSG5-NS3/4A(1bJ) (proteinase-active) or pSG5-NS3/4A(94AJ) (proteinase-inactive). Cell lysates were analyzed for CAT activities 48 h post transfection. CAT activity obtained in cells without NS4A expression (control) was expressed as 100%. Mean values ± SD of relative CAT activities from 3 independent experiments are shown. *, $P<0.05$; **, $P<0.01$. (B) HuH-7

cells were transfected with 1 μg of pSG5-p21 with or without 2 μg of pSG5-NS4B in the absence or presence of 2 μg of pSG5-Core, pSG5-NS3, pSG5-NS4A-F, pSG5-NS5A and pSG5-NS5B. Cell lysates were analyzed by immunoblotting 48 h post transfection. Relative intensity of the p21/Waf1 band for cells without NS4B expression (control) was expressed as 100%. Mean values ± SD from 3 independent experiments are shown. ***, $P<0.005$.

Fig. 6. Involvement of an N-terminal sequence of NS4A in the translational inhibition. Ltk^- cells were transfected with 1μg of pWWP-CAT and 10 μg of either pSG5-NS4A-F or its deletion mutants, such as pSG5-NS4A(1-40), pSG5-NS4A(18-54) and pSG5-NS4A(18-40). Cell lysates were analyzed for CAT activities 48 h post transfection. CAT activity obtained in cells without NS4A expression (control) was expressed as 100%. Mean values ± SD of relative CAT activities from 3 independent experiments are shown. *, $P<0.005$, compared with control.

Fig. 7. Inhibitory effects of NS4A and NS4B on synthesis of various cellular proteins. (A) HuH-7 cells were transfected with each of the expression plasmids for p21/Waf1, p53, CREB–RP(N), RNase L and 2'-5'-OAS in the absence or presence of pSG5-NS4A-F (1:1 ratio of transfected DNA) and analyzed by immunoblotting using antibodies to the respective cellular proteins. (B) Relative intensity of the bands in (A) was measured. The value for the target protein in cells without NS4A expression was arbitrarily expressed as 100%. Mean values ± SD from 3 independent experiments are shown. (C) The same analysis as above was done for NS4B. (D) Relative intensity of the bands in (C) was measured. Mean

values ± SD from 3 independent experiments are shown.

(Fig. 1) (Fig. 2) (Fig. 3) (Fig. 4) (Fig. 5) (Fig. 6) (Fig. 7) (Table 1) (Table 2)

Table 1

Primers used in this study

Primer name	HCV region	Sequence [a]	Nucleotide position
J4A-1-S	NS4A	5'-GGAGGAATTCGTC**ATG**AGCACCTGGGTGCT-3'	5301-5314
J4A-2-AS	NS4A	5'-GAGGAATTC**CTA**ACACTCTTCCATCTCATC-3'	5445-5462
J4A-18-S	NS4A	5'-TCTGGAATTCTAC**ATG**CTGACGACAGGCAG-3'	5352-5365
J4A-40-AS	NS4A	5'-AGAGGAATTC**CTA**GTCGGGAATAACAGCTG-3'	5404-5419
NS4B-1-S	NS4B	5'-CCGCGAATTCAAC**ATG**GCGTGGAGCAGTC-3'	5463-5475
NS4B-2-AS	NS4B	5'-GGGGAATTCGCC**ATG**GCGCCCATCACGGCCTAC-3'	6228-6245
J3-1-S	NS3	5'-GGGGAATTCGCC**ATG**GCGCCCATCACGGGCCTAC-3'	3408-3426
CAT-1-S	—	5'-TGTACCTATACCAGACCGT-3'	91-109
CAT-2-AS	—	5'-GCGGCATCAGCACCTTGT-3'	782-799
p21-1-S	—	5'-TATAGGATCCATGTCAGAACCGGCTGGG-3'	79-93
p21-2-AS	—	5'-TATAGAATTCAGGGCTTCCTCTTGGAGAA-3'	568-586
G3PDH-1-S	—	5'-ACCACAGTCCATGCCATCAC-3'	566-585
G3PDH-2-AS	—	5'-TCCACCACCCTGTTGCTGTA-3'	1017-1036

[a] The *Eco*RI recognition site is underlined. The translation initiation codon (ATG) and a complementary sequence of a stop codon (CTA) are written in boldface letters. S, sense; AS, antisense.

Table 2

Positions, sequences and values for the PEST motif in various cellular proteins

	PEST Region		
Protein	Position	Sequence	Value
p21/Waf1 (164)[a]	122-140	RSGEQAEGSPGGPGDSQGR	5.11
RNase L (741)	543-560	KAQSNEEVVQLSPDEETK	5.88
p53 (393)	24-65	KLLPENNVLSPLPSQAMDDLMLS PDDIEQWFTEDPGPDEAPR	4.71
CREB-RP(N) (392)	47-85	RCPEQDVPFDGSSLDVGMDVSPS EPPWELLPIFPDLQVK	1.06
2'-5'-OAS (367)	—	—	0

[a] Parenthesis indicates the total number of aa residues.

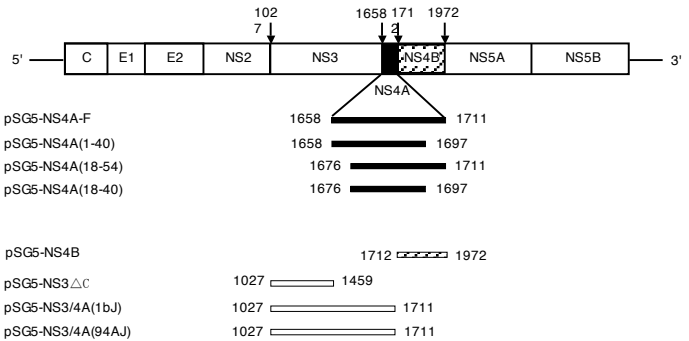

Fig. 1. Florese, R., et al.

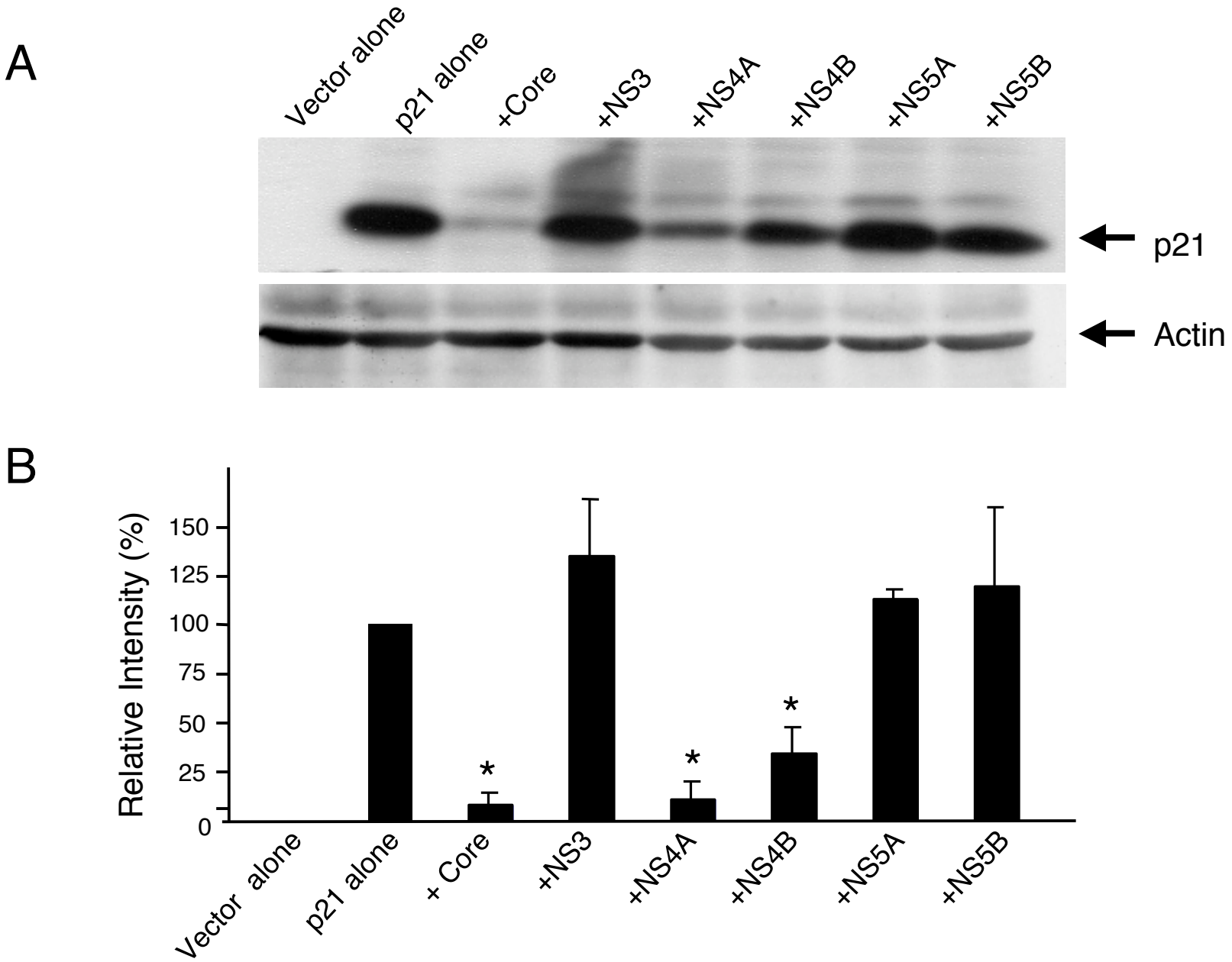

Fig. 2. Florese, R., et al.

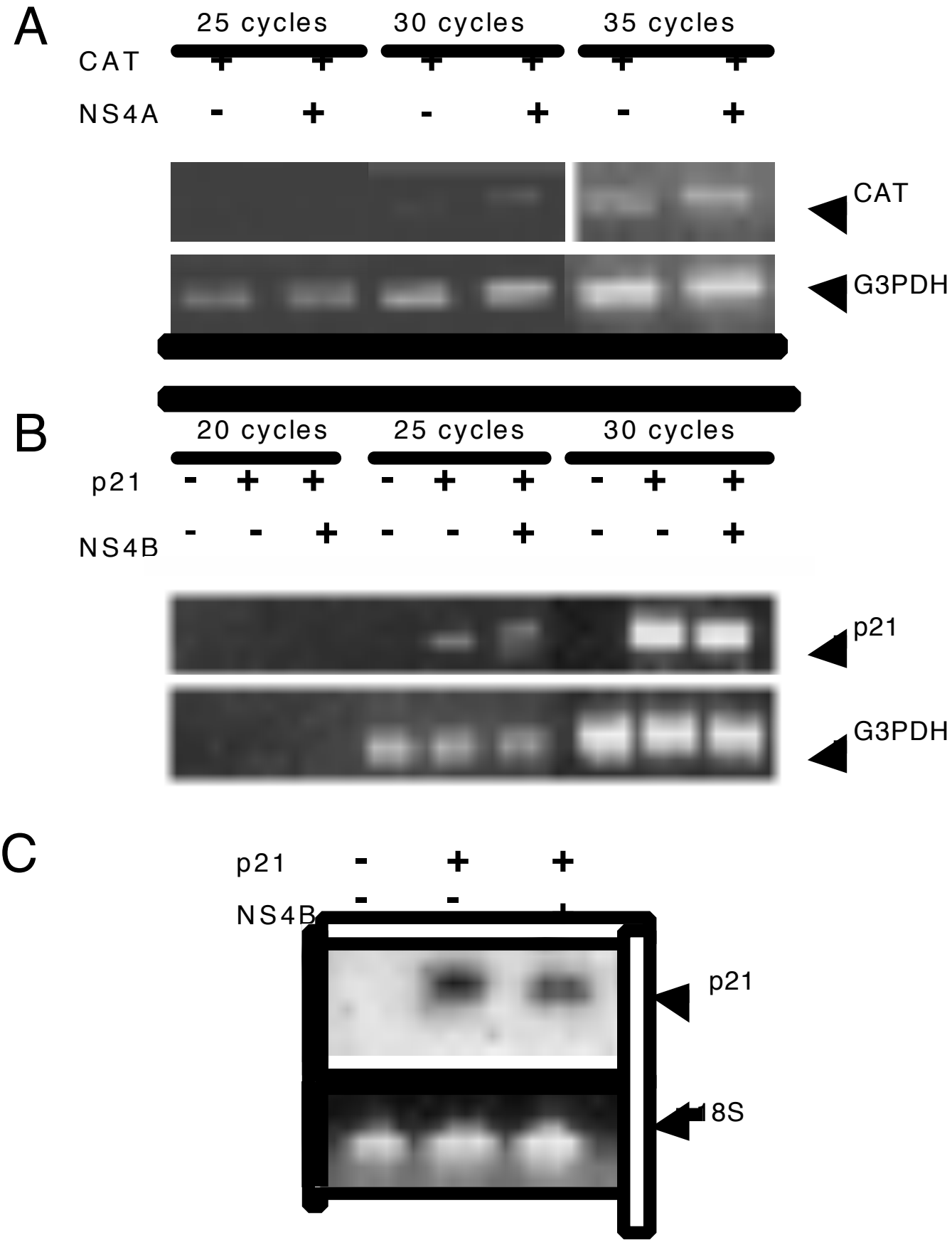

Fig.3. Florese, R., et al.

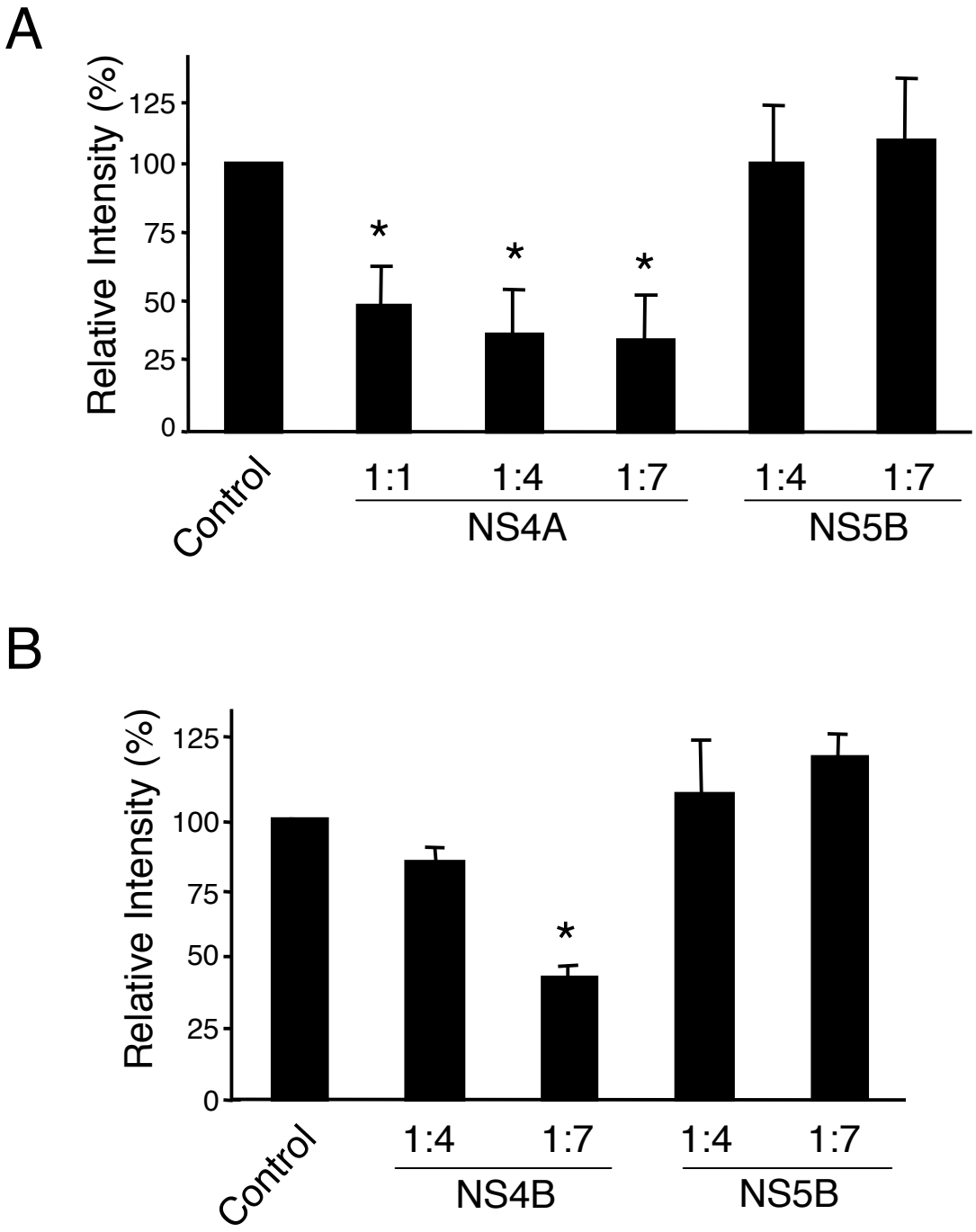

Fig. 4. Florese, R., et al.

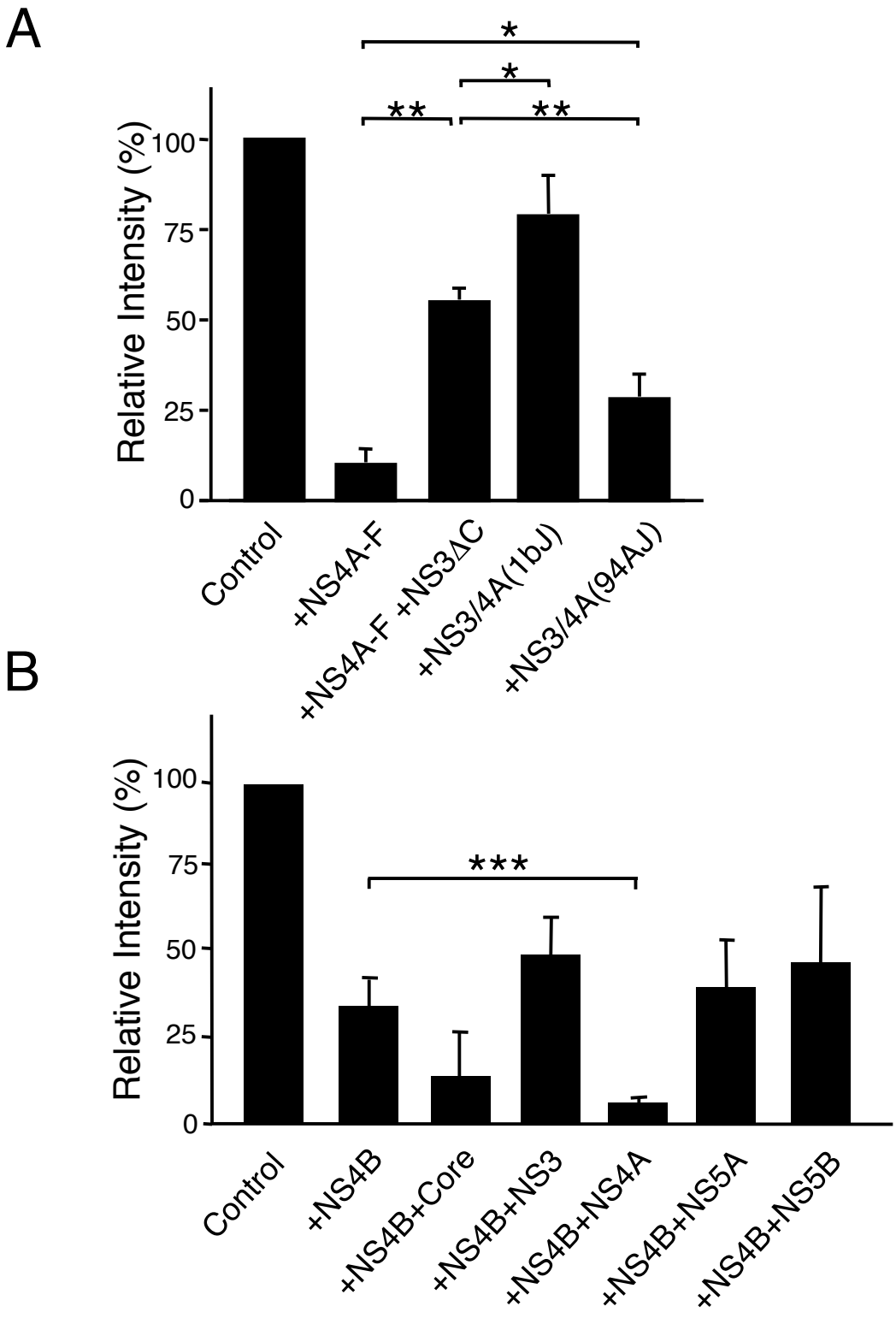

Fig. 5. Florese, R., et al.

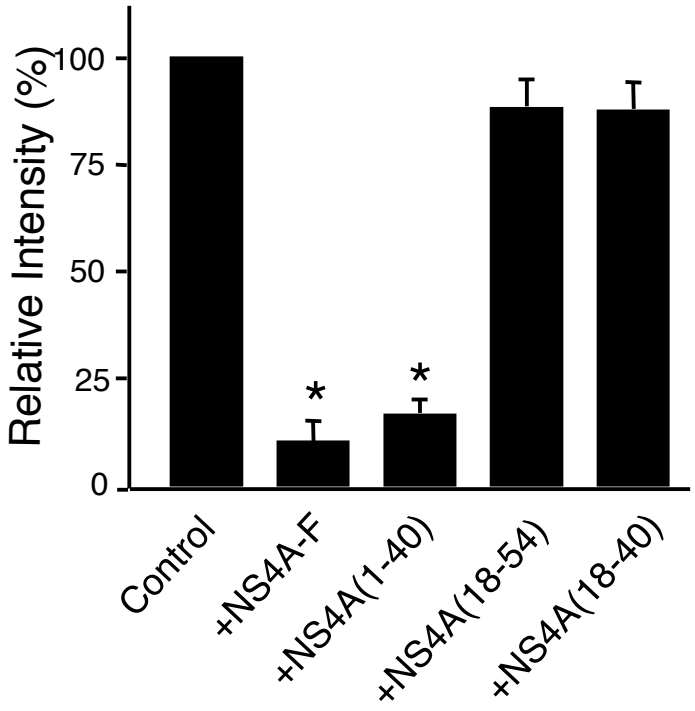

Fig. 6. Florese, R., et al.

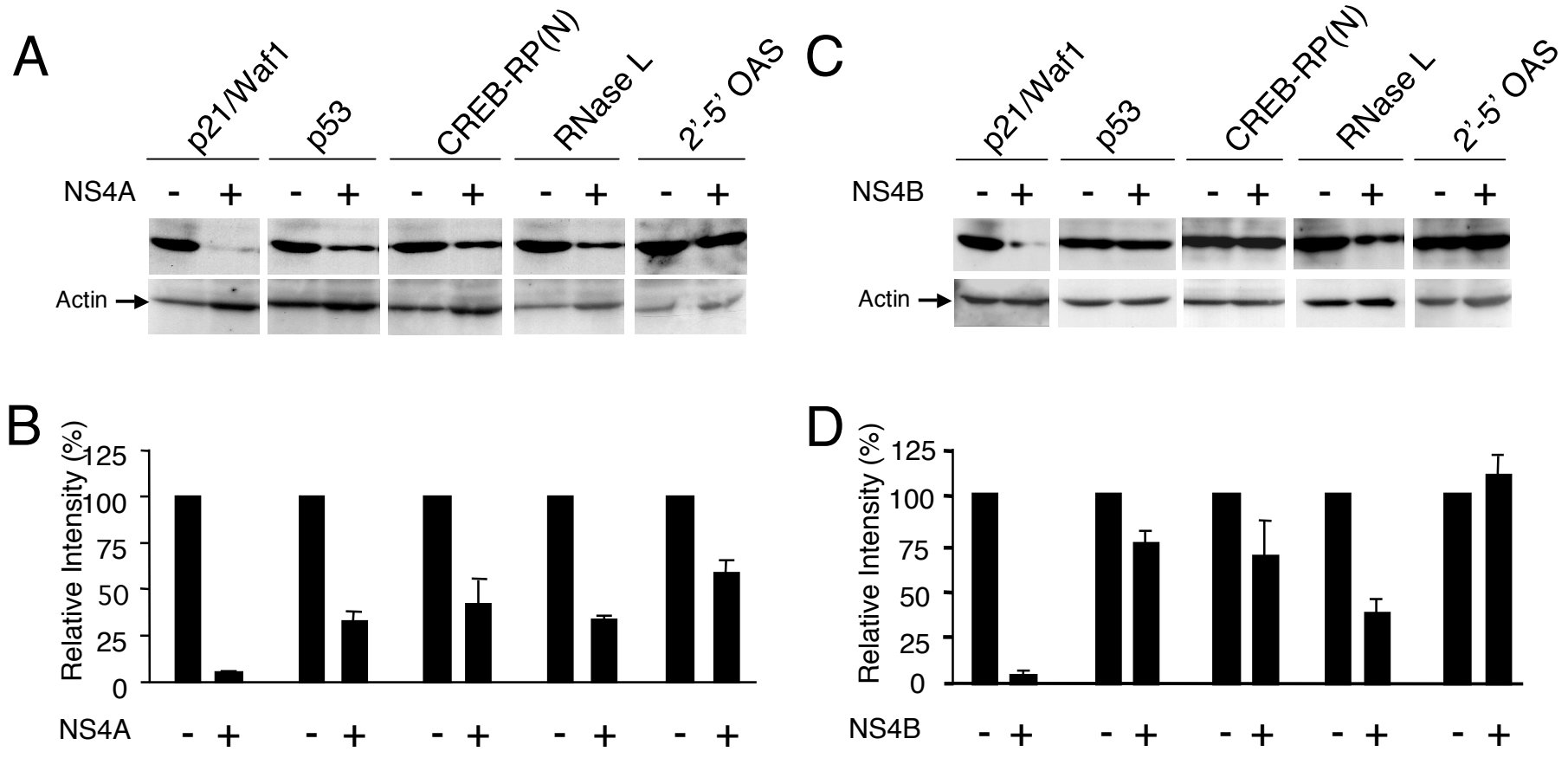

Fig. 7. Florese, R., et al.