

# Interferon- $\alpha$ inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway

Michael Frese,<sup>1</sup> Thomas Pietschmann,<sup>2</sup> Darius Moradpour,<sup>3</sup> Otto Haller<sup>1</sup> and Ralf Bartenschlager<sup>2</sup>

<sup>1</sup> Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Hermann-Herder-Str. 11, D-79104 Freiburg, Germany

<sup>2</sup> Institut für Virologie, Universität Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

<sup>3</sup> Abteilung Innere Medizin II, Medizinische Universitätsklinik Freiburg, Hugstetter Str. 55, D-79106 Freiburg, Germany

**Hepatitis C virus (HCV) persists in the majority of infected individuals and is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Chronic hepatitis C is currently treated with interferon (IFN)- $\alpha$  or with a combination of IFN- $\alpha$  and ribavirin. The availability of an HCV replicon system (Lohmann *et al.*, *Science* 285, 110–113, 1999) allowed the investigation of the effects of IFN on genuine HCV replication in cultured cells. It is shown here that IFN- $\alpha$  inhibits subgenomic HCV RNA replication in HuH-7 human hepatoma cells. Immunofluorescence, Western blot and Northern blot analysis revealed that levels of both HCV protein and replicon RNA were reduced after treatment with IFN- $\alpha$  in a dose-dependent manner. In further experiments, it was investigated whether MxA plays a role in the inhibition of HCV. The human MxA protein is an IFN-induced GTPase that has antiviral activity against various RNA viruses. However, HCV RNA replication was not affected in transfected HuH-7 cells that transiently overexpressed MxA. Moreover, a dominant-negative mutant of MxA did not interfere with the antiviral activity of IFN- $\alpha$  against HCV RNA replication. Taken together, these results demonstrate that IFN- $\alpha$  inhibits HCV replicons via an MxA-independent pathway.**

## Introduction

*Hepatitis C virus* (HCV) has been classified in the genus *Hepacivirus* of the family *Flaviviridae* (Robertson *et al.*, 1998). Different HCV isolates show a high degree of sequence diversity and phylogenetic analysis has revealed that six major genetic groups exist (Robertson *et al.*, 1998). Like all flaviviruses, HCV is an enveloped virus with a single-stranded RNA genome of positive polarity that expresses its proteins via translation of a single long open reading frame (reviewed in Reed & Rice, 2000; Bartenschlager & Lohmann, 2000). HCV is the most common aetiological agent of post-transfusion and sporadic non-A, non-B hepatitis (Choo *et al.*, 1989). In 1997, the World Health Organization estimated that more than 170 million people were infected with HCV worldwide (World Health Organization, 1997). Acute HCV infection is often subclinical or mild, but the virus persists in more than 75% of infected individuals. Chronic hepatitis C often progresses to

liver cirrhosis and eventually to hepatocellular carcinoma (reviewed in Hoofnagle, 1997; Theodore & Fried, 2000). Currently, chronic hepatitis C is treated with interferon (IFN)- $\alpha$  alone or in combination with ribavirin. Sustained response rates, however, are limited to 10–20% in the case of IFN- $\alpha$  monotherapy and 30–40% with IFN- $\alpha$  plus ribavirin combination therapy (Moradpour & Blum, 1999).

The antiviral effects of type I ( $\alpha/\beta$ ) IFNs are mediated by a number of effector proteins including double-stranded RNA-activated protein kinase (PKR), 2'-5' oligoadenylate synthetase (OAS) and Mx (reviewed in Stark *et al.*, 1998). Mx proteins are highly conserved, large GTPases that have been found in many mammalian, avian and fish species (reviewed in Arnheiter *et al.*, 1996; Leong *et al.*, 1998). The human MxA protein has antiviral activity against both negative- and positive-strand RNA viruses (for a review see Haller *et al.*, 1998). Transfected cells expressing MxA under the control of a constitutive promoter are highly resistant to various viruses of the families *Orthomyxoviridae* (Pavlovic *et al.*, 1990, 1992; Marschall *et al.*, 2000; Frese *et al.*, 1995), *Paramyxoviridae* (Schnorr *et al.*, 1993; Schneider-Schaulies *et al.*, 1994; Zhao *et al.*

**Author for correspondence:** Ralf Bartenschlager.

Fax +49 6131 393 5604. e-mail bartnsch@mail.uni-mainz.de

*al.*, 1996), *Rhabdoviridae* (Pavlovic *et al.*, 1990), *Bunyaviridae* (Frese *et al.*, 1996; Kanerva *et al.*, 1996) and *Togaviridae* (Landis *et al.*, 1998). Transgenic mice that constitutively express MxA are highly resistant to *Thogoto virus* (THOV), a tick-borne orthomyxovirus (Pavlovic *et al.*, 1995), and they exhibit increased resistance to *Influenza A virus*, *Vesicular stomatitis virus*, *La Crosse virus* and *Semliki Forest virus* (Pavlovic *et al.*, 1995; Hefti *et al.*, 1999), indicating that MxA plays an important role in IFN-induced antiviral defence against RNA viruses.

The mechanism(s) by which IFN- $\alpha$  inhibits HCV replication is presently not understood and may involve effects mediated by both the innate and the adaptive immune systems. Investigations have been hampered by the lack of efficient cell culture systems and small animal models permissive for HCV infection and replication. The recent development of selectable subgenomic RNAs (replicons) now allows studies on genuine HCV RNA replication in cell culture (Lohmann *et al.*, 1999). Here, we show that IFN- $\alpha$  efficiently inhibits the replication of HCV subgenomic RNAs in human hepatoma cells in a dose-dependent manner. Furthermore, we used the HCV replicon system to investigate whether MxA plays a role in the inhibition of HCV. Endogenous MxA that was expressed after stimulation with IFN- $\alpha$  as well as recombinant MxA that was transiently overexpressed in transfected cells did not inhibit HCV replicons. Therefore, we conclude that IFN-induced effector proteins other than MxA are responsible for the inhibition of HCV.

## Methods

■ **Cells and viruses.** HuH-7 human hepatoma cells and the HuH-7 cell clones 9-13, 5-15 and 11-7 (harbouring the HCV replicons I<sub>377</sub>/NS3-3', I<sub>389</sub>/NS3-3' and I<sub>377</sub>/NS2-3', respectively) have been described previously (Nakabayashi *et al.*, 1982; Lohmann *et al.*, 1999, 2001; Pietschmann *et al.*, 2001). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 200 U/ml penicillin G and 200 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. For cells that contained HCV replicons, the culture medium was additionally supplemented with 500–1000 µg/ml G418 (Geneticin, Life Technologies).

The Sicilian SiAr126 strain of THOV (Albanese *et al.*, 1972) was grown in BHK-21 cells. Stock virus contained  $8.3 \times 10^7$  p.f.u./ml.

■ **Plasmids.** The entire open reading frames of MxA (Aebi *et al.*, 1989) and MxA(T103A) (Ponten *et al.*, 1997) were amplified by PCR by using the forward primer 5'Hind-MxA (5' CGACAAAGCTTACCACCA-TGGTTGTTTCCGAAGTGGACATCG 3') and the reverse primer 3'Not-MxA (5' CGACAAAAGAGCGGCCGCTTAACCGGGGAAC-TGGGCAAG 3'). PCR products were digested with *Hind*III and *Not*I and inserted into the eukaryotic expression vector pSUPER-CATCH (kindly provided by C. M. Hovens, Institut für Medizinische Virologie, Universität Zürich, Switzerland) after restriction with the same enzymes. The resulting plasmids pSC-MxA and pSC-MxA(T103A) allow the expression of wild-type MxA and MxA(T103A) under the transcriptional control of the strong constitutive cytomegalovirus immediate-early promoter.

■ **Transfections.** Cells were transfected by using the Effectene reagent (Qiagen) as specified by the manufacturer. Note that the replication of HCV subgenomic RNAs was extremely sensitive to toxic or cytostatic effects caused by certain other transfection procedures (data not shown).

■ **Interferon treatment.** Recombinant human IFN- $\alpha$ 2 (kindly provided by K. Weyer and E. K. Weibel, Hoffmann-La Roche Ltd, Basel, Switzerland) and IFN- $\alpha$ B/D (a gift from Ciba Geigy Ltd, Basel, Switzerland) were used.

■ **Immunofluorescence analysis.** Cells grown on glass coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining was performed according to standard protocols. The NS5A protein of HCV was labelled by using the mouse monoclonal antibody (MAb) 11H (kindly provided by J. A. Hellings, Organon Teknika, Boxtel, The Netherlands). THOV proteins were labelled with the hyperimmune guinea pig antiserum gp457 (Jones & Nuttall, 1989) (kindly provided by P. A. Nuttall, NERC Institute of Virology and Environmental Microbiology, Oxford, UK) or with MAb 3D11, which is directed against the nucleoprotein (kindly provided by A. R. Filipe, Centre for Zoonoses Research, National Institute of Health, Lisbon, Portugal). MxA proteins were labelled by using either a polyclonal rabbit antibody directed against recombinant histidine-tagged MxA or MAb 2C12 (Staheli & Haller, 1985). Bound primary antibodies were visualized with goat antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or Cy3 (Dianova).

■ **Western blot analysis.** About  $8 \times 10^5$  parental HuH-7 cells and those of clone 9-13 were seeded into 8.5 cm diameter dishes. One day after seeding, the cell culture medium was replaced by medium containing 5000 U/ml IFN- $\alpha$ B/D (control cells were not stimulated with IFN but otherwise were treated identically). Three days after seeding, cells were harvested and total cell extracts were prepared in sample buffer (Laemmli, 1970). Protein samples were separated by SDS-PAGE, transferred to microporous PVDF membranes (Immobilon-P, Millipore) and immunostained according to standard protocols. The HCV proteins NS3, NS5A and NS5B were specifically labelled by using MAbs 1B6 (Wölk *et al.*, 2000), 11H (see above) and 5B-3B1 (D. Moradpour, unpublished results), respectively.

■ **Northern (RNA) blot analysis.** About  $3 \times 10^5$  cells of clone 9-13 were seeded into 6 cm diameter dishes and maintained in culture medium supplemented with 500 µg/ml G418. Three days after seeding, cells were washed once with PBS and the medium was replaced by medium without G418 but with 1000 U/ml IFN- $\alpha$ 2 (control cells were not stimulated with IFN but otherwise were treated identically). Cells were harvested at various time-points. Total RNA was prepared by the guanidinium thiocyanate-phenol-chloroform procedure (Chomczynski & Sacchi, 1987), denatured by treatment with 5.9% glyoxal in 50% DMSO and 10 mM sodium phosphate buffer, pH 7.0, separated by denaturing agarose gel electrophoresis and analysed by Northern blot following standard protocols (Ausubel *et al.*, 1997).

■ **Quantification of HCV replicon RNA.** Northern blot analysis was performed as described above. Prior to hybridization, the membrane was stained with methylene blue and cut roughly 1 cm below the 28S rRNA band. The upper part of the blot containing the HCV replicon RNA was hybridized with a <sup>32</sup>P-labelled, negative-sense riboprobe complementary to the internal ribosome entry site (IRES) of HCV and the *neo* gene. The lower strip was hybridized with a <sup>32</sup>P-labelled, antisense riboprobe to detect  $\beta$ -actin mRNAs. HCV- and  $\beta$ -actin-specific signals were quantified by phosphorimaging with a BAS 2500 scanner (Fuji). HCV signals were

corrected for total amounts of RNA loaded in each lane of the gel and compared to a serially diluted standard of *in vitro* transcripts of the HCV replicon in order to calculate the number of replicon molecules.

## Results

### IFN- $\alpha$ inhibits HCV subgenomic RNA replication

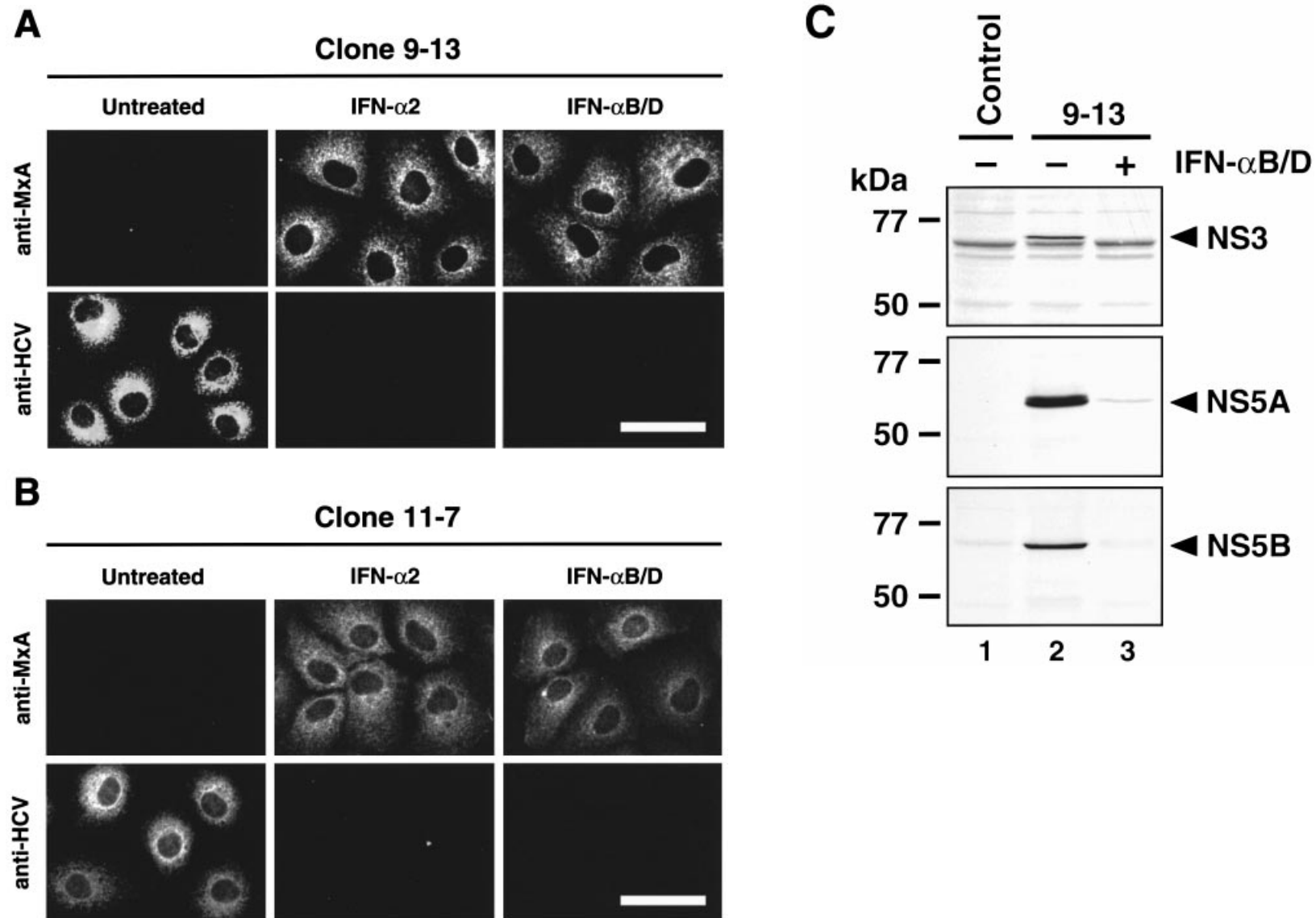
Recently, HCV replicons were transfected into human hepatoma (HuH-7) cells and several cell clones were selected that support high levels of HCV RNA replication and expression of HCV proteins (Lohmann *et al.*, 1999). For example, cells of clone 9-13 contain the HCV I<sub>377</sub>/NS3-3' replicon, which is composed of the HCV 5' non-translated region plus nucleotides 342–377 of the core coding region, the *neo* gene, encoding neomycin phosphotransferase, the IRES of *Encephalomyocarditis virus* (EMCV), the coding region of the HCV non-structural proteins NS3 to NS5B and the HCV 3' non-translated region. This RNA carries a cell culture-adaptive glycine-for-arginine substitution in NS5B at position 2884 of the HCV polyprotein that strongly increases the yield of replicon-harboring cell colonies and that enhances RNA replication (Lohmann *et al.*, 2001; N. Krieger, V. Lohmann and R. Bartenschlager, unpublished results).

In order to determine whether type I IFNs inhibit the replication of HCV subgenomic RNAs, cells of clone 9-13 were treated with 5000 U/ml IFN- $\alpha$ 2 or IFN- $\alpha$ B/D, fixed and double-immunostained for NS5A and MxA. Fig. 1(A) shows that NS5A expression was reduced dramatically in cells treated with IFN- $\alpha$ , whereas more than 95% of untreated control cells produced large amounts of the HCV protein (bottom panels). However, in a small number of IFN-treated cells (estimated to be fewer than 1%), weak NS5A-specific staining was observed (data not shown). In this experiment, MxA was used as a marker to assess the biological activity of the IFN preparations. As expected, treatment with IFN- $\alpha$  induced the expression of MxA in nearly 100% of the cells (upper middle and right panels). In contrast, MxA was not detectable in untreated, control cells (upper left panel). This indicates that the replication of subgenomic HCV RNAs did not induce the synthesis of type I IFN to a level sufficient to induce the expression of MxA. To exclude the possibility that the observed inhibition of HCV replicons was a peculiarity of cell clone 9-13, two additional cell clones were tested: (i) cell clone 11-7, carrying the HCV I<sub>377</sub>/NS2-3' replicon, which has the NS2–NS5B coding sequence with two adaptive mutations in NS3 and NS4B, at positions 1261 and 1846 (V. Lohmann and R. Bartenschlager, unpublished results); (ii) cell clone 5-15, carrying the HCV I<sub>389</sub>/NS3-3' replicon, which contains a part of the core coding sequence slightly longer than that in HCV I<sub>377</sub>/NS3-3' (Lohmann *et al.*, 1999). The replicon in this cell line has one adaptive mutation in NS5A, at position 2197 (N. Krieger, V. Lohmann and R. Bartenschlager, unpublished results). When tested for inhibition by IFN- $\alpha$ , a reduction in HCV protein expression similar to that observed with cell

clone 9-13 was found with cell clones 11-7 (Fig. 1B) and 5-15 (data not shown). Synthesis of HCV proteins was further assessed by the Western blot technique. Cells of clone 9-13 were treated with 5000 U/ml IFN- $\alpha$ B/D and total cell extracts were analysed with antibodies against NS3, NS5A and NS5B. None of the proteins were detectable after the IFN- $\alpha$  treatment, whereas untreated, control cells expressed easily detectable levels of NS3, NS5A and NS5B (Fig. 1C). These results indicate that HCV protein synthesis in general, and not only that of NS5A, is impaired after IFN- $\alpha$  treatment.

The effect of IFN- $\alpha$  on the replication of HCV subgenomic RNAs was also analysed by Northern blotting. Fig. 2(A, B) shows the results of a time-course experiment in which the number of HCV replicon molecules was measured at various time-points after treatment with IFN- $\alpha$ 2. Cells of clone 9-13 were trypsinized, split and seeded at low densities into new cell culture dishes. After 3 days of cultivation, the cells contained about  $2 \times 10^8$  HCV replicon molecules per  $\mu$ g total RNA (Fig. 2A; lanes 4 and 9). From then on, the cells were treated with 1000 U/ml IFN- $\alpha$ 2 or were left untreated. Within the next 48 h, the amount of replicon RNA nearly doubled in untreated, control cells, and then decreased slightly over the following 48 h (Fig. 2B). In contrast, a sharp decline in the amount of HCV RNA was found in IFN-treated cells within the first 24 h of treatment, followed by a slower reduction during the next 2 days. At this time, the number of replicon molecules corresponded to  $0.2 \times 10^8$  per  $\mu$ g total RNA, which is 1/10 of the original amount and about 1/20 of the amount found in untreated, control cells. It should be noted that the increase in replicon RNA levels observed in untreated cells is due to a tight coupling between RNA replication and host cell growth. We found that HCV protein expression and replication are highest in growing cells and lowest in resting cells (Pietschmann *et al.*, 2001). To substantiate our findings, we also analysed the replication of HCV I<sub>377</sub>/NS2-3' and HCV I<sub>389</sub>/NS3-3' replicons. As expected, the amounts of these HCV RNAs also dropped after IFN- $\alpha$  treatment, with kinetics similar to those observed with the HCV I<sub>377</sub>/NS3-3' replicon (data not shown).

Dose-response curves for IFN- $\alpha$  were established in order to determine the efficiency with which IFN- $\alpha$  induced the inhibition of replication of HCV subgenomic RNAs. Cells that had been seeded 3 days before the experiment were incubated with various concentrations of IFN- $\alpha$ 2 for 48 h and the amount of HCV replicon RNA was analysed by Northern blotting. As exemplified for clone 9-13, dose-dependent inhibition of the HCV replicon was found (Fig. 2C). Interestingly, quantitative analysis of the data revealed a biphasic decline in replicon RNA levels (Fig. 2D). Drastic effects on HCV RNA levels were already observed with 25 U/ml, whereas a further increase in the IFN- $\alpha$ 2 concentration resulted in only moderate further inhibition of the HCV replicon. Similar response curves were observed with replicons in cell lines 5-15 and 11-7 and when cells were treated with IFN- $\alpha$  under conditions used for



**Fig. 1.** HCV protein synthesis is inhibited in HuH-7 cells after treatment with IFN- $\alpha$ . (A)–(B) Double immunofluorescence analysis of NS5A and MxA expression in HuH-7 cells harbouring HCV replicons after treatment with IFN- $\alpha$ . Cells that contain the HCV I<sub>377</sub>/NS3-3' replicon (clone 9-13) (A) or the HCV I<sub>377</sub>/NS2-3' replicon (clone 11-7) (B) were seeded onto glass coverslips, cultured for 12 h and incubated for a further 60 h in the absence of IFN (Untreated; left panels), in the presence of 5000 U/ml human IFN- $\alpha$ 2 (middle panels) or in the presence of 5000 U/ml human IFN- $\alpha$ B/D (right panels). Cells were then fixed and immunostained for NS5A (upper panels) or MxA (bottom panels) using the NS5A-specific MAb 11H or a rabbit polyclonal antibody directed against MxA. Bars, 50  $\mu$ m. (C) Detection of HCV proteins by Western blot analysis. Parental HuH-7 control cells (lanes 1) and cells of clone 9-13 (lanes 2 and 3) were seeded into cell culture dishes. After 24 h, the cells were treated with 5000 U/ml IFN- $\alpha$ B/D (lanes 3) or were left unstimulated (lanes 1 and 2). Cell extracts were prepared 3 days later and samples of 20  $\mu$ g protein were analysed. HCV proteins NS3, NS5A and NS5B were detected with the MAbs 1B6 (upper panel), 11H (middle panel) and 5B-3B1 (lower panel), respectively. Positions of molecular mass markers are shown on the left.

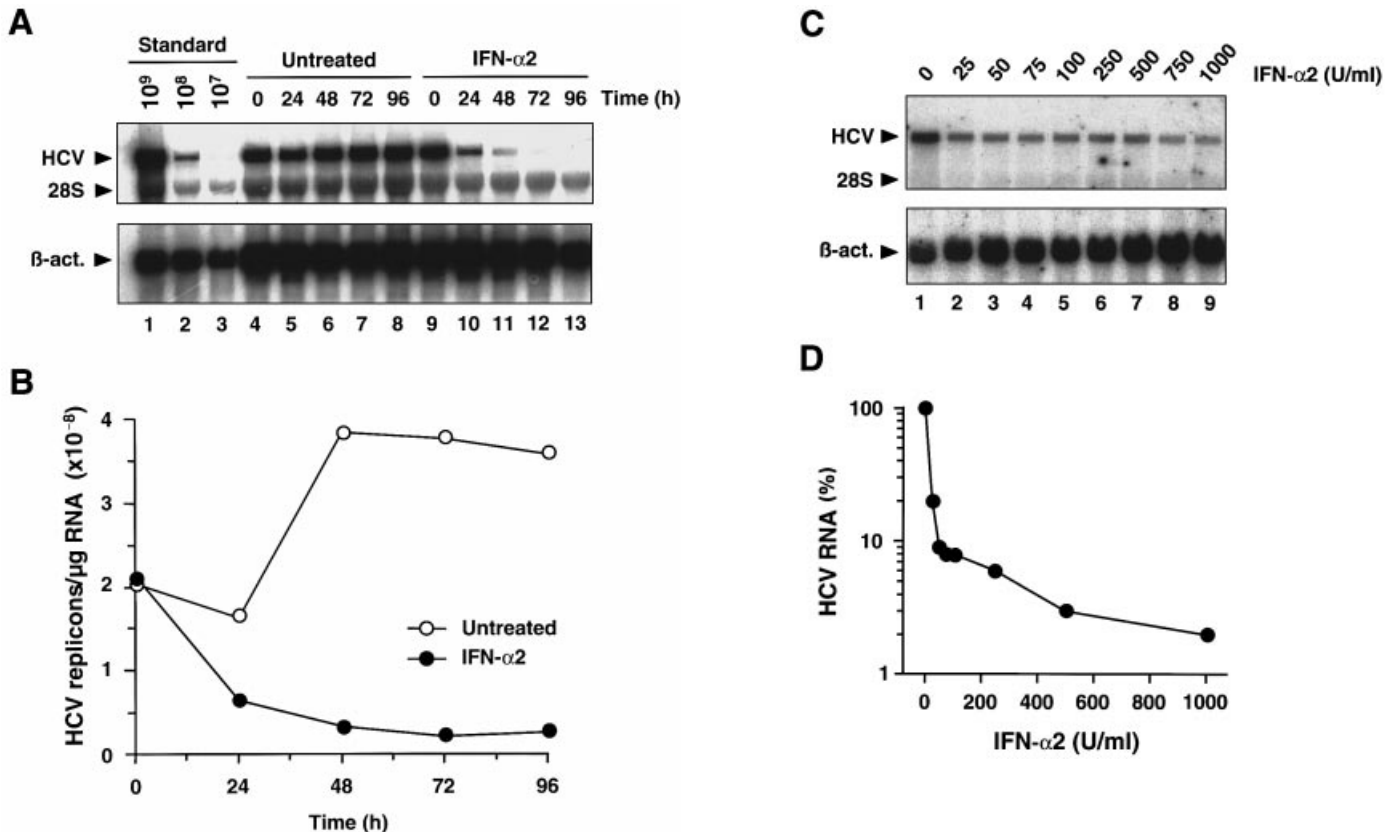


Fig. 2. Inhibition of HCV replicons by IFN- $\alpha$ . (A) Northern blot analysis of HCV replicon RNAs in cells of clone 9-13 at various time-points after treatment with IFN- $\alpha$ . Cells that had been seeded 3 days before the experiment were incubated for 0, 24, 48, 72 and 96 h in the absence (lanes 4–8) or in the presence (lanes 9–13) of 1000 U/ml IFN- $\alpha$ 2. Total RNA was prepared and 2  $\mu$ g RNA per lane was analysed with a <sup>32</sup>P-labelled, negative-sense riboprobe complementary to the IRES of HCV and the *neo* gene (upper panel) or to  $\beta$ -actin (lower panel). For comparison, known quantities of HCV replicon *in vitro* transcripts were analysed (lanes 1–3). Note that this standard RNA is a mixture of *in vitro* transcripts and 2  $\mu$ g total RNA from untransfected HuH-7 cells without HCV replicons. The positions of HCV replicon RNA, 28S rRNA and  $\beta$ -actin mRNA ( $\beta$ -act.) are indicated on the left. (B) HCV- and  $\beta$ -actin-specific bands of the Northern blots shown in (A) were quantified by phosphorimaging and the number of replicon molecules was determined by comparison with the serial dilution of *in vitro* transcripts. The number of HCV replicon molecules per  $\mu$ g total RNA was plotted against the time of treatment. (C) Northern blot analysis of HCV replicon RNAs in cells of clone 9-13 after treatment with various concentrations of IFN- $\alpha$ . Cells that had been seeded 3 days before the experiment were incubated for 48 h in the absence (lanes 1) or in the presence (lanes 2–9) of 25–1000 U/ml IFN- $\alpha$ 2. RNA was prepared and analysed as described above. (D) Quantification of the Northern blots shown in (C). HCV replicon RNA levels were corrected for the amounts of total RNA using the  $\beta$ -actin hybridization signal. Subsequently, the HCV replicon RNA levels of untreated control cells were compared with that found in cells after treatment with IFN- $\alpha$ . The percentage reduction was calculated and plotted against the IFN- $\alpha$  concentration.

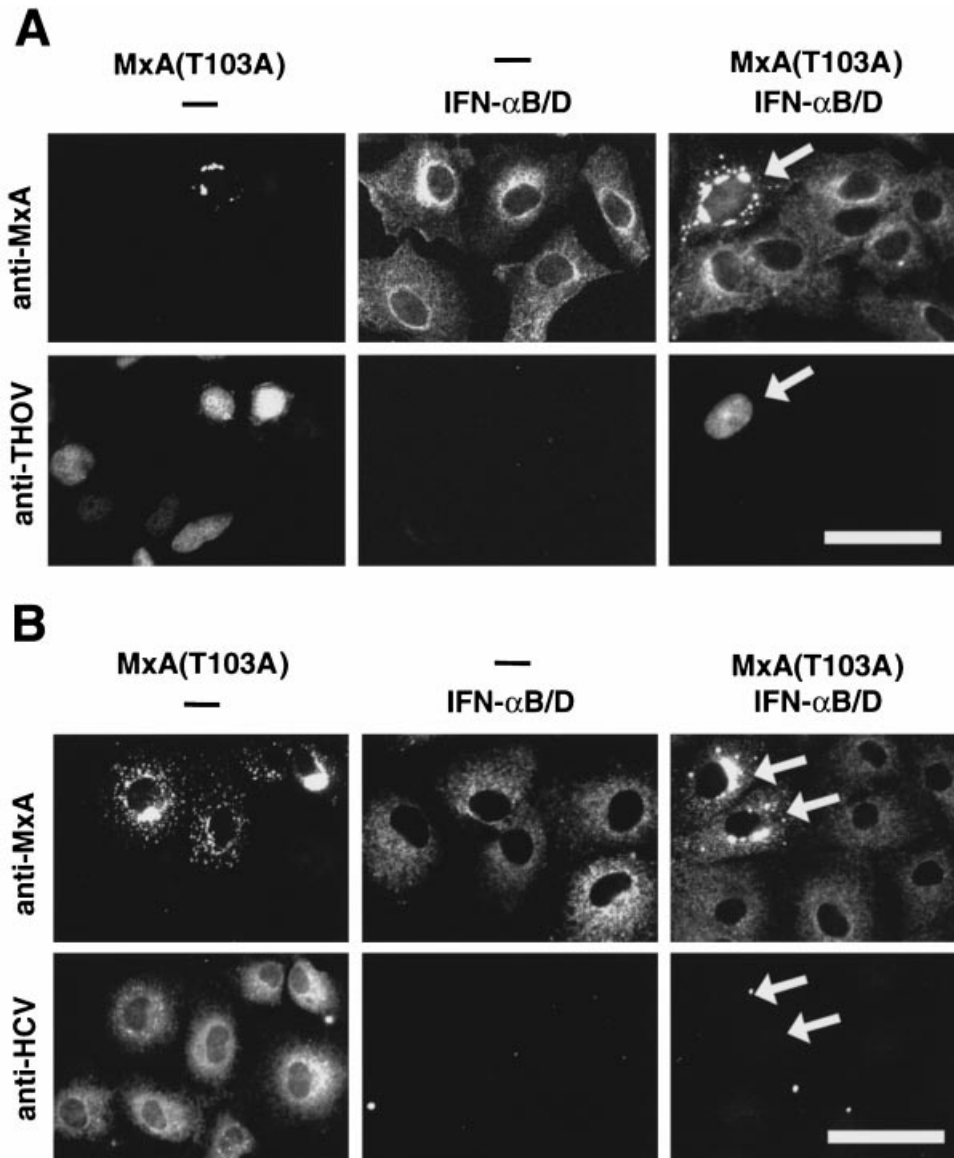
Western blot analysis (data not shown). Taken together, our results demonstrate that the replication of HCV subgenomic RNAs is highly sensitive to type I IFN-mediated cellular defence mechanisms.

### MxA does not inhibit the replication of HCV subgenomic RNAs

The ability of Mx proteins to inhibit the multiplication of various RNA viruses (Haller *et al.*, 1998) prompted us to investigate their role in cellular defence against HCV. Keskinen *et al.* (1999) reported that HuH-7 cells express MxA and MxB after stimulation with high doses of IFN- $\alpha$ . To confirm their findings, HuH-7 cells were treated with 1000 U/ml IFN- $\alpha$ B/D,

total cell extracts were prepared 12, 24 and 36 h later and the expression of MxA and MxB was analysed by Western blotting. As expected, HuH-7 cells responded quickly to stimulation with IFN- $\alpha$ . MxA and MxB expression had already reached maximum levels 12 h after IFN was added to the cell culture (data not shown). During the next 36 h, only a slight reduction in the amount of both Mx proteins was observed, indicating that Mx proteins are not rapidly degraded in HuH-7 cells (data not shown).

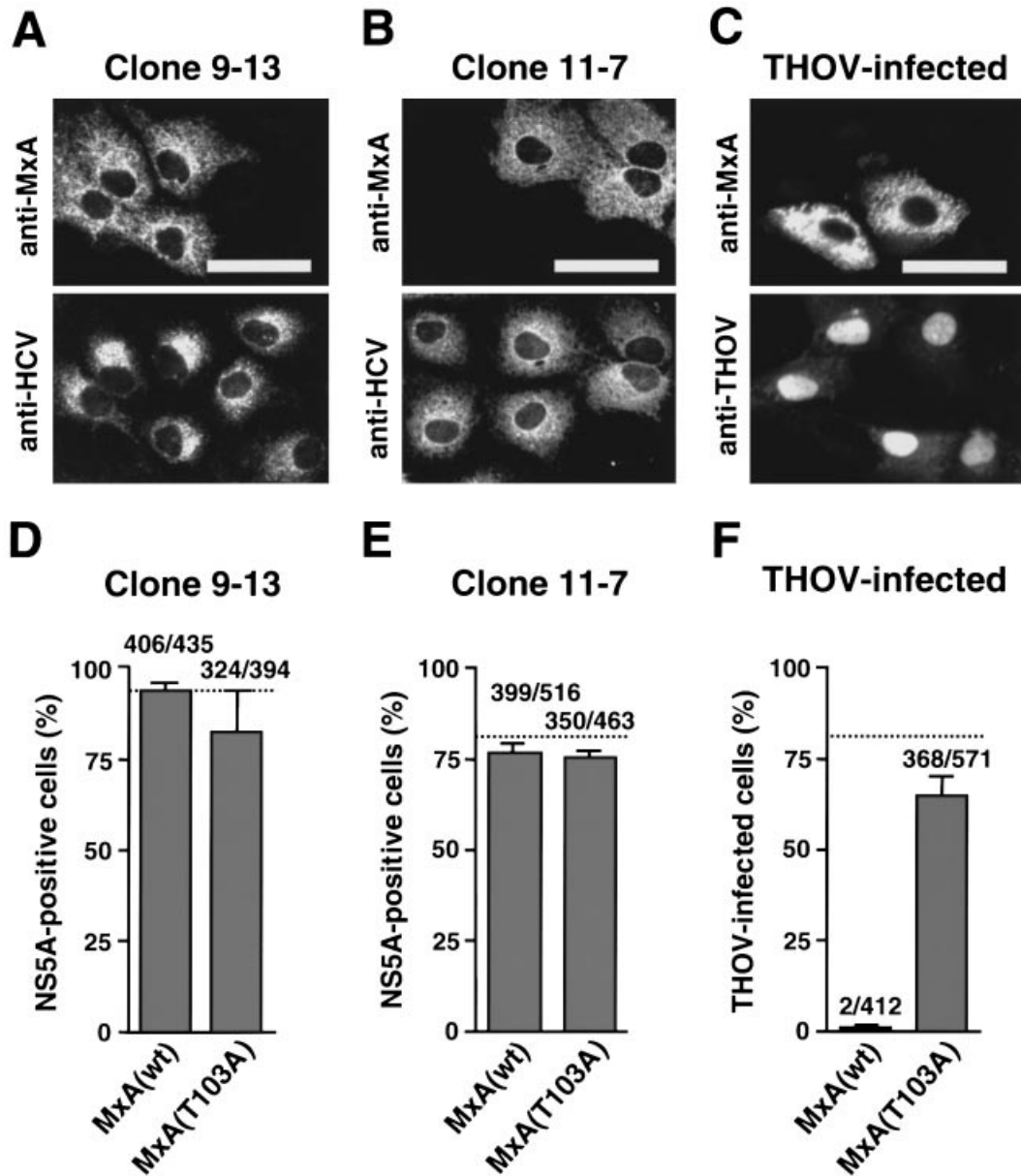
We investigated the role of MxA in the IFN-mediated inhibition of HCV RNA replication by blocking its antiviral activity by expression of a dominant-negative mutant. For that purpose, we used MxA(T103A), which contains a point mutation located between the first and second GTP-binding



**Fig. 3.** Dominant-negative effect of MxA(T103A) on wild-type MxA. (A) Expression of the dominant-negative mutant MxA(T103A) inhibits the antiviral activity of MxA against THOV. Parental HuH-7 cells were transfected with an expression vector encoding MxA(T103A) (left and right panels) or were left untransfected (middle panels). About 24 h later, cells were trypsinized, seeded onto glass coverslips and cultured for another 24 h. Cells were incubated for a further 16 h in the presence of 5000 U/ml IFN- $\alpha$ B/D (middle and right panels) or were left untreated (left panels). Cells were then infected with THOV at an m.o.i. of about 50 p.f.u./cell, fixed 7 h post-infection and double-immunostained for MxA (upper panels) and the nucleoprotein of THOV (lower panels) by using a rabbit polyclonal antibody directed against MxA and the THOV nucleoprotein-specific MAb 3D11. Arrows in the right panels point to a cell(s) expressing both the dominant-negative mutant MxA(T103A) and wild-type MxA. Bar, 50  $\mu$ m. (B) Expression of MxA(T103A) does not destroy the antiviral defence against HCV that is induced by IFN- $\alpha$ . HuH-7 cells containing the HCV<sub>1377</sub>/NS3-3' replicon (clone 9-13) were transfected with an expression vector encoding MxA(T103A) (left and right panels) or were left untransfected (middle panels). About 24 h later, cells were trypsinized, seeded onto glass coverslips and cultured for 12 h. Cells were incubated for a further 72 h in the presence of 5000 U/ml IFN- $\alpha$ B/D (middle and right panels) or were left untreated (left panels). Cells were then fixed and double-immunostained for MxA (upper panels) and NS5A (lower panels) by using a rabbit polyclonal antibody directed against MxA and the NS5A-specific MAb 11H. Arrows in the right panels point to cells expressing both the dominant-negative mutant MxA(T103A) and wild-type MxA. Bar, 50  $\mu$ m.

consensus motifs (Ponten *et al.*, 1997). MxA(T103A) produced in *E. coli* and highly purified does not bind GTP and, as a consequence, has no GTPase activity (Ponten *et al.*, 1997).

When overexpressed transiently in mouse 3T3 cells, the mutant protein forms large aggregates in the cytoplasm and does not show any antiviral activity (Ponten *et al.*, 1997).



**Fig. 4.** Overexpression of MxA in HuH-7 cells does not inhibit HCV replicons but blocks THOV replication. (A)–(B) HuH-7 cells containing the HCV replicons  $I_{377}/NS3-3'$  (clone 9-13) (A) or  $I_{377}/NS2-3'$  (clone 11-7) (B) were transfected with an expression vector encoding MxA, seeded onto glass coverslips 24 h post-transfection, fixed 72 h later and double-immunostained for MxA (upper panels) and NS5A (lower panels) by using a rabbit polyclonal antibody directed against MxA and MAb 11H, respectively. Bars, 50  $\mu$ m. (C) Parental HuH-7 cells were transfected with an expression vector encoding MxA, seeded onto glass coverslips 24 h post-transfection and subsequently infected with THOV at an m.o.i. of about 25 p.f.u./cell. The cells were fixed 9 h post-infection and immunostained for MxA (upper panels) and THOV proteins (lower panels) by using MAb 2C12 and the hyperimmune serum gp457, respectively. Bar, 50  $\mu$ m. (D)–(F) Quantitative analysis of the respective experiments described in parts (A)–(C), including additional control experiments with MxA(T103A). The percentage of cells expressing both virus proteins and Mx proteins is given in relation to the total number of Mx-expressing cells. Columns and error bars represent mean values of at least three independent experiments and 95% confidence intervals, respectively. The number of cells expressing virus proteins and Mx proteins/total number of cells expressing Mx is indicated above each column. NS5A was detected in about 90% of non-transfected cells of clone 9-13 and in about 80% of non-transfected cells of clone 11-7 (indicated as dotted lines); THOV proteins were found in about 80% of non-transfected HuH-7 cells (indicated as dotted line).

Furthermore, co-expression of wild-type MxA and MxA(T103A) in mouse 3T3 cells leads to the formation of antivirally inactive heterooligomers (Ponten *et al.*, 1997). To

demonstrate that recombinant MxA(T103A) also blocks the antiviral activity of endogenous MxA that is expressed in human hepatoma cells after IFN- $\alpha$  treatment, HuH-7 cells were

transiently transfected with an expression vector encoding MxA(T103A), treated with 5000 U/ml IFN- $\alpha$ B/D and subsequently infected with THOV. We chose THOV for this experiment because this virus is extremely sensitive to MxA (Frese *et al.*, 1995). The dominant-negative effect of recombinant MxA(T103A) on endogenous MxA protein was analysed by double immunofluorescence with specific antibodies directed against MxA and virus proteins (Fig. 3A). Transfected cells expressed large amounts of MxA(T103A) that accumulated in characteristic cytoplasmic aggregates (upper left panel). In contrast, endogenous wild-type MxA protein accumulated in the form of small granules in the cytoplasm of cells that were treated with IFN- $\alpha$  (upper middle panel). As expected, THOV replicated unhindered in transfected cells expressing MxA(T103A) (bottom left panel) but not in cells expressing MxA (bottom middle panel). However, in cells co-expressing both MxA(T103A) and MxA (upper right panel), THOV antigens were easily detectable (bottom right panel), indicating virus replication. This experiment proved that MxA(T103A) is indeed useful for blocking the antiviral activity of the endogenous MxA of HuH-7 cells. Next, we expressed MxA(T103A) in cells of clone 9-13, subsequently stimulated the cells with 5000 U/ml IFN- $\alpha$ B/D and analysed MxA and HCV protein expression by double immunofluorescence (Fig. 3B). Note that expression of MxA(T103A) alone did not inhibit NS5A synthesis, indicating that neither the transfection procedure itself nor the expression of the inactive mutant had any deleterious effects on HCV replication (Fig. 3B, left panels). If endogenous MxA was indeed the effector protein that mediated the IFN-induced inhibition of HCV replicons, MxA(T103A) would be expected to interfere in a dominant-negative way and restore HCV protein expression in these cells. This was clearly not the case (Fig. 3B, right panels), suggesting that IFN- $\alpha$  most likely acts through an MxA-independent mechanism.

In order to determine the activity of MxA against HCV replicons directly, we overexpressed MxA transiently in cells of clone 9-13 and analysed the cells 4 days later for expression of MxA and NS5A by double immunofluorescence. Despite the fact that transiently transfected HuH-7 cells expressed much more MxA protein than did IFN-stimulated cells (data not shown), HCV protein synthesis was not inhibited in transfected cells expressing recombinant MxA (Fig. 4A). The cell clones 11-7 and 5-15 were also analysed to demonstrate that resistance to MxA was not only a property of the NS3-3' replicon in cell line 9-13. Cells of both clones were transfected with an expression plasmid encoding MxA and stained 4 days later for MxA and NS5A. No difference in HCV protein synthesis between untransfected cells and cells expressing MxA was found with clone 11-7 (Fig. 4B). The same observation was made with cells of clone 5-15 (data not shown). As a control, we overexpressed MxA transiently in parental HuH-7 cells and subsequently infected the cells with THOV. As expected, MxA-expressing cells were highly

resistant to THOV (Fig. 4C), proving that recombinant MxA has antiviral activity in HuH-7 cells. In order to substantiate these findings, a quantitative analysis of the experiments described above was performed (Fig. 4D-F). Furthermore, the antivirally inactive mutant MxA(T103A) was expressed in parallel to demonstrate that the transfection procedure itself did not affect virus replication (Fig. 4D-F). Taken together, these results indicate that MxA is not responsible for the inhibition of HCV subgenomic RNA replication.

## Discussion

Since the first report on the beneficial effects of IFN- $\alpha$  in chronic hepatitis C, by Hoofnagle *et al.* (1986), thousands of patients have been treated with IFN- $\alpha$ . The success of this therapy is often correlated with the genotype of HCV (reviewed in Trepo, 2000). In a meta-analysis of 15 trials evaluating short treatment regimes of IFN- $\alpha$ , the sustained response rate was significantly lower among patients with genotype 1b (18.1%,  $n = 536$ ) than among patients with other genotypes (54.9%,  $n = 288$ ) (Davis & Lau, 1997). Another study, which analysed the outcome of long-term treatment with IFN- $\alpha$  and ribavirin, revealed a similar picture. Patients with HCV genotype 1 achieved a sustained response rate of only 28% ( $n = 166$ ) compared with 66% ( $n = 61$ ) in patients that were infected with other genotypes (McHutchison *et al.*, 1998). In this context, it is interesting to note that the HCV replicons used in the present report were derived from a genotype 1b consensus sequence obtained from a chronically infected patient who had undergone liver transplantation (Lohmann *et al.*, 1999). Unfortunately, nothing is known about the sensitivity of the 'parental' virus to IFN- $\alpha$  treatment *in vivo*. Given the fact that most genotype 1 viruses are rather resistant towards IFN- $\alpha$  therapy, our observation that the replication of HCV genotype 1b replicons is highly sensitive to IFN- $\alpha$  might be surprising. Moreover, the interferon sensitivity-determining region of NS5A of this isolate corresponds to that of a non-responder (Enomoto *et al.*, 1995, 1996). However, it should be kept in mind that some patients infected with genotype 1 viruses show a complete response (although this is rare) and, even in the case of non-responders, a drop in viraemia is found occasionally during the initial phase of treatment (for review see Foster & Thomas, 2000). The data presented here demonstrate that genotype 1b replicons can indeed be inhibited by IFN- $\alpha$ , but it remains to be seen whether replicons derived from other genotypes behave differently and whether the inclusion of the structural proteins that are missing in the subgenomic replicons would contribute to IFN- $\alpha$  resistance. Furthermore, it would be interesting to know whether sensitivity to IFN- $\alpha$  is a general feature of all HCV replicons or whether replicons that contain sequences of therapy-resistant HCV variants show the phenotype of the 'parental' virus.

Most recently, the HCV replicons of the cell clones 9-13, 5-15 and 11-7 have been recloned and sequenced. Cell culture-

adaptive mutations that enhance the number of G418-selectable, replicon-harboring cell colonies as well as RNA replication have been identified at various positions of the HCV polyprotein. These are located in NS5B at position 2884 (Arg → Gly) in the case of the replicon in cell line 9-13 (Lohmann *et al.*, 2001), in NS5A at position 2197 (Ser → Pro) with the replicon in cell line 5-15 and in both NS3 and NS4B at positions 1261 (Thr → Ser) and 1846 (Lys → Thr), respectively, in the case of the replicon in cell line 11-7 (N. Krieger, V. Lohmann and R. Bartenschlager, unpublished results). As shown here, these mutations do not counteract the IFN-induced antiviral response of the host cell.

One potential drawback of the HCV replicon system used in this study is the bicistronic design of the constructs. The original IRES of HCV directs the translation of the *neo* gene, whereas the expression of the HCV non-structural genes is mediated by the IRES of EMCV. Therefore, we cannot exclude the possibility that the observed inhibition of HCV RNA replication after IFN- $\alpha$  treatment is due to a block in EMCV IRES activity reducing HCV protein synthesis and, as a consequence, most likely also reducing RNA replication. In order to exclude this possibility, we have recently developed cell lines harbouring monocistronic replicon RNAs in which the HCV IRES directs translation of a hygromycin-ubiquitin-NS3-to-NS5B fusion protein (N. Krieger, V. Lohmann and R. Bartenschlager, unpublished results). In this construct, the heterologous protein sequences are removed from the HCV proteins by host cell enzymes via the ubiquitin-dependent pathway, circumventing the problem of inserting a second, heterologous IRES element. Preliminary data show that translation/replication of this replicon is also inhibited by IFN- $\alpha$  (N. Krieger, V. Lohmann and R. Bartenschlager, unpublished). Thus, the EMCV IRES is not responsible for the IFN- $\alpha$ -mediated inhibition of HCV replicons.

The application of IFN- $\alpha$  to patients with chronic hepatitis C boosts both their innate and adaptive immune systems. It was previously not known which part of the antiviral defence is more important in clearing HCV. However, our finding that IFN- $\alpha$  inhibits the replication of HCV replicons in cell culture indicates that IFN-induced effector proteins of the innate immune system are in the 'front line' against HCV. But who is doing the job? It was conceivable that MxA inhibited HCV because (i) this protein is known to inhibit the replication of various other RNA viruses (Haller *et al.*, 1998) and (ii) a single nucleotide polymorphism in the first IFN-stimulated response element of the *MxA* gene promoter has been reported to correlate with the response of hepatitis C patients to IFN- $\alpha$  treatment (Hijikata *et al.*, 2000). Although it has been shown that MxA expression is not induced in PBMCs during the acute phase of HCV infection (Jakschies *et al.*, 1994), increased MxA levels have been found in PBMCs of patients with chronic hepatitis C (Antonelli *et al.*, 1999; Fernández *et al.*, 1999). Furthermore, MxA levels were monitored in PBMCs of hepatitis C patients during IFN- $\alpha$  therapy. Once the treatment

had been started, MxA levels increased further and remained high until the end of therapy (Fernández *et al.*, 1999). Most recently, MxA expression was also analysed in cells of the liver. Biopsies that were taken from hepatitis C patients prior to IFN treatment showed elevated MxA expression levels in hepatocytes and/or macrophages in 82% ( $n = 28$ ) of the samples, indicating that HCV is able, at least in most cases, to persist in the presence of MxA (MacQuillan *et al.*, 2000). These findings are in line with our observation that MxA fails to inhibit HCV replicons in cell culture. Thus, we conclude that IFN-induced effector proteins other than MxA are responsible for the inhibition of HCV replication. OAS and PKR, two other proteins that contribute to IFN-induced antiviral defence, might interfere with the replication of HCV (Korth & Katze, 2000; Taylor, 2000). In addition, other, as yet unknown, IFN-induced proteins with antiviral activity exist (Zhou *et al.*, 1999). Thus, it will be challenging to identify the IFN-induced effector proteins that inhibit the replication of HCV. In summary, our results demonstrate that replicon-harboring cell lines are powerful tools in investigating the complex interaction between HCV and the IFN-induced antiviral defence system of the host. Further studies on that subject are needed urgently in order to improve chronic hepatitis C therapy.

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