

Hepatitis C Virus Core Protein Induces Cell Proliferation and Activates ERK, JNK, and p38 MAP Kinases Together with the MAP Kinase Phosphatase MKP-1 in a HepG2 Tet-Off Cell Line

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Hepatitis C virus (HCV) core protein is a multifunctional protein interacting with cellular and viral proteins and promoters. A tetracycline-regulated system was used to generate a HepG2 Tet-Off cell line allowing regulated expression of a full-length (191 aa) and an N_c-truncated core protein (160 aa). In this system HCV core protein expression activates extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinase, induces MAP kinase phosphatase MKP-1 expression, and increases cell proliferation. This was accompanied by an activation of c-Jun and ATF-2, but not Elk-1 and c-Fos. Furthermore, AP-1 activation was independent of c-Fos. Full-length and N_c-truncated HCV core proteins exerted similar effects. © 2002 Elsevier Science

Key Words: virus; hepatitis C; MAP kinase; core protein; proliferation; Tet-Off.

INTRODUCTION

Hepatitis C virus (HCV) infection is a common disease, affecting about 3% of the world population (WHO, 1997). Hepatitis C accounts for approximately 20% of cases of acute hepatitis and 70% of cases of chronic hepatitis. Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma (Marcellin, 1999).

HCV is a member of the *Flaviviridae* family and was first isolated in 1989. The positive-strand RNA genome contains a large open reading frame of about 9.5 kb encoding a large precursor polyprotein, which is cleaved by both cellular and viral proteases into structural and nonstructural proteins (Choo *et al.*, 1989). Core protein is encoded at the 5'-terminal region of the ORF and is composed of a basic, RNA-binding amino-terminal domain and a highly hydrophobic carboxy-terminal region (Santolini *et al.*, 1994). Mature 21-kDa HCV core protein is produced by cleavage between aa 191 and 192 of the precursor polyprotein. HCV core protein products of 19 and 16 kDa have further been described (Santolini *et al.*, 1994). The core protein is associated with the cytoplasmic side of the endoplasmic reticulum (Hijikata *et al.*, 1991; Santolini *et al.*, 1994) but has also been detected in the nucleus (Lo *et al.*, 1995).

In addition to encapsidation of viral RNA, recent studies have suggested a possible role of the HCV core protein in host cell growth, apoptosis, and carcinogenesis. The transforming potential of the HCV core protein

has been demonstrated in rat embryo fibroblasts (Chang *et al.*, 1998; Ray *et al.*, 1996a) and transgenic mice (Moriya *et al.*, 1998). *In vitro* studies suggested that the HCV core protein has regulatory functions acting as either a *trans*-activator or a *trans*-suppressor for different cellular and viral genes (Ray *et al.*, 1997, 1998; Srinivas *et al.*, 1996; Shih *et al.*, 1995). Pro- and anti-apoptotic functions of the HCV core protein have been reported (Ray *et al.*, 1996b; Ruggieri *et al.*, 1997; Marusawa *et al.*, 1999). However, the precise role of the HCV core protein in apoptotic cell death is still unclear. Furthermore, the HCV core protein has been shown to interact with apolipoprotein II, tumor necrosis factor (TNF) receptor lymphotoxin β -receptor, 14-3-3 protein, DEAD box protein DD3, and heterogeneous ribonucleoprotein K (Aoki *et al.*, 2000; Zhu *et al.*, 1998; Hsieh *et al.*, 1998; Matsumoto *et al.*, 1997; Owsianka and Patel, 1999; Sabile *et al.*, 1999).

Although it is likely that the HCV core protein has an important role in the regulation of cell growth, apoptosis, or carcinogenesis, the mechanisms by which the HCV core protein influences these processes remain to be determined. The MAP (mitogen-activated protein) kinase cascade has emerged as a key signaling pathway regulating cell growth, differentiation, and transformation through intracellular phosphorylation (Lewis *et al.*, 1998; Schmidt *et al.*, 1997; Mansour *et al.*, 1994). In mammalian cells, three distinct subtypes of MAP kinase pathways have been identified, all sharing a threonine-X-tyrosine phosphorylation motif: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK.

We investigated the potential role of the HCV core

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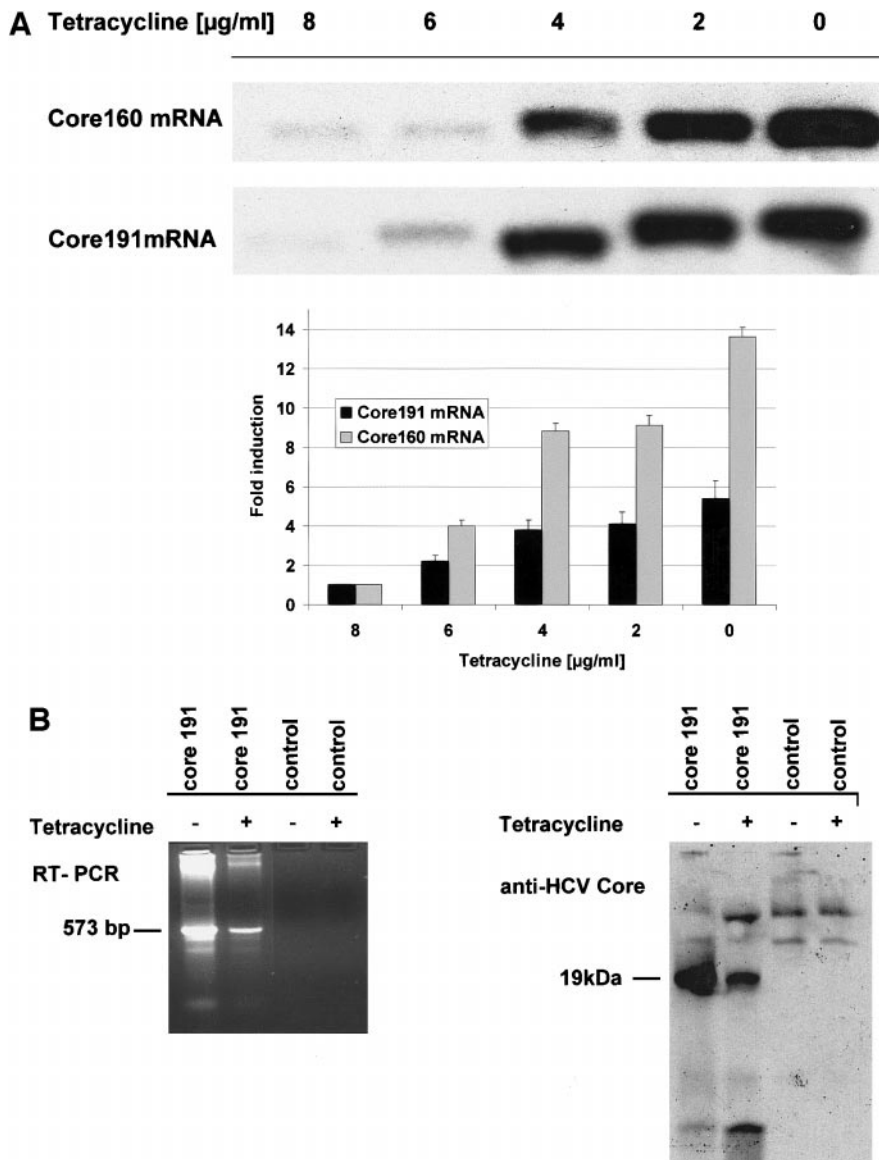


FIG. 1. Tetracycline regulated HCV core expression in HepG2 Tet-Off cells. (A) 20 μg of cellular RNA isolated from clones HepG2-191-1B and HepG2-160-B cultured in the presence of 0, 2, 4, 6, or 8 $\mu\text{g/ml}$ of tetracycline was analyzed for HCV core transcription by Northern blot analysis as described under Materials and Methods. The equal loading of mRNA was determined by GAPDH (not shown). (B) The mRNA transcripts of HCV core 160 and 191 were detected in total RNA isolated from HepG2 transfectants by RT-PCR. Protein lysates (100 μg) prepared from the clones HepG2-191-1B and HepG2-160-B and pBI-L transfected control cells, cultured in the presence or in the absence of tetracycline, were separated by 12% SDS-PAGE and analyzed by immunoblotting using a polyclonal anti-HCV core antibody.

protein in the intracellular signal transduction pathway of MAP kinases. In order to analyze the effect of the HCV core protein on the MAP kinase signaling pathway at different expression levels we established a HepG2 Tet-Off cell line with regulated expression of the HCV core protein. The HepG2 Tet-Off cell line is based on regulatory elements of the tetracycline-resistance operon of *Escherichia coli* with a tetracycline-controlled transactivator (tTa) and a tTA-dependent promoter which is virtually silent in the presence of tetracycline and is activated in the absence of tetracycline (Gossen and Bujard, 1992).

RESULTS

Characterization of HepG2 Tet-Off with regulated expression of HCV core proteins and control cells

Inducibility and expression of the HCV core gene was determined by luciferase activity, Northern blot analysis (Fig. 1A), Western blot analysis, and reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1B). Twelve hours after withdrawal of tetracycline, induction rates of about 1:370 were found for both the HepG2 clone 191-1B and the HepG2 clone 160-B. In time course ex-

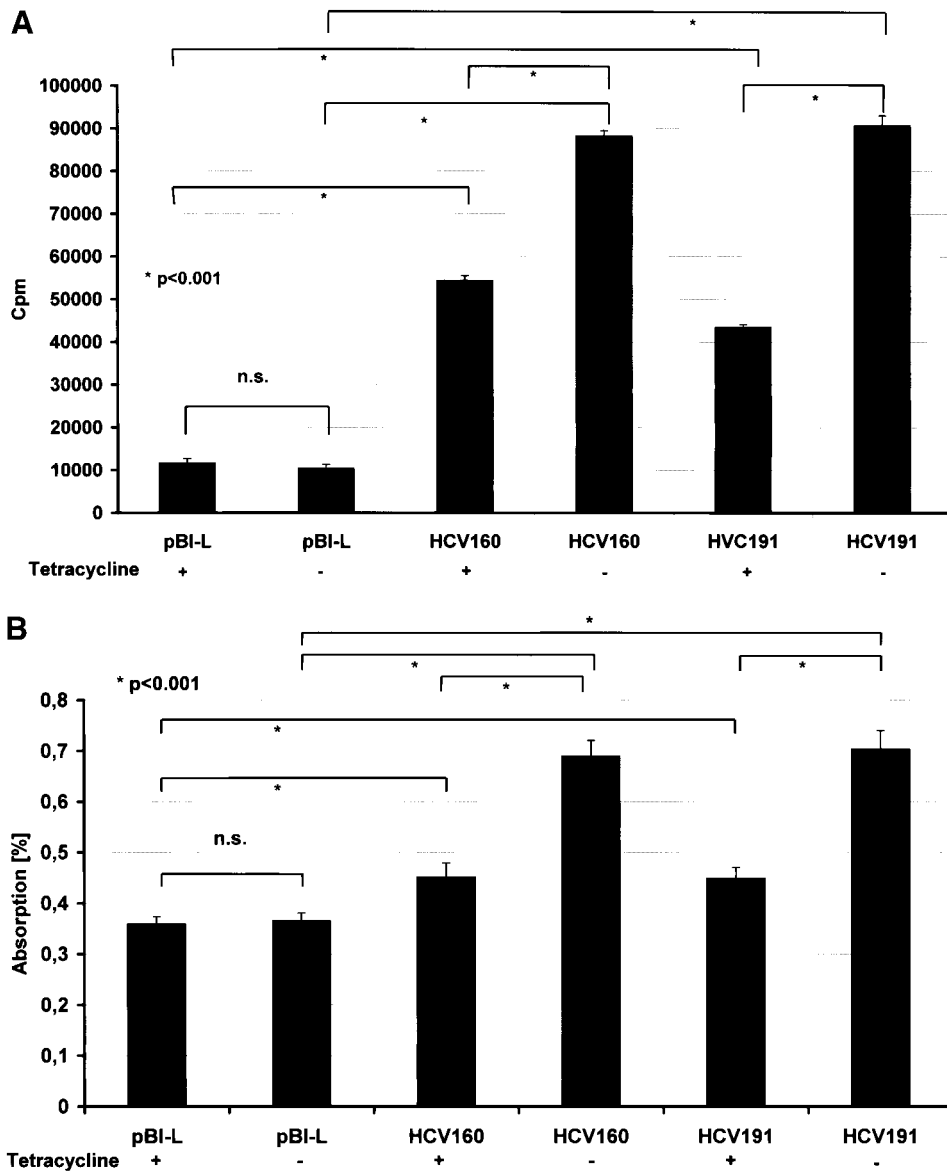


FIG. 2. Effect of HCV core on cell proliferation. (A) [^3H]Thymidine uptake in the presence (+) and in the absence (-) of 8 $\mu\text{g}/\text{ml}$ tetracycline. (B) Measurement of cell proliferation by MTT assay in the presence (+) and in the absence (-) of 8 $\mu\text{g}/\text{ml}$ tetracycline. Mean values and standard deviation of six independent experiments are given. * P value < 0.001.

periments a significant increase in luciferase activity was observed 30–40 min after withdrawal of tetracycline. In all experiments a baseline level of HCV core expression was maintained that could not be suppressed further by increasing tetracycline concentrations.

HepG2 Tet-Off cells (pBI-L) with a luciferase gene under control of a CMV minimal promoter without the HCV core insert were taken as controls for all experiments.

Measurement of cytotoxicity and cell proliferation

In all cells expressing HCV core proteins, a significant induction of cell proliferation was noticed as determined by [^3H]thymidine uptake (Fig. 2A). [^3H]Thymidine uptake

and MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) metabolism were more pronounced in cells with high HCV core protein expression than in cells with low HCV core protein expression (Figs. 2A and 2B). The activities of LDH (lactate dehydrogenase) in the cell culture supernatant were taken as an indicator of cytotoxicity. No significant differences were noticed between control cells and cells expressing the HCV core protein independent of the addition of tetracycline. Tetracycline itself did not alter cell viability (not shown) and was without effect on [^3H]thymidine uptake in control cells ($12,070 \pm 1563$ cpm for control cells with tetracycline and $11,090 \pm 310$ cpm for cells without tetracycline; ns).

Effect of HCV core protein on the activity and protein expression of MAP kinases (JNK, ERK, and p38)

As shown in Figs. 3A–3C withdrawal of tetracycline from the culture medium for 12 h led to a 3.7 ± 0.2 -, 4.2 ± 0.8 -, and 2.8 ± 0.2 -fold increase of the basal activity of JNK, ERK, and p38 in the clone HepG2-191-1B and a 4.1 ± 0.2 -, 4.2 ± 0.9 -, and 2.8 ± 0.1 -fold increase in the clone HepG2-160-B when compared to the control cells. The HCV core proteins 191 and 160 induced protein expression of JNK and p38 1.9 ± 0.1 - and 1.8 ± 0.2 -fold (clone HepG2-191-1B) and 2.1 ± 0.1 - and 2.0 ± 0.2 -fold (clone HepG2-160-B) compared to the control cells. No significant increase was observed in the protein expression of ERK1/2 either in the clone HepG2-191-1B or in the clone HepG2-160-B compared to control cells. No significant effect of tetracycline itself was observed on JNK, ERK, or p38 MAP kinase activity in control cells.

Effect of HCV core protein on proto-oncogenes c-Jun and c-Fos

In the absence of tetracycline, mRNA expression of c-jun in the clones HepG2-191-1B and HepG2-160-B increased 5.9 ± 0.8 - and 6.1 ± 0.6 -fold by 12 h, compared to control cells (Fig. 4A). In immunoblot analysis, the protein expression remained unchanged in control cells within 3 to 12 h, whereas a 8.2 ± 1.5 - and 9.2 ± 2.2 -fold increase in the protein expression after the withdrawal of tetracycline from the culture medium was noted within 12 h in the clones HepG2-191-1B and HepG2-160-B (Fig. 4A). Withdrawal of tetracycline from the culture medium did not induce significant changes at the mRNA or protein level of c-Fos compared to control cells (Fig. 4B).

Effect of HCV core protein on AP-1 activity

Withdrawal of tetracycline increased the basal activity of the transcription factor AP-1 within 12 h by 5.2 ± 1.2 - and 4.9 ± 0.9 -fold in the clones HepG2-191-1B and HepG2-160-B compared to control cells (Fig. 5A). There was no effect of tetracycline itself on AP-1 activation in control cells: the absolute ODs were $14,428 \pm 1552$ (pBI-L without tetracycline) and $14,712 \pm 1250$ (pBI-L with tetracycline). The authenticity of the AP-1 band following incubation of the nuclear extracts with antibodies raised against c-Jun and c-Fos proteins was ascertained by supershift assay. Antibodies against the c-Jun protein shifted the band to a higher molecular mass, whereas antibodies to c-Fos did not shift the AP-1 band (Fig. 5B).

Effect of HCV core protein on Elk-1, ATF-2, and NF- κ B activation

Elk-1 activation was tested by EMSA using an SRE (serum response element)-specific sequence with HepG2 cells grown in the presence ($8 \mu\text{g/ml}$) and ab-

sence of tetracycline. No significant changes in the activity of the transcription factor Elk-1 were seen. The activity of the transcription factor ATF-2 was investigated by EMSA with a CRE-specific sequence. The withdrawal of tetracycline from the culture medium significantly increased the basal activity of ATF-2 in the clone HepG2-191-1B and in the clone HepG2-160-B 3.9 ± 0.2 - and 3.6 ± 0.4 -fold compared to control cells (Fig. 6). IL-1 stimulated NF- κ B expression was reduced in all cells expressing HCV core protein when compared to controls (Fig. 7). Some effect of tetracycline was found in both control and HCV core expressing cells.

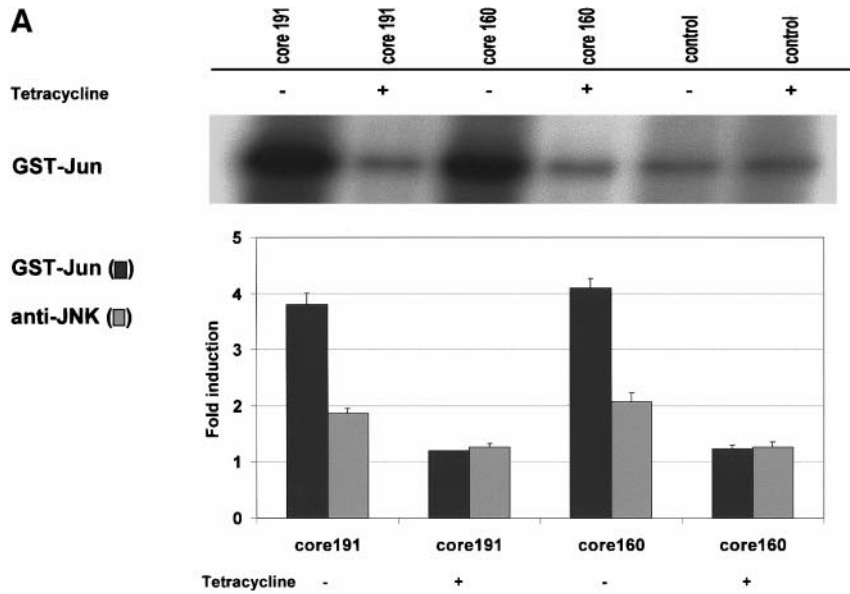
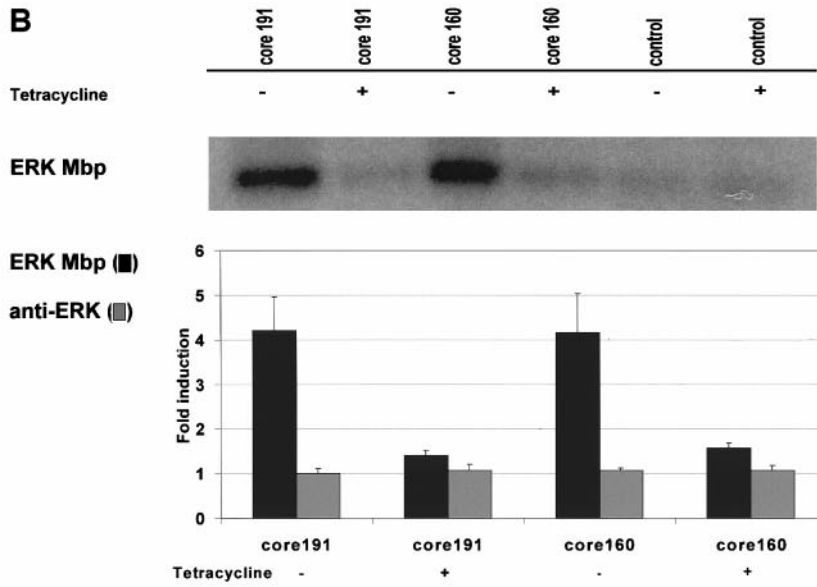
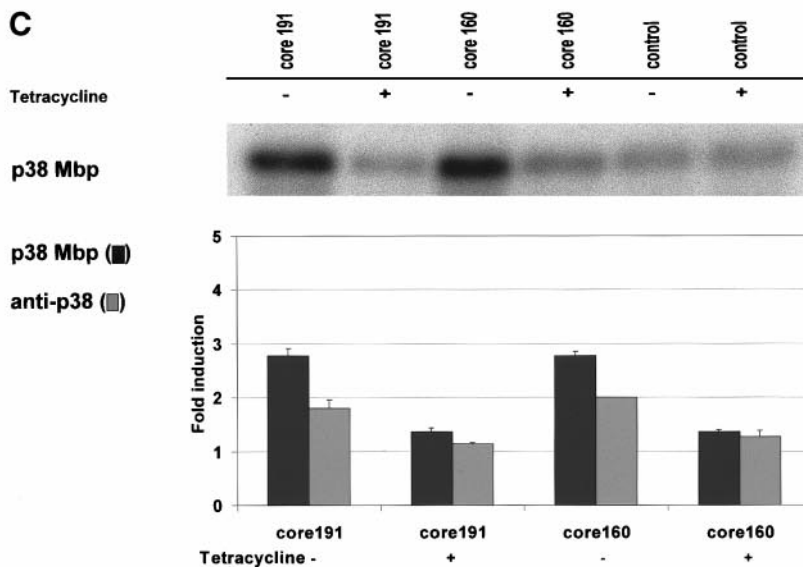
Effect of HCV core protein on MKP-1 expression

Northern blot analysis revealed a significant induction of MKP-1 (5.8 ± 1.7 and 4.1 ± 0.9) in HepG2-191 and HepG2-160 cells compared to control cells after withdrawal of tetracycline (Fig. 8). The increase in mRNA levels in HepG2 core transfected cells went in line with a marked increase in the protein levels of MKP-1, reaching a mean induction of 6.2 ± 2.7 (HepG2-191) and 4.8 ± 1.8 (HepG2-160) 12 h after withdrawal of tetracycline.

DISCUSSION

Protein kinases and phosphatases play a key role in the regulation of cell growth. There is increasing evidence that the development of hepatocellular carcinoma might be associated with activation of the Ras/Raf/MAP kinase pathway (Ito *et al.*, 1998; Schmidt *et al.*, 1997). On the other hand, there are numerous clinical and *in vitro* studies suggesting an important role of the HCV, especially of the HCV core protein, in liver carcinogenesis (Moriya *et al.*, 1998; Ray *et al.*, 1996a, 2000; Chang *et al.*, 1998). Thus one may speculate that the HCV core protein plays a key role in the development of human liver disease through activation of the MAP kinase pathway. Indeed, activation of ERK MAP kinases has been reported for the HCV core protein (Hayashi *et al.*, 2000). However, the influence of the HCV core protein on MAP kinases and the influence of MAP kinases on disease progression are not fully understood. Major findings of the study presented here are the increased cell proliferation in HepG2 Tet-Off cells stably transfected with HCV core proteins. Furthermore an activation of ERK, JNK, and p38 MAP kinases and MKP-1 was found. The Tet-Off system was established in order to investigate the effects of the HCV core proteins even at low expression levels. However, even in the Tet-Off system, HCV core protein overexpression cannot be ruled out.

Although transforming and anti-apoptotic properties have been attributed to the HCV core protein alone or in synergy with H-ras (Chang *et al.*, 1998; Jin *et al.*, 2000; Ray *et al.*, 1996a), cell proliferation has not yet been studied. Increased cell proliferation was found in the present study for HepG2 cells expressing HCV core

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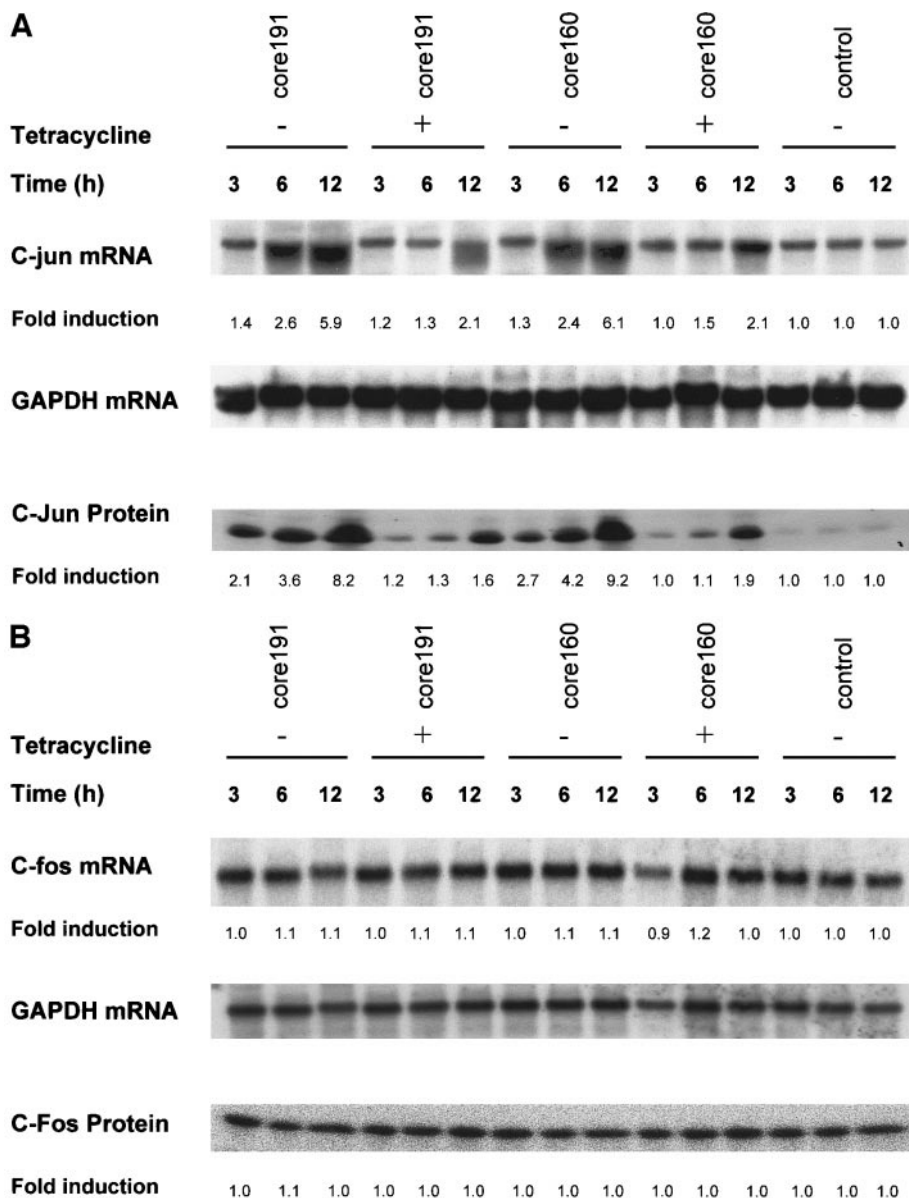


FIG. 4. Effect of HCV core protein on the expression of c-jun (A) and c-fos (B). Twenty micrograms of total RNA and 50 μ g nuclear extract isolated from the clones HepG2-191-1B and HepG2-160-B cultured in the absence (–Tc) and in the presence (+Tc) of 8 μ g/mL tetracycline and from empty-vector (pBI-L) transfected control cells, harvested at 3, 6, and 12 h, were analyzed by Northern blot and Western blot as described under Materials and Methods. The equal loading of RNA was determined by GAPDH. For calculation of gene and protein induction, empty-vector controls were taken as reference. A representative blot and the mean induction of five (c-jun) and three (c-fos) independent experiments are given.

proteins compared to control cells. The transcription factor AP-1 plays a key role in cell proliferation (Leppa and Bohmann, 1999). In line with previous studies (Kato *et al.*, 2000; Shrivastava *et al.*, 1998) a strong activation of AP-1 by the HCV core protein was found. Activation of

AP-1 was accompanied by an induction of the proto-oncogene c-Jun at the protein and mRNA levels.

Although the ERK cascade plays an important role in the regulation of cell growth and differentiation (Krontiris, 1995) its contribution to HCV core-induced cell prolifer-

FIG. 3. Effect of HCV core protein on MAP kinase (JNK, p38, and ERK) activity and protein expression. JNK activity (A), ERK activity (B), and p38 activity (C) were measured by immune complex kinase assay with GST-c-Jun or myelin basic protein as substrate. The clones HepG2-191-1B and HepG2-160-B and the empty-vector (pBI-L) transfected control cells were prepared as described under Materials and Methods. A representative blot and mean inductions of four (JNK) and three (ERK and p38) independent experiments are given. Kinase activity is represented by black bars, and protein expression is shown by gray bars. For calculation of kinase activity and protein expression, empty-vector controls were taken as references. The total protein expression was determined by Western blot analysis using specific antibodies as mentioned under Materials and Methods.

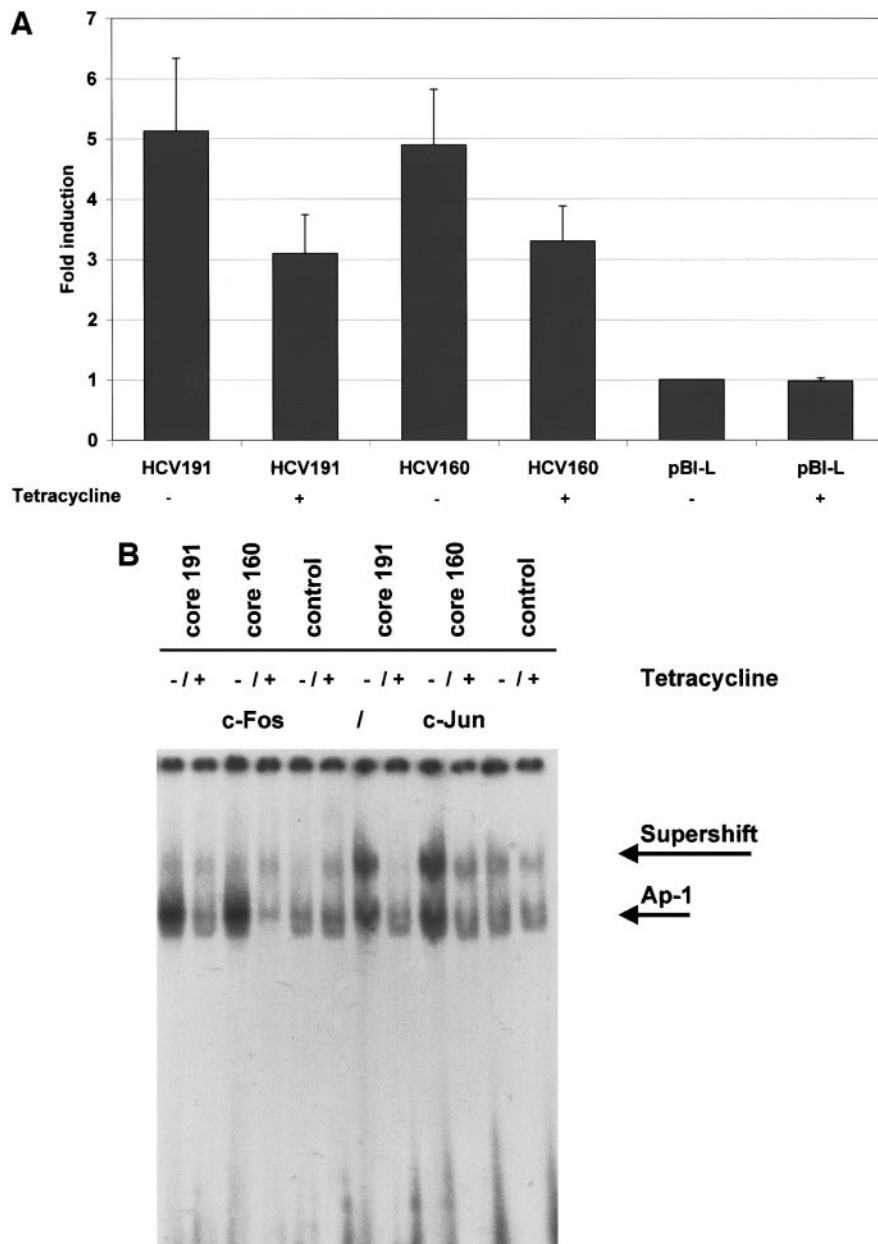


FIG. 5. Effect of HCV core protein on AP-1 activation. (A) Nuclear extracts (4 μ g) were incubated with 8 fmol of [γ - 32 P]ATP end-labeled double-stranded AP-1-specific sequence as described under Materials and Methods. Control cells and HCV core DNA transfected cells cultured in the presence of 8 μ g/ml tetracycline (+) and in the absence of tetracycline (-) were analyzed for AP-1 activity by gel-shift assay. Results of three independent experiments are given as means and SEM. Empty-vector controls were taken as references. (B) Supershift assay was performed with specific antibodies to c-Jun or c-Fos.

ation appears unlikely. This is supported by the finding that HCV core-induced ERK activation was not accompanied by activation of c-Fos or Elk-1. Further, c-Fos protein was not required for the formation of the AP-1 complex. The present results based on the investigation of the endogenous SRE/Elk-1 activity are in contrast to studies using a reporter assay demonstrating a significant increase of the Elk-1 activity (Hayashi *et al.*, 2000; Kato *et al.*, 2000). However, the possibility cannot be excluded that HCV core proteins influence degradation of Elk-1 and c-Fos proteins as degradation of c-Fos is

influenced by MAP kinases, c-Jun, and cell growth (Salvat *et al.*, 1999; Papavassiliou *et al.*, 1992). There is evidence that the kinetics of ERK activation drives cells toward either proliferation (transient activation) or differentiation (sustained activation) (Cowley *et al.*, 1994). A long-lasting activation (12 h) was found in our cells, supporting further the idea that ERKs are not the main mediators for cell proliferation in our HepG2 cells.

Increased cell proliferation may also be due to activation of ATF-2, as seen in our study. It has been reported that ATF-2 might interact with the AP-1 complex and

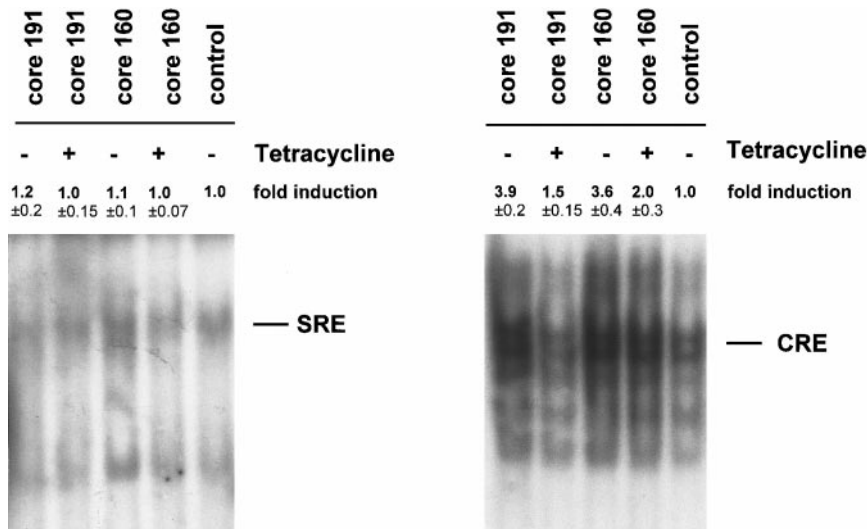


FIG. 6. Effect of HCV core protein on Elk-1 and ATF-2 activation. Four micrograms of nuclear extract of HepG2 Tet-Off cells stably transfected with pBI-L empty vector (controls), pBCore160, and pBCore191 cultured in the presence of 8 μ g/ml tetracycline (+) and in the absence of tetracycline (–) were analyzed by gel-shift assay with 8 fmol of [γ - 32 P]ATP end-labeled double-stranded SRE-specific or CRE-specific sequence for Elk-1 or ATF-2 as described under Materials and Methods. For calculation of Elk-1 and ATF-2 activation, empty-vector controls were taken as references. Fold induction and SEM of three independent experiments are given.

contribute to enhanced cell proliferation (van Dam *et al.*, 1995). ATF-2 is activated by p38 MAP kinase, which was induced in our HCV core transfected HepG2 cells. Besides the activation of ATF-2, cytosolic phospholipase A₂, and MAP kinase-activated protein (MAPKAP), p38

MAP kinase plays an important role during liver regeneration (Spector *et al.*, 1997). The p38 MAPK is further known to be activated in response to inflammatory signals, cell hydration, and stress and plays a role in cell repair, apoptosis, proteolysis, cell cycle regulation, and activation of immune responses (Kyriakis and Avruch, 1996; Häussinger *et al.*, 1999). Although p38 MAPK might contribute to enhanced cell proliferation, the role of p38 MAPK in hepatitis C virus infection remains to be further elucidated. One interesting feature might be a modulation of the JAK/STAT pathway by the HCV core protein via p38 MAPK activation and upregulation of SOCS (suppressors of cytokine signaling) (Bode *et al.*, 1999). The JAK/STAT signal transduction pathway plays an important role in mediating interferon signaling (Darnell, 1997).

The MAP kinase phosphatase MKP-1 is an immediate-early gene able to dephosphorylate phosphoserine/threonine as well as phosphotyrosine residues. There is evidence that MKP-1 inhibits apoptosis via preferential inactivation of JNK/p38 MAP kinases and to a lesser degree via ERKs (Magi-Galluzzi *et al.*, 1998), suggesting a role of MKP-1 in cell proliferation and carcinogenesis. MAP kinase activity results from the kinase/phosphatase balance (Camps *et al.*, 2000). A simultaneous activation of MAP kinases and MKP-1 was observed in our cells. The simultaneous activation of MAP kinases and MKP-1 suggests that MKP-1 induction may not be sufficient for inactivation of MAP kinases in Hep G2 cells. A different cellular localization of MKP-1 (nuclear localization) and MAP kinases, which are found in the nucleus and the cytoplasm, might be a further possible explanation. It has been shown in addition that ERK-1,2 can function as

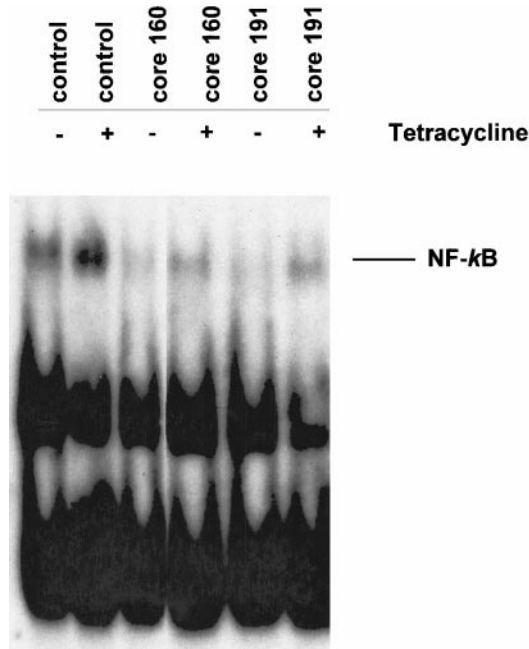


FIG. 7. Effect of HCV core protein on NF- κ B activation. HepG2 Tet-Off cells stably transfected with pBI-L empty vector (controls), pBCore160, and pBCore191 cultured in the presence of 8 μ g/ml tetracycline (+) and in the absence of tetracycline (–). HepG2 cells were preincubated for 12 h with 50 U/liter IL-1. Nuclear extracts were investigated for NF- κ B activation by EMSA.

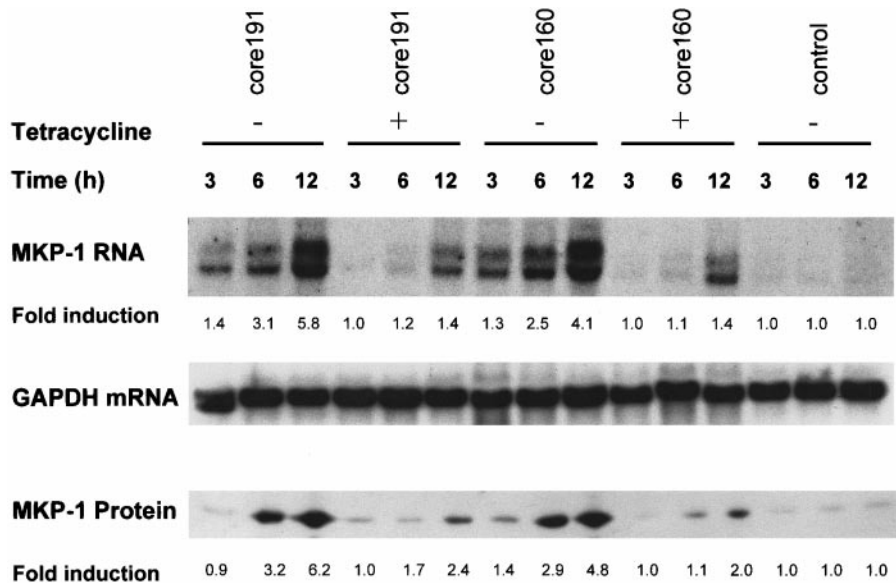


FIG. 8. Effect of HCV core protein on the expression of MKP-1. Twenty micrograms of total RNA isolated from the clones HepG2-191-1B and HepG2-160-B cultured in the absence (–Tc) and in the presence of (+Tc) of 8 $\mu\text{g}/\text{ml}$ tetracycline and from empty-vector (pBI-L) transfected control cells was analyzed by Northern blot as described under Materials and Methods. The equal loading of RNA was judged by GAPDH. Protein lysates (50 μg) were analyzed by Western blot with specific antibodies as described under Materials and Methods. For calculation of MKP-1 expression empty-vector controls were taken as references. Mean induction levels of five independent experiments are given.

positive feedback regulators of MKP-1, by inducing MKP-1 and increasing the half-life of MKP-1 (Brondello *et al.*, 1997, 1999). Given this complexity, kinetic studies will be required to clarify the function of MKP-1 in its interplay with MAP kinases.

It has been demonstrated that accumulation of viral proteins can induce an endoplasmic reticulum (ER) overload response and thus interfere nonspecifically with intracellular signal transduction pathways (Pahl and Baeuerle, 1995). ER overload requires the activation of NF- κB . As NF- κB activation was reduced upon expression of the HCV core protein in our HepG2 cells an activation of MAP kinases due to ER overload seems unlikely.

Different HCV core protein products have been described with molecular masses of 21, 19, and 16 kDa (Lo *et al.*, 1995). Due to a localization of the proteins at the endoplasmic reticulum (p21, p19) and in the nucleus (p16), different biological functions have been proposed. However, in the present study only qualitative differences were found for full-length and N_c-truncated HCV core proteins with regard to MAP kinase signals.

MATERIALS AND METHODS

Plasmid constructs

The plasmid pcDNA3 (a gift from J. Wands, Providence, RI) containing the entire HCV-1b core genomic region was used as template in a standard PCR to amplify the full-length HCV core (aa 1–191) and the 3'-end truncated HCV core (aa 1–160) by using the oligonucleotide sense

primer 5'-CCC AAG CTT GGG ACC ATG AGC ACG AAT CCC and the antisense primers 5'-CCC AAG CTT GGG GTC CTC CAG AAC CCG GA-3' (HCV core 160) and 5'-CCC AAG CTT GGG CGG GGA AGC TGG AAT GG-3' (HCV core 191) with integrated restriction sites of the restriction enzyme *Hind*III. The digested DNA fragments were inserted into the *Hind*III site of the pBI-L vector (Clontech, Palo Alto, CA) using standard cloning procedures (Fig. 9) (Sambrook *et al.*, 1989). The pBI-L vector contains a bidirectional tTA-dependent promoter (in Fig. 9, TRE denotes tetracycline-responsive element) allowing the regulated expression of a luciferase reporter gene and the gene of interest. The constructed plasmids were transformed into *E. coli* Top F'10 (Invitrogen, NV Leek, The Netherlands) according to standard methods (Sambrook *et al.*, 1989) and the resulting clones were sequenced using the primer pair B-IL sense (5'-CAAT-TCGAGCTCGGTACCCG) and B-IL antisense (5'-CAA-GGGTCCCCAACTCACC) by direct cycle sequencing with dye-labeled dideoxynucleotides according to the manufacturer's instructions (Perkin-Elmer, Foster City, CA) on an ABI Prism 310 sequencer.

Establishment of HepG2 Tet-Off cell lines

HepG2 is a well-characterized human cell line (American Tissue Culture Collection HB 8065, Rockville, MD). In order to develop a Tet-Off cell line from the HepG2 cells, about 40 μg of the regulator plasmid pTet-Off was mixed together with the cell suspension at a density of 2×10^6 cells/ml and electroporated in the Easyject Plus

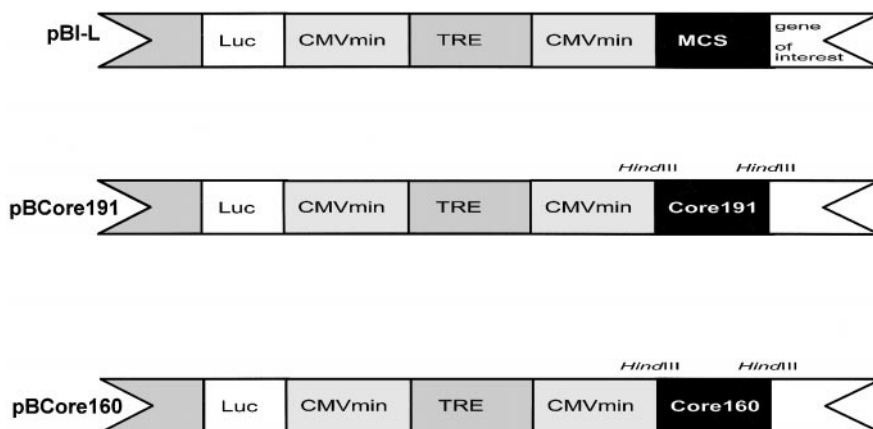


FIG. 9. Schematic representation of the plasmid constructs used for transfection of HepG2 Tet-Off cells. pBI-L, control vector; pBCore160, vector with HCV core insert of 160 aa; pBCore191, vector with HCV core insert of 191 aa; Luc, luciferase; TRE, tetracycline responsive element; CMVmin, cytomegalovirus minimal promoter; MCS, multiple cloning site; Core191, HCV core insert of 191 aa; Core 160, HCV core insert of 160 aa.

D2000 electroporator (Eurogentec, Serain, Belgium) at 1050 μ F and 260 V ($t = 20$ ms). The cells were allowed to divide twice before the addition of geneticine (400 μ g/ml; Gibco BRL, Grand Island, NY) to the culture for selection. After transient transfection with the pBI-L vector 16 of 30 clones displayed luciferase activity upon withdrawal of tetracycline. Clones with the best signal-to-noise ratio were chosen for the development of a double stable Tet-Off cell line by cotransfection with the constructs pTK-Hyg and pBCore160, pBCore191, or pBI-L. After 48 h cells were selected by addition of hygromycin at a concentration of 300 μ g/ml. Tetracycline was added to the medium in order to suppress the expression of the HCV core protein during the selection process. Resistant colonies were transferred to individual wells and screened for expression of the HCV core protein by luciferase assay. Screening of 40 HepG2-Tet-Off clones (stably-transfected with the plasmids pBCore191 and pBCore160 comprising the cDNA encoding regions for the full-length HCV core protein 191 and carboxy-terminal truncated HCV core 160, respectively) revealed 21 positive clones, 8 of which allowed inducible expression of HCV core protein 191 and 11 of which allowed inducible expression of HCV core protein 160, as assessed by luciferase assay. Clones termed HepG2-191-1B and HepG2-160-B showed the best signal-to-noise ratios, were characterized further, and were chosen for the subsequent experiments. HepG2 cells stably transfected with the pBI-L vector without the HCV core insert were used as controls for all experiments.

Luciferase assays

Luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Relative light units (RLU) were measured in an illuminometer (EG&G, Berthold, Germany).

Measurement of cell proliferation and cytotoxicity

Measurement of cell viability and proliferation was performed by [3 H]thymidine uptake assay and in addition by the MTT assay (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The MTT assay is based on the reduction of tetrazolium salt by viable cells. The reaction produces a water-insoluble formazan salt, which must be solubilized by a suitable solvent. Reduced MTT was assessed at $A_{450\text{ nm}} - A_{620\text{ nm}}$ using an ELISA reader (Anthos Reader 2001, LaborTec Instruments, Salzburg, Austria). Experiments were repeated six times.

For thymidine uptake, HepG2 Tet-Off cells, cultured in the presence or in the absence of tetracycline, were incubated with 0.5 μ Ci [3 H]thymidine for 24 h. [3 H]Thymidine incorporation after incubation with 5% TCA and washing was determined by liquid scintillation using a Microbeta Trilux 1450 plate reader (EG&G Wallac, Turku, Finland). Experiments were repeated six times.

Cytotoxicity was determined by measurement of LDH release. The LDH activity was determined according to the manufacturer's protocol in five separate experiments (Roche Diagnostics).

RT-PCR

First-strand cDNA was made using the first cDNA synthesis kit (Roche Diagnostics) by mixing 2 μ g of denatured total RNA with 5 pmol of specific antisense primer (160-AS, 5'-CCCAAGCTTGGGGTCTCCAGAACC CGGA, and 191-AS, 5'-CCCAAGCTTGGGCGGGGAAGCTGGAATGG). Reverse transcription was carried out in a total volume of 20 μ l under standard conditions using 20 U AMV reverse transcriptase with an incubation at 25°C for 10 min and 42°C over 60 min. PCR was performed in a total volume of 100 μ l containing 5 μ l of the first-strand cDNA mix, 2.5 U *Taq* polymerase (Sigma,

St. Louis, MO), 0.2 mM dNTP mix, 20 pmol specific primers, and 1× PCR supplied PCR buffer. For generation of the HCV core 160-nt fragment and the HCV core 191 fragment, the combination of the sense primer 5'-AACATGACCACGAATCCC with either the antisense primer 5'-CTCCTCCAGAACCCGGA or the antisense primer 5'-CGGGGAAGCTGGAATGG was used.

Northern blotting

Total RNA was isolated from HepG2 cells using the RNeasy Total RNA Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). RNA separation was carried out by denaturing agarose gel electrophoresis [1.2% agarose, 12.3% formaldehyde, 20 mM MOPS-acetate (pH 7.0), 1 mM EDTA, 0.5% ethidium bromide] according to standard protocols (Sambrook *et al.*, 1989). RNA was transferred to nylon membranes (Hybond-N nylon membranes, Amersham Pharmacia Biotech, Braunschweig, Germany) by the capillary elution method in 7.5 mM NaOH and fixed at 80°C for 2 h. The membranes were hybridized with [α -³²P]dCTP 5' random labeled (approximately 10⁶ cpm/ml) specific cDNA probes in 50% formamide, 0.25 M NaPO₄ (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS, and 200 μ g/ml salmon sperm DNA at 43°C overnight. After being washed twice with 2× SSC and twice with 1× SSC, blots were exposed to Kodak AR X-Omat films at -70°C with intensifying screens. The loading of RNA was determined by GAPDH. Specific signals of at least three independent experiments were analyzed by Reystest software (Reystest, Straubenhardt, Germany).

Western blotting

Cells were washed twice with cold PBS and lysed by lysis buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 20 mM β -glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X, and a protease inhibitor cocktail (Roche Diagnostics). Separation of proteins was carried out by SDS-polyacrylamide gel electrophoresis (10–15% gel) according to standard protocols (Sambrook *et al.*, 1989). Transfer of protein to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) from SDS-PAGE was accomplished in a Hoefer TE 62X Transphor II unit. Membranes were blocked with TBS (Tris-buffered saline) buffer with 5% bovine serum albumine (BSA) overnight at 4°C. Blots were incubated with the primary antibody of interest at a concentration of 1:200 to 1:5000 in TBS buffer containing 1% BSA and 0.1% Tween 20. The primary antibodies were directed against c-Fos (Sc-2356, Santa Cruz Biotechnologies, Santa Cruz, CA), c-Jun (Sc-44, Santa Cruz Biotechnologies), ERK-1,2 (Sc-44, Santa Cruz Biotechnologies), JNK-1 (SC-474, Santa Cruz Biotechnologies), p38 MAPK (Sc-535, Santa Cruz Biotechnologies), and MKP-1 (Sc-1102, Santa Cruz Biotechnolo-

gies). The polyclonal anti-HCV core antibody was a gift from Dr. Bartenschlager (University of Mainz). After being washed in TBS-0.1% Tween, blots were incubated with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnologies) at a concentration of 1:2000 to 1:30,000 at RT for 1 h. Specific signals were detected by use of the ECL Western blotting reagents (Amersham Pharmacia Biotech, Braunschweig, Germany). Blots were exposed to Kodak AR X-Omat films for 1–5 min. Experiments were performed at least three times.

Immune complex MAP kinase assays

HepG2 cells were lysed in a buffer containing 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β -glycerolphosphate, 25 mM MgCl₂, 2 mM sodium orthovanadate, 1 mM DTT, and 1% NP-40. The cell lysate was transferred to a microcentrifuge tube and centrifuged for 15 min at 15,000 rpm at 4°C. Aliquots of the supernatant containing 100 μ g protein were incubated with 2 μ g of an antibody against ERK-1,2, JNK-1, or p38 MAPK for 1 h at 4°C, respectively. The sample was mixed with 20 μ l of agarose conjugate (Protein A-agarose, Protein G PLUS-agarose, or Protein A/G PLUS-agarose) and allowed to incubate at 4°C under constant agitation overnight. The immunoprecipitates were collected by centrifugation at 1000 rpm for 3 min at 4°C. After being washed three times in kinase reaction buffer [12.5 mM MOPS (pH 7.5), 12.5 mM β -glycerolphosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM sodium vanadate] the pellet was resuspended in 15 μ l of kinase buffer and incubated with 1 mg/ml myeline basic protein (for the ERK assay and p38 MAPK assay) or with 0.1 mg/ml glutathione S-transferase-c-Jun peptide (for the JNK assay), and 10 μ Ci [γ -³²P]ATP for 30 min at 37°C. The reaction was terminated by addition of 15 μ l of SDS-PAGE sample buffer, and activities of ERK-1,2, JNK-1, and p38 MAPK were detected by autoradiography after SDS-polyacrylamide gel electrophoresis (12% gel). Experiments were repeated at least three times.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from HepG2 cells by the addition of cell lysis buffer [20 mM HEPES (pH 7.9), 10 mM NaCl, 0.2 mM EDTA, 2 mM DTT, 1 mM Na Vanadate, and 1 mM proteinase inhibitor], and nuclei were precipitated by centrifugation and subsequent addition of nuclear lysis buffer [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 1 mM Na Vanadate, 420 mM NaCl, 0.2 mM EDTA, 2 mM DTT, 25% glycerol, and 1 mM protease inhibitor]. For determination of NF- κ B signals, cells were preincubated for 12 h with 50 U/liter of IL-1. Specific oligonucleotides for binding of AP-1 (5'-CGCTTGATGACTCAGCCGGAA), Elk-1 (5'-GGATGTCCATATTAGGACATCT), ATF-2 (5'-ACCACCCCTGACCTAACTCCG), and NF- κ B (5'-AGTTGAGGGGACTT-TCACAGGC) were prepared by end labeling of the 5'-

terminus with [γ - 32 P]ATP. Reactions were performed by incubation of 4 μ g nuclear extract with 8 fmol of γ - 32 P-labeled oligonucleotide for 30 min at RT in a buffer containing 5% glycerol, 2 mM MgCl₂, 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 60 mM KCl, and 50 μ g/ml of poly(dI-dC). The DNA-protein complex was separated from free oligonucleotide by electrophoresis on a 4% native polyacrylamide native gel and visualized after exposure to a Kodak AR X-Omat film at -70°C with intensifying screens. Experiments were performed at least three times.

Statistics

Results were compared by Student's *t* test using SPSS software (SPSS, Munich, Germany). *P*-values < 0.05 were considered statistically significant.

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