Western blotting analysis and quantitation

Cells were washed with ice cold PBS and lysed in lysis buffer (2%NP-40; 0.5% sodium deoxycholate; 0.2% SDS; 25 mM Tris-HCl, pH 7.5; 50 mM NaCl; proteases inhibitor cocktail). Protein concentration in cellular extracts was determined with Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.). 30 to 50 μg of proteins were separated by 10% or 12.5% SDS-PAGE for *c-myc* analysis, p53 and p21; separated proteins were then electrotransferred to polyvinylidene difluoride membrane (PVDF membranes, Immobilon, Millipore, Badford, U.S.A.) by a semidry system. After blocking overnight at 4°C the membranes were probed with the indicated monoclonal antibody for 1–2 h at 37°C or overnight at room temperature, followed by incubation with secondary antibody conjugated (anti-mouse IgG/horseradish peroxidase, Amersham, Buckinghamshire, U.K.). Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham).

Equal protein loading and transfer were verified by staining the membranes with Ponceau Red. The immunoblots were quantitated by densitometry and protein levels were normalized with respect to β-actin level. Protein expression changes two-fold or more than that of the control levels, as indicated by densitometric analysis of a series of blots (a minimum of two), were considered to be significant.

Analysis of c-myc protein stability

For the *c-myc* protein turnover experiments, cell lines were plated in six wells plates at 24 h before treatment with cycloheximide (70 μg/ml) to stop protein synthesis. At defined time points, cells were harvested and processed for immunoblotting. Intensities of the band were quantitated by densitometric analysis and normalisation to the β-actin level. Levels of *c-myc* at various times of treatment were expressed as the relative percentage of the *c-myc* protein at zero time.

Flow cytometric analysis

The level of p53 and p21 in parental and in established cell lines was measured by flow cytometric analysis as described [18]. Briefly, cells were collected from dishes and washed in PBS after fixation in 2% paraformaldehyde for 20 min at room temperature. Cells were then permeabilized with a solution containing 0.5% TritonX-100 for 10 min at room temperature. Fixed cells were then incubated for 45 min at room temperature with primary antibodies to p53 and p21, followed by incubation with corresponding FITC-conjugated secondary antibodies for 30 min at room temperature. After washing in PBS, the antigen density was measured by using Becton Dickinson FACScalibur Flow Cytometer and percentage of positive cells as well as intensity level of both antigens were determined by Cell Quest software.

Results

The effect of HCV core protein on cell growth

The effect of the core protein on cell growth was examined by the evaluation of proliferation rate and population doubling time in four independent clones of Hep39 cell lines expressing the core protein in comparison with vector control and parental HepG2 cell lines. According to the daily cell counts, core transfectants showed a faster proliferation rate than the parental HepG2 or Hepswx cells (Fig. 1A). Consistently, the cell doubling time was decreased from 28.4 hours and 33.7 hours in parental HepG2 and in Hepswx cell lines respectively to average

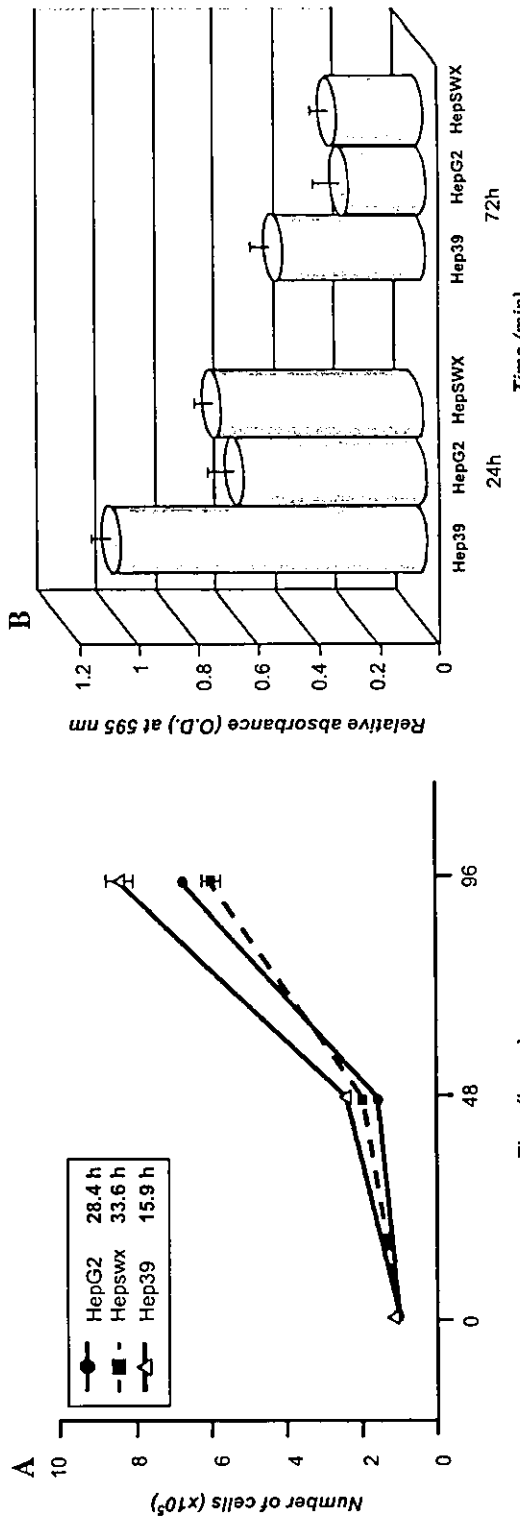


Fig. 1. Effect of HCV core protein expression on cell proliferation rate. A Growth curve was obtained by daily viable cell counts with the trypan blue exclusion method. The plots were obtained from mean values of two independent clones of Hepswx and four different clones of Hep39 cell lines. Duplicate wells from two independent experiments were counted at each time point and the population doubling time obtained from day 1 to day 4 is indicated. B MTT assay for viable cells. The plots were obtained from the mean O.D. values of three experiments

16 hours in Hep39 cells. Accordingly, results of the MTT assay (Fig. 1B) indicated faster proliferation of Hep39 cell lines, whose relative absorbance was twice than HepG2 and Hepswx cells lines in the time intervals analysed.

HCV core protein expression is suggested to be involved in faster growth character of hepatoblastoma cell lines.

The effect of HCV core protein on cell cycle

To correlate the increased cell growth of HCV core expressing cells with cell cycle progression, distribution was examined in non synchronised Hep39 cell lines in comparison with mock transfected. The percentage of cells in each stage of cell cycle was determined after propidium iodide staining on the basis of DNA content (Fig. 2). Consistent with the growth rate, exponentially growing core

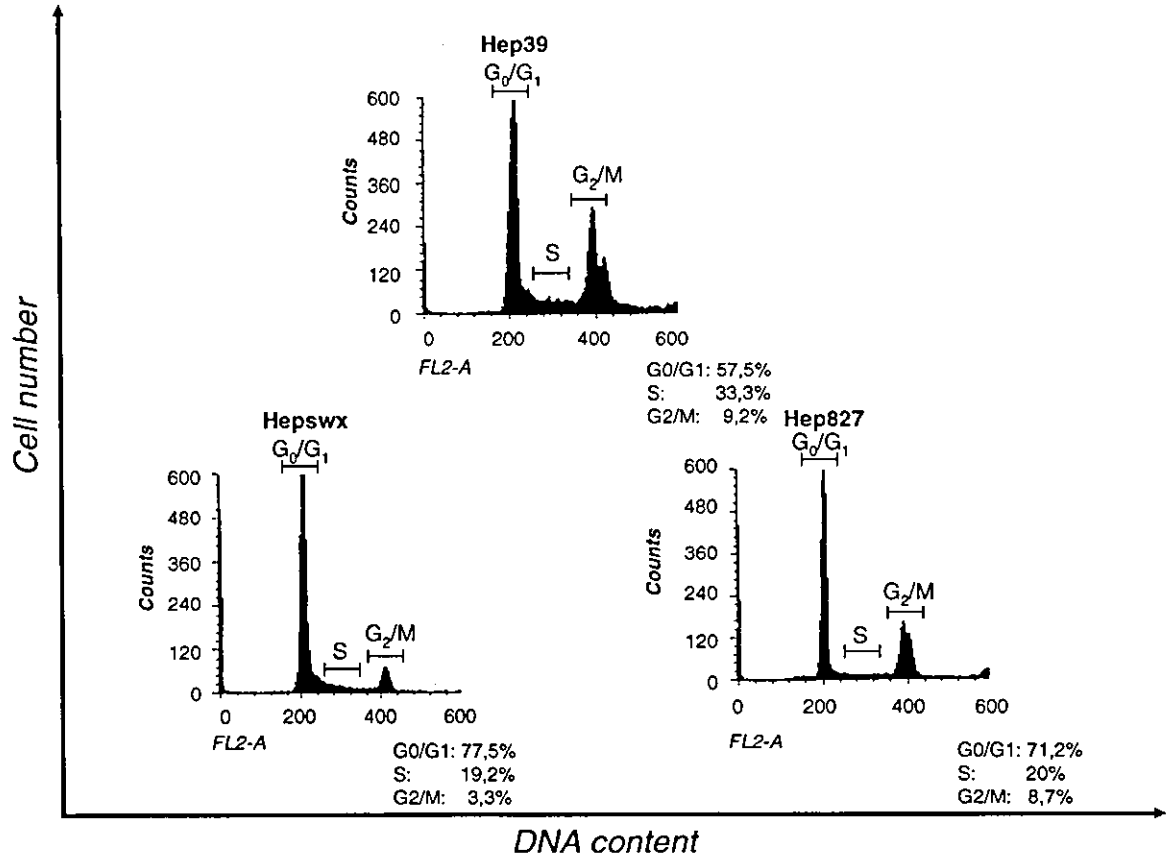


Fig. 2. Cell cycle analysis of HepG2 cells expressing HCV core protein (Hep39), E1-E2 proteins (Hep827) and mock transfected (Hepswx) cell lines. Flow cytometry analysis of the cell cycle was done on exponentially growing cell lines by CellQuest software; the DNA distribution was examined by ModFit 3.0 software. Mean percentage of cells in G0/G1, S and G2/M phase of the cell cycle is reported for each established cell line from two independent experiments. (The bars are for illustration purposes and are not to scale)

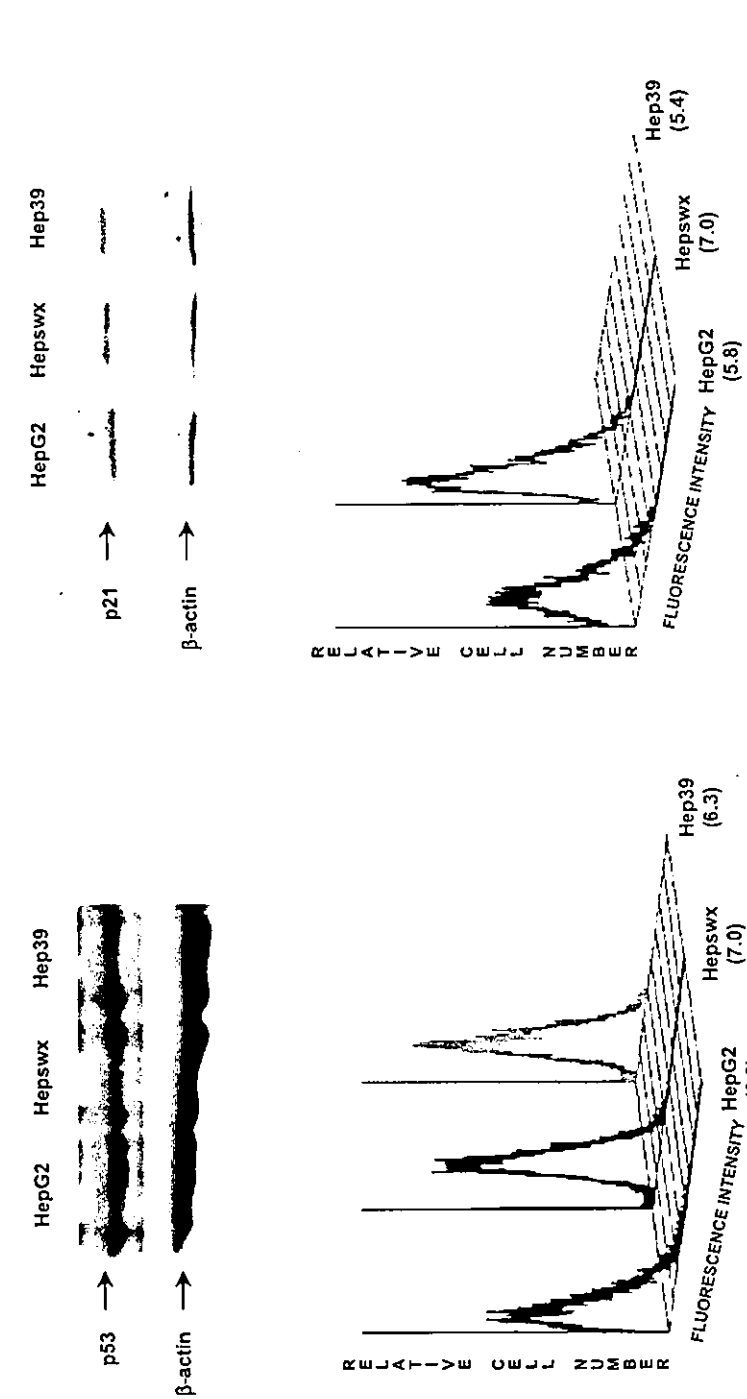


Fig. 3. Quantitative evaluation of endogenous p53 and p21 proteins expression in HepG2, Hepswx and Hep39 cell lines. Western blotting and Flow cytometry analysis indicates no significant changes in expression of p53 and p21 proteins in core expressing cell lines. Level of p53 and p21 was analysed by CellQuest software by acquisition of 10 000 events and intestine of p53 and p21 is indicated by numbers in parenthesis

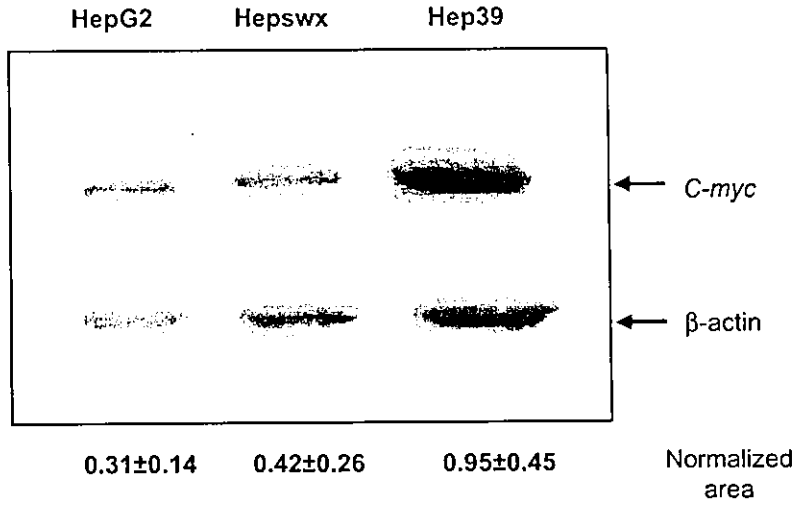


Fig. 4. *c-myc* protein expression in Hep39 cell line by immunoblot analysis. Total cell lysates (40 μg) from each cell line were subjected to 10% SDS-PAGE and probed with the specific monoclonal antibody as described in Materials and methods. The numbers represent mean values (area) of densitometric determination normalized to the level of β-actin, detected on the same blot, from three independent experiments, in which at least two different autoradiography were quantitated

transfected cell lines had a greater percentage of cells in S phase compared to that of Hepswx cells. An average of about two-fold increased S phase fractions was measured in Hep39 compared to the vector transfected cells. Mean percentage of cells in S phase quantitated by Modfit 3.0 software from two independent experiments were: 33.27% in Hep39 cells vs. 19% in mock transfected cell lines.

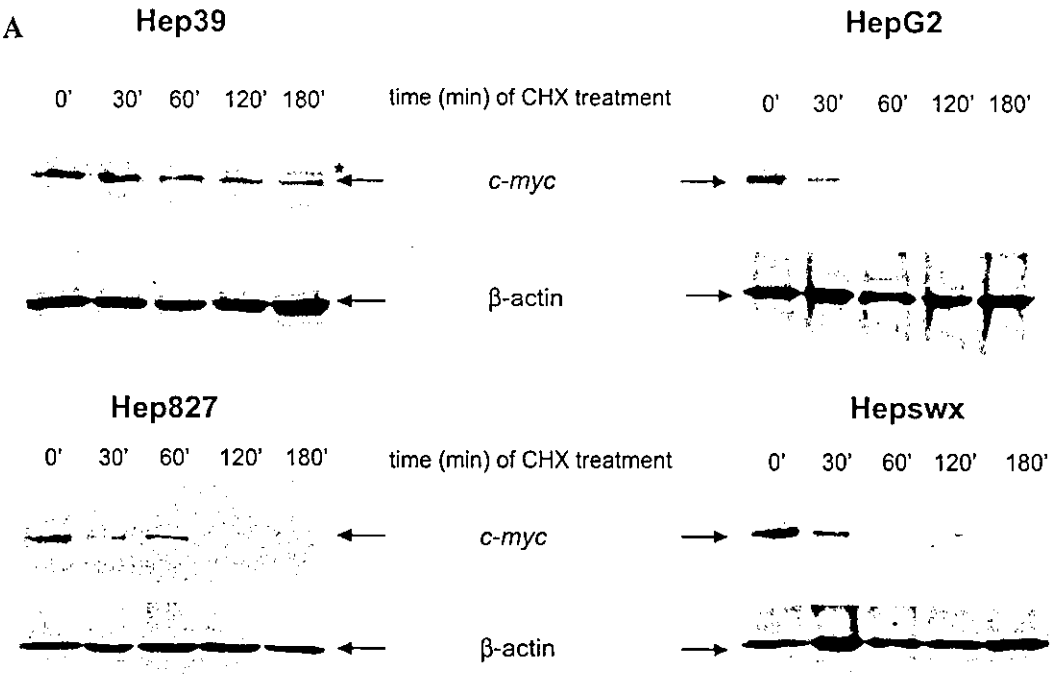
The S phase increase was also tested in E1-E2 expressing cell lines (Hep827) to exclude a potential non specific effect due to the general expression of HCV proteins in our cell system. The results showed that percentage of S phase cells in Hep827 population did not significantly differ from the vector control (Fig. 2). The data indicated that the core protein expression caused S phase cellular accumulation in non synchronised cell line of human liver origin.

Molecular mechanism for perturbation of the cell cycle

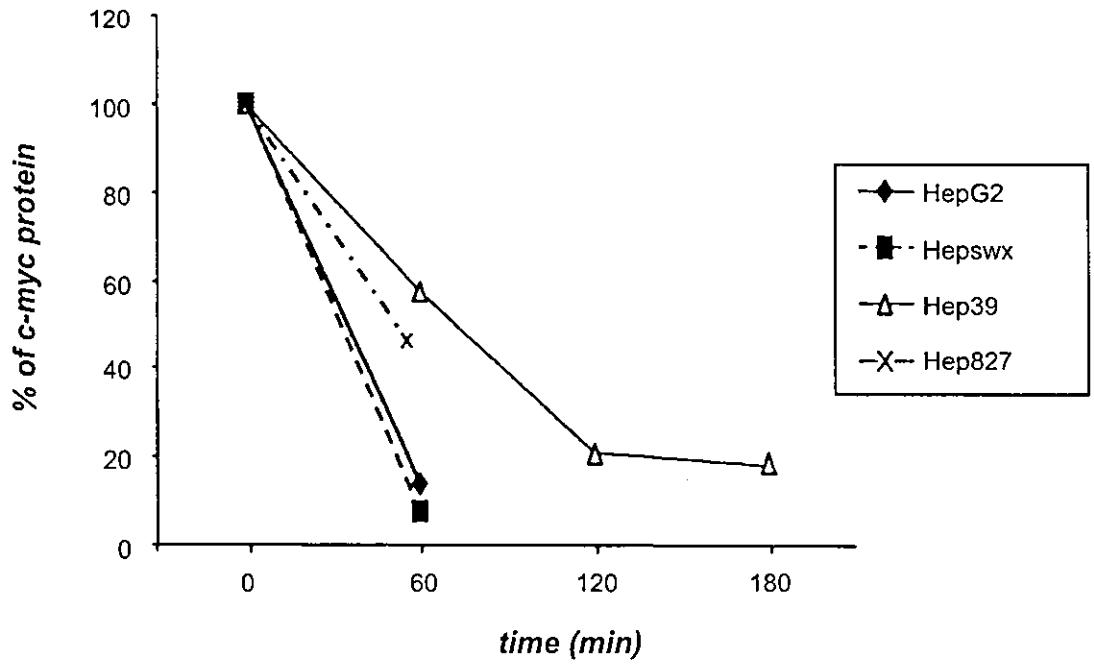
Cell cycle progression is regulated by various cellular proteins including p53, p21, p*Rb*, cyclins (from D1 to D3), cyclin E. Some transcription factors as E2F-1 and *c-myc* regulate cell cycle G1/S transition.

Fig. 5. Prolonged stability of *c-myc* protein in Hep39 cell lines. A Cells were treated with cyclohexamide (70 μg/ml) to block protein synthesis, lysed at the indicated times and processed for immunoblotting using anti-*c-myc* monoclonal antibody. B Densitometric quantitation of the blotting was used to determine the *c-myc* protein remaining at various times. The data obtained from densitometric analysis were expressed as the relative percentage of the amount of *c-myc* protein at the zero time point and plotted as a function of time

A


Hep39
HepG2
0' 30' 60' 120' 180'
time (min) of CHX treatment
0' 30' 60' 120' 180'
c-myc
β-actin
Hep827
Hepswx
0' 30' 60' 120' 180'
time (min) of CHX treatment
0' 30' 60' 120' 180'
c-myc
β-actin

B


120
100
80
60
40
20
0
% of c-myc protein
0
60
120
180
time (min)
HepG2
Hepswx
Hep39
Hep827

HCV core protein has been reported to have opposing effects on p53 and p21 expression and on their relative promoter activity depending on the expression system and the intracellular localization of the mature or truncated core protein [30, 39]. We analysed the effect of the core protein on p53, p21 and *c-myc* level to detect whether mechanisms mediated by these molecules might be responsible for cell cycle perturbation in Hep39 cells. In core stable transfectants of HepG2 cells endogenous expression of p53 and p21 proteins did not result to be affected by the core protein expression. In fact, no significant variation in p53 and p21 level could be detected by western blotting. Moreover immunofluorescence and FACS analysis did not indicate different percentage of cells expressing p21 and p53 or variation in their expression levels among Hep39 cell population compared to HepG2 and Hepswx cells (Fig. 3).

Conversely, immunoblotting analysis and densitometric quantitation of endogenous *c-myc* protein indicated an average two-fold increase of *c-myc* protein in Hep39 cells with respect to mock transfected (relative intensities after normalisation to the β-actin: 0.95 in Hep39 cells vs. 0.43 in Hepswx cells) (Fig. 4). The HCV core protein may influence endogenous *c-myc* expression in HepG2 cell line.

c-myc protein stability

We sought to examine the possibility that a prolonged half-life of *c-myc* protein could be responsible for the increased level of *c-myc* protein in Hep39 cells and contribute to the behaviour of core transfectants toward cell cycling. To this aim the rate of degradation of *c-myc* protein was measured following treatment of cell cultures with cycloheximide to block protein synthesis. Results reported in Fig. 5A indicated half-life values 4–5 times more stable in Hep39 cells expressing HCV core protein. *c-myc* half-life in Hep39 cells was two hours longer than HepG2 and Hepswx cell lines (Fig. 5B). This result suggests a close relation between *c-myc* protein stability and HCV core protein expression in human hepatoblastoma cell lines.

We could not identify the additional minor band (marked with an asterisk in Fig. 4A) appearing at 180 min during pulse-chase analysis detected by anti-c-myc specific mAb in Hep39 cells. Since Hep827 cell lines expressing E1-E2 proteins of HCV did not show such prolonged half-life of *c-myc* protein the core protein is indicated to be responsible for stabilization of the *c-myc* protein.

Discussion

The results from present study indicate that HCV core protein promotes cell proliferation and increases the S phase fraction in core stable transfectants of unsynchronized human hepatoblastoma cell line. Furthermore, the effect on cell growth was associated to increase of *c-myc* protein stability, which is one of the regulatory molecules that direct cell cycle progression. The HCV core protein is a component of viral nucleocapsid [34] with a number of reported regulative functions playing role(s) in the pathogenesis and oncogenesis associated to HCV

infection [21, 22]. The core protein can influence expression of cellular factors involved in cell growth, cellular DNA transcription [30, 39] and cell transformation [22, 23, 29]. Further studies indicate that HCV core protein can either promote or suppress apoptosis induced by various stimuli, depending on different cellular and expression systems [20, 28, 31, 39].

A role of HCV core protein in cell cycle regulation has been previously suggested by studies from two groups [5, 14]. Consistently with present results these reports described the effect of the core protein in the promotion/acceleration of S phase progression in various cell systems and growth conditions. Cho et al. showed a promotion of cell proliferation by HCV core protein mediated through upregulation of cyclin E in stable transfected Rat-1 cells [5]. Honda et al. reported accelerated progression through the S phase in synchronous culture of stable CHO transformants upon serum starvation by upregulation of *c-myc* [14]. Although these latter Authors used a cell system different from our un-synchronous un-starved HepG2 transfectants, however in both systems a pro-proliferation effect of the HCV core protein through increase of the S phase of the cell cycle could be detected.

The cell cycle regulation involves a number of regulatory proteins (p53, p21, *c-myc* cyclins, cDKs) that direct cell's progression through the stages of the cell cycle. Cell proliferation and apoptosis are tightly coupled processes and cell cycle regulators can influence both cell proliferation and death. Moreover some cellular factors involved in the cell cycle are cell-type specific and their expression or level may be diverse in different cell lines. Unequally from the above cited studies present data were obtained by the core protein expression in a human hepato-blastoma cell line, HepG2, which is biosynthetically and biochemically similar to human hepatocytes thus being suitable for studying viral-cell interactions of hepatotropic viruses, such as HCV. Moreover, we examined the core protein effect on cell cycle in non synchronized cell lines, which more appropriately reproduce conditions of the liver *in vivo*. Consistently with the cited studies, present results indicated that also in unsynchronised HepG2 cells, HCV core protein expression caused a perturbation of cell cycle, with increased fraction of cells in S phase.

A wealth of biological data support the role of *c-myc* oncoprotein as a central regulator of cell proliferation, apoptosis and cell cycle progression through induction of S phase entry in quiescent cells [3, 16]. In addition, *c-myc* oncogene has been reported to transform primary cells in cooperation with *ras* oncogene and when overexpressed *c-myc* may also induce apoptosis [2, 7, 37].

HCV core protein has been previously reported to affect *c-myc* expression by transactivation of *c-myc* promoter in transiently transfected cell lines [27] or by increasing expression of *c-myc* transcripts in stable CHO cells [14]. In our study endogenous *c-myc* mRNA was only slightly increased by intracellular expression of the core protein (data not shown). However, *c-myc* protein half-life was significantly prolonged in cell lines expressing HCV core protein (Fig. 4). The lack of the increase in percentage of cells in S phase and of extended *c-myc* protein half life in HepG2 cells expressing HCV envelope proteins suggested that these effects were strictly related to the core protein expression.

The mechanisms for the regulation of *c-myc* protein stability have been reported [33] and stabilisation of *c-myc* protein as possible result of defective proteolysis has been shown in several Burkitt's lymphoma-derived cell lines [10]. As the *c-myc* and core proteins are not known to interact each other, the effect is presumably indirect. One possibility is that *c-myc* protein could be modified so that it is less susceptible to proteolytic degradation. Alternatively, HCV core may affect pathways involved in *c-myc* turnover. Actually, a partial shift to a slower migrating form of *c-myc* was observed in stable cell lines expressing the core protein. This control mechanism of cellular protein expression levels by viral protein is in accordance with the reported multifunctional activities of HCV core protein. Furthermore some other viral proteins have been shown to act by stabilising cellular proteins involved in apoptosis and cell growth regulation. For example, adenovirus E1A protein induces stabilisation of p53 protein in some cell lines in which E1A expression is accompanied by apoptosis [19]; bovine papilloma E2 gene stabilises p53 in HeLa cells inducing p21 expression and blocking cells in G1 phase of the cell cycle [7].

p53 and functionally coupled p21 proteins may be modulated by several viral products [33] and are able to affect both apoptosis and cell cycle progression. HCV core protein has been reported to have opposing effects on p53 and p21 expression and on their relative promoters activity. In present study, flow cytometric and immunoblot analysis of core expressing cells indicated that p53 and p21 are not involved in the increased proliferation of Hep39 cell lines. Nevertheless it cannot be ruled out that HCV core protein could affect functional activity of p53 and p21 without modulating their expression levels.

In conclusion, HCV core protein increases the S phase fraction in HepG2 cells, by stabilising endogenous *c-myc* protein. This may indicate a potential role of HCV core protein in hepatocarcinogenesis.

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Identification of Basal Promoter and Enhancer Elements in an Untranslated Region of the TT Virus Genome

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The regulation of TT virus (TTV) gene expression was characterized. Transient-transfection assays using reporter constructs revealed that a 113-nucleotide (nt) sequence within the untranslated region, proximal to the transcription initiation site and containing a TATA box motif, has a basal promoter activity. This sequence is well conserved among different TTV genotypes. Upstream stimulating factor bound to a consensus binding motif within this region and positively regulates TTV transcription. Furthermore, a 488-nt region upstream of the basal promoter exhibited enhancer activity, presumably in a cell type-specific manner. This study illustrates some of the mechanisms involved in the transcriptional regulation of TTV.

TT virus (TTV), which was discovered in a patient with acute hepatitis, is an unenveloped, single-stranded, circular DNA virus, with a genome of approximately 3.8 kb (6). TTV is thought to be a new member of the *Circoviridae* family of viruses, and it was recently proposed that the virus be named Torque Teno virus (6). The TTV genome includes an untranslated region (UTR) of approximately 1.2 kb and a coding region of approximately 2.6 kb, including two major open reading frames which are sandwiched by the TATA box and polyadenylation signal motifs (11, 13, 15). Analyses of TTV transcripts have revealed three spliced mRNA species of 3.0, 1.2, and 1.0 kb with common 5′ and 3′ termini (9, 14). However, the molecular mechanisms controlling TTV transcription are still unknown. In this study, the basal promoter and enhancer of a TTV isolate, SANBAN of genogroup 3 (5, 18), were identified and functionally characterized.

First, we determined the transcription initiation sites of the TTV genome by 5′ rapid amplification of cDNA end (5′-RACE) analysis (Marathon cDNA amplification kit; Clontech) using poly(A)-rich RNA from a human hepatocellular carcinoma cell line, HepG2, transfected with a cloned TTV genome. The 5′-RACE PCR products were cloned and sequenced. We observed two potential transcription initiation sites, which map at nucleotides (nt) 121 and 110 (numbered according to the sequence deposited in DDBJ/GenBank/EMBL databases under accession number AB025946). Although transcription may be initiated at both sites, the upper site was designated position +1 in this study.

The UTR of the TTV genome contains a TATA box element between positions −40 and −35, as well as a number of putative transcription factor-binding motifs (Fig. 1A). Despite considerable genetic diversity throughout the whole genome, the UTR sequence was relatively conserved among the different TTV genotypes, presumably reflecting its functional constraints (15, 16). Thus, we analyzed transcriptional regulation of the UTR sequence.

To characterize TTV promoter activity, a firefly luciferase reporter plasmid, p(−890/+115), was constructed by subcloning the TTV sequence from positions −890 to +115, which was amplified by PCR using appropriate primers with restriction sites at the 5′ ends, into the promoterless pGL3-Basic (Promega). Eleven different cell lines were transfected with p(−890/+115), along with a *Renilla* luciferase expression vector, pRL-TK, as an internal standard for determining transfection efficiency. Luciferase activities in cell lysates prepared after 16 h of transfection were determined (2). It is of interest that the 1.0-kb fragment demonstrated a pronounced promoter activity in all the hepatocellular carcinoma cell lines tested. Human (Huh7, HepG2, and FLC4 [1, 2]) and mouse (Hepa1-clc7) hepatocellular carcinoma cells were tested (Fig. 1B). This fragment demonstrated the greatest activity in Huh7 cells (~10-fold greater than in other cells). We observed substantial promoter activity in GL37 (African green monkey kidney) and CHO (Chinese hamster ovary) cells, whereas limited activity was observed in Caco2 (human colon carcinoma), MOLT4 (human T-cell leukemia), CV1 (African green monkey kidney fibroblast), 3T3 Swiss (mouse fibroblast), and CMT93 (mouse rectal carcinoma) cells. These results indicate that the UTR of the TTV genome functions as a promoter in a cell type-specific manner.

To assess basal, proximal promoter activity in the UTR, a series of 5′ deletions fused to the luciferase gene were constructed and transfected into Huh7 and HepG2 cells (Fig. 2). A deletion extending to nt −601 [p(−601/+115)] enhanced promoter activity in both cell lines (by ~1.5-fold), while deletion of another 274 nt [p(−327/+115)] decreased promoter activity by more than 80%, suggesting that there is a negative regula-

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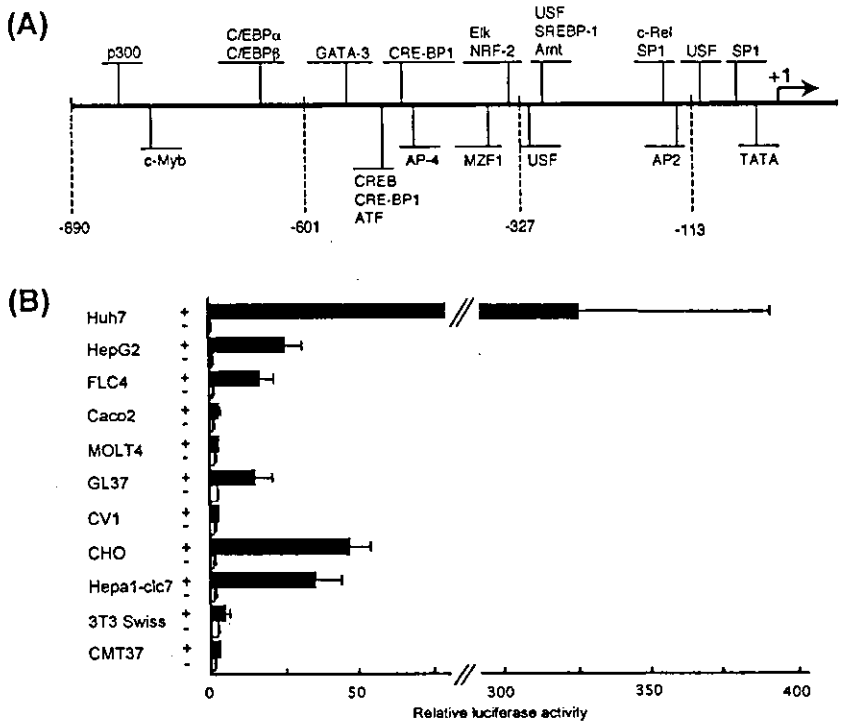

FIG. 1. Functional analysis of the TTV promoter in the viral UTR. (A) Schematic representation of the 1.0-kb UTR sequence. The TATA box element and putative transcription factor-binding sites are shown. Transcription factor-binding sites were identified using the TRASFAC database and a search program (http://motif.genome.ad.jp/). The transcription initiation site (+1) is indicated and corresponds to nt 121 (AB025946). The numbers at the bottom of vertical dotted lines indicate the start points of the full-length promoter construct and deletion mutants of the promoter constructs used in Fig. 1B and 2. (B) Cell type specificity of the TTV promoter activities. Cells were transfected with p(−890/+115) (+) or promoter-less pGL3-Basic (−) together with pRL-TK (*Renilla* luciferase). Cell extracts were prepared 16 h after transfection, and luciferase activities in the extracts were determined using a dual-luciferase reporter assay system (Promega) with the Lumat LB9501 luminometer (Berthold). All values were normalized to *Renilla* luciferase activities and are shown as means ± standard deviations (error bars) of three independent samples.

tory element between nt −890 and −601. A deletion extending to nt −113 [p(−113/+115)] resulted in a slight to moderate reduction in activity, but promoter activity still remained greater than that observed with p(+15/+113), in which the TATA box and the transcription start sites were deleted. These findings suggest that the 113 nt immediately upstream of the transcription initiation site contains a basal promoter region critical for TTV gene expression.

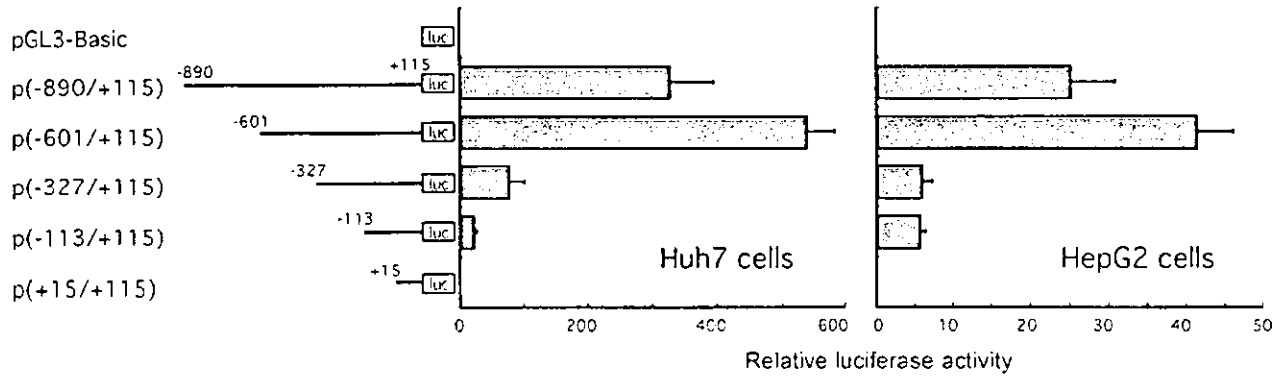

FIG. 2. Deletion analysis of the TTV promoter in Huh7 and HepG2 cells. The structures of the luciferase reporter constructs containing various lengths of the TTV UTR sequence are shown to the left. A series of DNA fragments with 5' deletions of the TTV promoter were amplified by PCR using the full-length TTV DNA of SANBAN isolate as a template with the same reverse primer and various forward primers. The fragments were cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples.

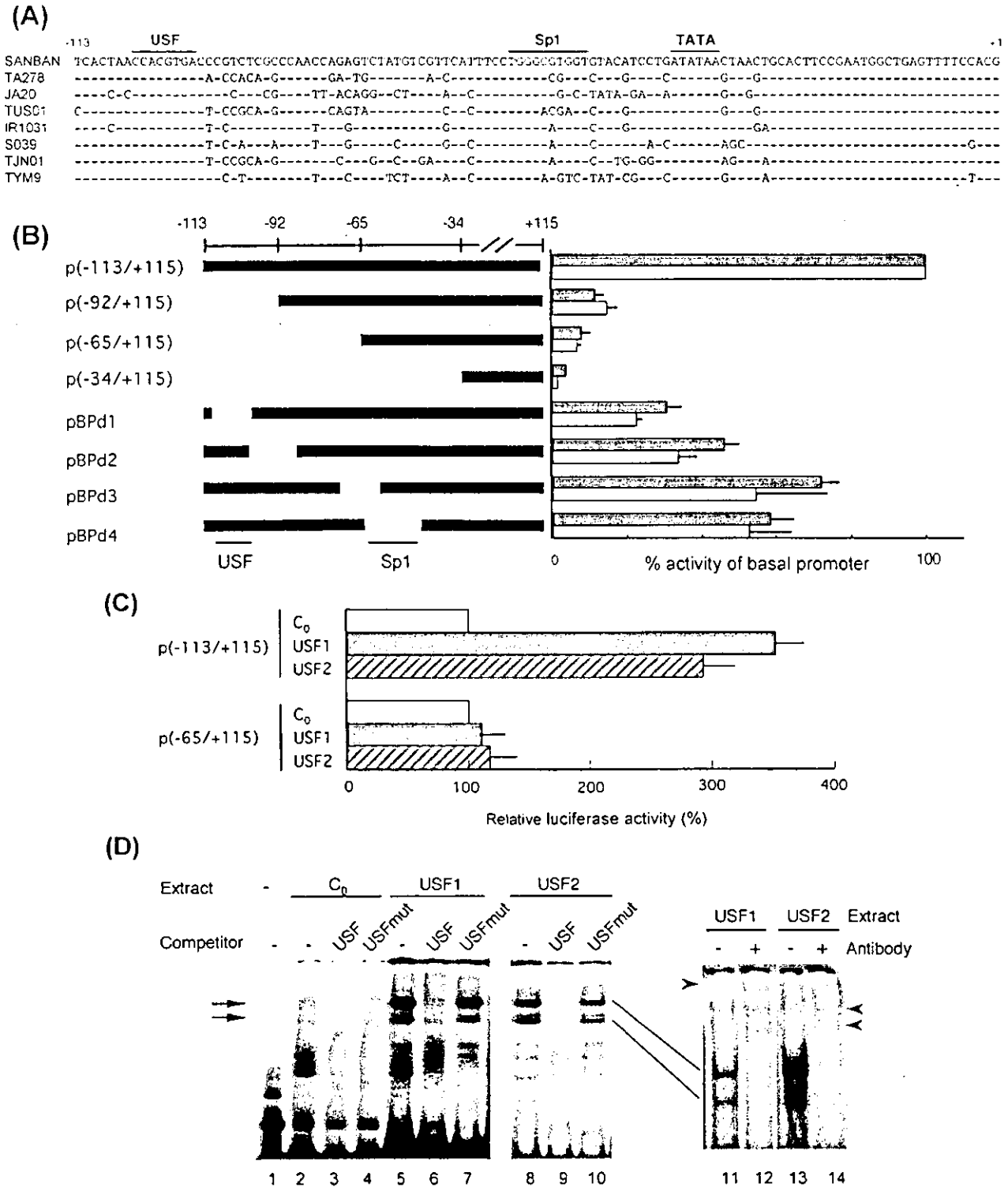

FIG. 3. Basal promoter activity of TTV regulated by USF. (A) Alignment of the putative basal promoter regions from TTV isolates SANBAN (DDBJ/GenBank/EMBL accession number AB025946), TA278 (AB017911), JA20 (AF122914), TUS01 (AB017613), IR1031 (AB038619), S039 (AB038620), TJN01 (AB028668), and TYM9 (AB050448). The transcription initiation site is numbered +1. The TATA box and positions of putative binding sites for USF and SP1 are indicated. Nucleotides that are identical to those in the SANBAN isolate (−) are indicated. (B) Effect of deleting DNA from the basal promoter region on the TTV basal promoter activity. A series of DNA fragments with 5′ or internal deletions of the basal promoter region were amplified by PCR and cloned into pGL3-Basic at XhoI and HindIII sites. The indicated constructs were transfected into Huh7 cells (gray bars) or HepG2 cells (white bars). Relative luciferase activity in each transfectant was determined as described in the legend

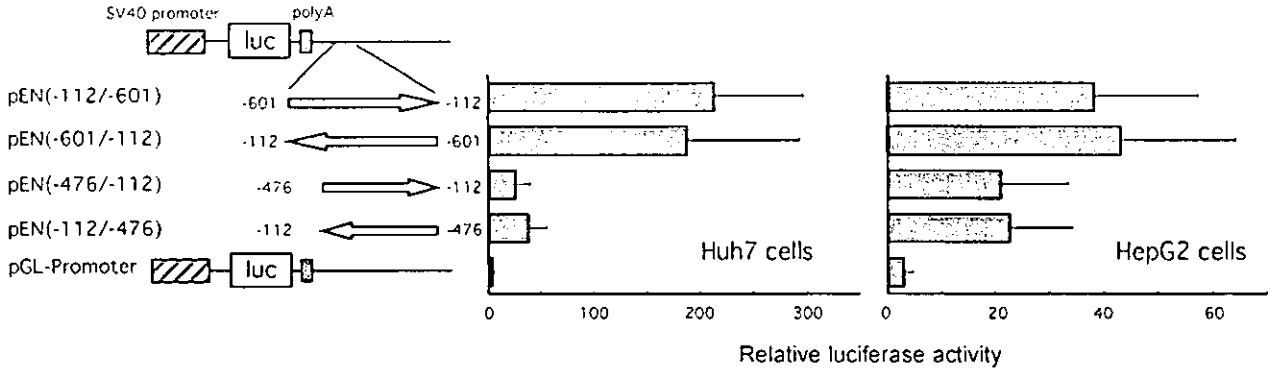

FIG. 4. Enhancer activity of the 488-nt fragment (−601/−114) of the TTV UTR. The 488-nt (−614/−114) and 363-nt (−476/−114) fragments were PCR amplified using primers with 5′ overhangs containing BglII (sense) and BamHI (antisense) sites. The fragments were then cloned into pGL3-Promoter at the BamHI site. The indicated constructs (left panel) were transfected into Huh7 cells or HepG2 cells. Relative luciferase activity in each transfectant was determined as described in the legend to Fig. 1B. Results are shown as means ± standard deviations (error bars) of three independent samples. SV40, simian virus 40.

Computer-assisted analysis of this basal promoter region identified potential binding sites recognized by upstream stimulating factor (USF) and Sp1, which are conserved among TTV genotypes (Fig. 3A). To determine whether these sequences contribute to TTV promoter activity, 5′ or internal deletion mutations were introduced into p(−113/+115), which was then examined for dual luciferase activity. Deletions, those found in p(−92/+115) and pBPd1, reduced promoter activity by 70 to 85%, suggesting that the USF-binding sequence is crucial for TTV promoter activity (Fig. 3B). Deletion of a Sp1-binding sequence (pBPd4) also conferred a decrease in promoter activity, although their effects were relatively moderate, suggesting that the Sp1-binding motif and/or its encompassing sequence may play a role in regulating TTV promoter activity by maintaining the structural integrity of the transcriptional machinery.

In genes where USF regulates transcription, cotransfection of USF expression vectors with reporter genes stimulates reporter activities. To further investigate the effect of USF on TTV promoter activity, we cotransfected USF1 or USF2 expression vectors (pCMV-USF1 and pCMV-USF2) (7) with p(−113/+115) into HepG2 cells. The cotransfection significantly increased promoter activity (by threefold), suggesting that USF proteins regulate TTV transcription (Fig. 3C).

USF is a family of basic-helix-loop-helix-leucine zipper transcription factors, initially identified by their ability to bind to the 5′-CACGTG-3′ sequence within the adenovirus major late promoter (3, 4, 10). USF1 and USF2 have been subsequently shown to bind to the promoters of various cellular and viral genes. To determine whether the TTV basal promoter was capable of USF binding, gel mobility shift assays were performed on an end-labeled oligonucleotide (nt −113 to −84) containing the putative USF-binding motif (Fig. 3D). DNA-protein-binding complexes were observed in nuclear extracts from cells transfected with pCMV-USF1 (Fig. 3D, lane 5), pCMV-USF2 (lane 8), and the empty vector (lane 2). An excess of unlabeled homologous probe competed with the protein binding (lanes 3, 6, and 10), whereas a mutated USF sequence failed to compete (lanes 4, 7, and 10). The addition of anti-USF antibodies to the binding reaction mixture supershifted the DNA-protein complexes (lanes 12 and 14). The combined data demonstrate that USF binds to its binding motif within the TTV basal promoter to up-regulate viral transcription.

On the basis of the results of the luciferase assays using 5′ deletions of the TTV UTR (Fig. 2), the positive regulatory element appears to be located immediately upstream of the basal promoter. To ascertain whether the 488-bp fragment between nt −601 and −113 functions as the enhancer region, this fragment or a 5′ deletion of this fragment was placed downstream of the polyadenylation signal in pGL3-Promoter (Promega), driven by the simian virus 40 promoter, in either

to Fig. 1B. Results are shown as a percentage of the activity in cells transfected with p(−113/+115); values are shown as means ± standard deviations (error bars) (n = 3 per group). (C) Effects of USF overexpression on basal promoter activity. HepG2 cells were cotransfected with each reporter construct with pCMV-USF1 (USF1), pCMV-USF2 (USF2), or empty pC_0 vector (C_0). Luciferase activity was determined 48 h after transfection. For each reporter construct, relative luciferase activity is presented as a percentage of the activity in pC_0-transfected cells. (D) Binding of USF proteins to the region from nt −113 to −84 in the TTV basal promoter. The electrophoretic mobility shift assays were performed as described previously (19). A double-stranded oligonucleotide corresponding to the TTV sequence from nt −113 to −84 was used as a probe. Nuclear extracts from the cells transiently transfected with pCMV-USF1 (USF1; lanes 5, 6, 7, 11, and 12), pCMV-USF2 (USF2; lanes 8, 9, 10, 13, and 14), or pC_0 (C_0; lanes 2 to 4) or no extract (lane 1) were mixed with ^{32}P-labeled probe for the binding reaction mixtures. Competitors, unlabeled probe (USF), and a mutant with the USF-binding motif (USFmut) were added at a 25-fold molar excess. The sense sequence (5′-TCACTAAC CAATTGACCCGTCTCGCCCAAC [the mutated nucleotides are underlined]) and complementary sequence of the mutant with the USF-binding motif were added. A supershift experiment was also performed by incubating antibody against USF1 (lane 12) or USF2 (lane 14) (+) with the nuclear extracts before the probe was added. The positions of specific binding complexes (arrows) and supershifted complexes (arrowheads) are indicated.

the sense or antisense orientation. Luciferase activity of constructs containing the 488-bp fragment [pEN(−601/−114) and pEN(−114/−601)] led to 50- and 10-fold stimulation in Huh7 and HepG2 cells, respectively. The 5′ deletion extending to nt −476 [pEN(−476/−114) and pEN(−114/−476)] reduced enhancer activity (Fig. 4). No enhancement was observed by transfection of MOLT4 cells with pEN(−601/−114) and pEN(−114/−601) (data not shown). These results demonstrate that the 488-bp region upstream of the basal promoter contains an enhancer element, suggesting cell-specific transcription of the TTV genome. It is noteworthy that the enhancer element is conserved among TTV genotypes. For example, 72% homology has been observed between clones SANBAN and TA278, and the database search has revealed at least 20 potential transcription factor-binding sites within this element, including CREB and CRB, which are activated upon cyclic AMP signaling-dependent phosphorylation (12, 17).

While the manuscript was being prepared, Kamada et al. reported the promoter and enhancer activities in the UTR of TTV, clone VT416 whose genome is 98% similar to that of TA278, and its cell tropism (8). However, they did not identify transcription factors that bind to the region and regulate TTV transcription. In summary, the findings reported by Kamada et al. and the findings of our present study emphasize the important role of the UTR as a basal promoter and enhancer within the UTR. Other areas of interest for further study include the identification of additional factors involved in tissue-specific TTV transcription and determining the significance of polymorphism of the regulatory elements.

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Down-regulation of viral replication by adenoviral-mediated expression of siRNA against cellular cofactors for hepatitis C virus

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Abstract

Small interfering RNA (siRNA) is currently being evaluated not only as a powerful tool for functional genomics, but also as a potentially promising therapeutic agent for cancer and infectious diseases. Inhibitory effect of siRNA on viral replication has been demonstrated in multiple pathogenic viruses. However, because of the high sequence specificity of siRNA-mediated RNA degradation, antiviral efficacy of siRNA directed to viral genome will be largely limited by emergence of escape variants resistant to siRNA due to high mutation rates of virus, especially RNA viruses such as poliovirus and hepatitis C virus (HCV). To investigate the therapeutic feasibility of siRNAs specific for the putative cellular cofactors for HCV, we constructed adenovirus vectors expressing siRNAs against La, polypyrimidine tract-binding protein (PTB), subunit gamma of human eukaryotic initiation factors 2B (eIF2Bγ), and human VAMP-associated protein of 33 kDa (hVAP-33). Adenoviral-mediated expression of siRNAs markedly diminished expression of the endogenous genes, and silencing of La, PTB, and hVAP-33 by siRNAs substantially blocked HCV replication in Huh-7 cells. Thus, our studies demonstrate the feasibility and potential of adenoviral-delivered siRNAs specific for cellular cofactors in combating HCV infection, which can be used either alone or in combination with siRNA against viral genome to prevent the escape of mutant variants and provide additive or synergistic anti-HCV effects.

Keywords: Small interfering RNA; Hepatitis C virus; Cellular factor; Antiviral agents

Introduction

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis (Choo et al., 1989), which frequently leads to liver cirrhosis and hepatocellular carcinoma (Saito et al., 1990). It has been estimated that more than 3% of the world population is infected with HCV. The only current medical therapy for HCV infection is IFN either alone or in combination with ribavirin. Unfortunately, only a small fraction of treated patients develop a sustained remission. Thus, development of new therapies for HCV infection is of great clinical and economic significance.

RNA interference (RNAi) is an evolutionarily conserved phenomenon of posttranscriptional gene silencing (PTGS) that has been described in plants, invertebrates, and vertebrates. In this process, double-stranded RNA (dsRNA) is cleaved into small interfering RNAs (siRNAs) of 21–23 nt by an RNaseIII-like enzyme known as Dicer, followed by incorporation of siRNA into a RNA-induced silencing complex (RISC) that recognizes and cleaves the target. In mammals, however, it has been reported that long dsRNAs (larger than 30 nt in length) induce an antiviral interferon response and, in turn, lead to the nonspecific translational shutdown. Thus, its application to mammalian cells is largely limited. A crucial insight came from Elbashir et al. (2001), who found that specific gene silencing in mammalian cells can be mediated by siRNAs of 21 nt, which can bypass dsRNA-induced nonspecific interferon response. This significantly facilitated the use of siRNA technology in mammalian cells. As a powerful reverse genetic approach, siRNA contributes greatly to linking of gene sequence with biological function. Additionally, the potential of using siRNA for prevention and treatment of viral infection has also proved to be promising. A couple of

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laboratories have demonstrated inhibitory effect of siRNAs on respiratory syncytial virus (Bitko and Barik, 2001), HIV (Jacque et al., 2002), poliovirus (Gitlin et al., 2002), and more recently on HCV (Randall et al., 2003). Although these studies have taken us to a fascinating new field in antiviral therapy, one outstanding drawback of the approach using siRNA direct against viral genome is that genetic heterogeneity due to high mutation rates of viruses may lead to emergence of escape variants resistant to siRNA. Indeed, it was shown that a single point mutation in the siRNA target region conferred escape in poliovirus (Gitlin et al., 2002). Alternatively, cellular factors involved in the viral life cycle provide more attractive candidates for siRNA targeting to ensure a sustained antiviral effect. For example, it has been reported that siRNAs targeted to the HIV-1 main receptor, CD4 (Novina et al., 2002), or coreceptor, CCR5 (Qin et al., 2003), can suppress the entry and replication of HIV-1.

In this study, we investigated the therapeutic feasibility of siRNAs specific for the putative cellular cofactors for HCV, including La, polypyrimidine tract-binding protein (PTB), subunit gamma of human eukaryotic initiation factors 2B (eIF2Bγ), and human VAMP-associated protein of 33 kDa (hVAP-33). Our results demonstrate that knockdown of the endogenous La, PTB, or hVAP-33 with adenoviral-delivered siRNA efficiently inhibits HCV RNA replication in cultured cells.

Results

Silencing of the endogenous genes in Huh-7 cells

Although the exact significance is not fully understood, there is increasing experimental evidence that showed specific interaction between various cellular factors and HCV RNA or viral protein, implicating potential roles of these proteins in HCV RNA replication and/or translation. Among these cellular factors, La antigen was shown to bind to the 5′-untranslated region (UTR) of HCV (Ali and Siddiqui, 1997), PTB was shown to interact with both 5′- and 3′-UTR (Ali and Siddiqui, 1995; Tsuchihara et al., 1997), and hVAP-33, a SNARE-like protein, was found to specifically bind to NS5A and NS5B proteins, suggesting that it may be responsible for the association of the HCV replication complexes with the membrane (Tu et al., 1999). Additionally, eIF2Bγ was identified as a cofactor of HCV cap-independent translation (Kruger et al., 2000). To explore whether these presumed HCV cofactors can be used as functional targets for therapeutic siRNA, we first screened the RNAi activity of several synthetic siRNA molecules for each target gene (data not shown) and the most potent one was further used to construct siRNA expression plasmids (Fig. 1A). The small-hairpin RNAs are transcribed from each of these plasmids under the control of human U6 promoter. Huh-7 cells were transfected with each siRNA-expressing plasmid, and the expression level of each endogenous gene in the transfected cells was assessed by Northern blot analysis. When compared with the empty vector pShuttle, siRNA-expressing plasmid reduced the mRNA level of their respective target genes, albeit with different degree (Fig. 1B). The mRNA level of GAPDH, which served as an internal control for RNA loading, remained constant in each lane. Expectedly, each siRNA had no effect on the expression level of the irrelevant genes, confirming a specific RNAi effect induced by expressed siRNA.

Effects of the endogenous gene silencing on HCV internal ribosome entry site-dependent translation

Both La and PTB were shown to bind to HCV RNA in 5′-UTR, which constitutes internal ribosome entry site (IRES), indicating their potential roles in regulating HCV IRES-mediated translation. However, while Siddiqui and co-workers reported that La antigen (Ali et al., 2000) and PTB (Anwar et al., 2000) greatly enhance HCV IRES-mediated translation, Kaminski et al. (1995) contradicted the role of PTB in translation of HCV RNA, and Isoyama et al. (1999) found low requirement of La protein for HCV IRES activity. Translation is one of the processes in viral replication, and authentic cofactors for HCV IRES function are promising targets for therapeutic siRNA. To further clarify the conflicting issue on the functional role of these proteins in IRES-mediated translation, HepT cells stably expressing T7 RNA polymerase (Zhang et al., 1999) were transfected with each pShuttlesiRNA and the reporter vector pNC371RL (Zhang et al., 2002), which contains a T7 promoter, nt 1–371 of HCV sequence fused in frame with the Renilla luciferase (Rluc) gene. The cell lysates were assayed for the HCV IRES-dependent Rluc expression 48 h following transfection. To analyze the influence of the siRNA-mediated gene silencing on cap-dependent translation, pGL3-Control vector was cotransfected and the firefly luciferase (Fluc) activity was measured simultaneously. Compared with the Rluc activity in cells transfected with the empty vector pShuttle (100%), the Rluc activities in cells transfected with pShuttlesiLa, pShuttlesiPTB, pShuttlesieIF2B, and pShuttlesihVAP were 57.8%, 62.6%, 98.2%, and 66.0%, respectively (Fig. 2A). While the cap-dependent Fluc expression was comparable in these transfectants, except a marginal reduction in cells transfected with pShuttlesieIF2B. To test the specificity of inhibition by siRNAs, reporter assay with pEMCVRL, in which the translation of Rluc gene is directed by encephalomyocarditis virus (EMCV) IRES identical to that inserted in the HCV replicon, was also conducted as described above. As shown in Fig. 2B, the EMCV IRES-directed Rluc expression was not significantly affected by expression of siLa, siPTB, and sieIF2B, whereas a moderate inhibition of EMCV IRES-dependent translation was observed in cells expressing sihVAP. These results provide further evidence of La and

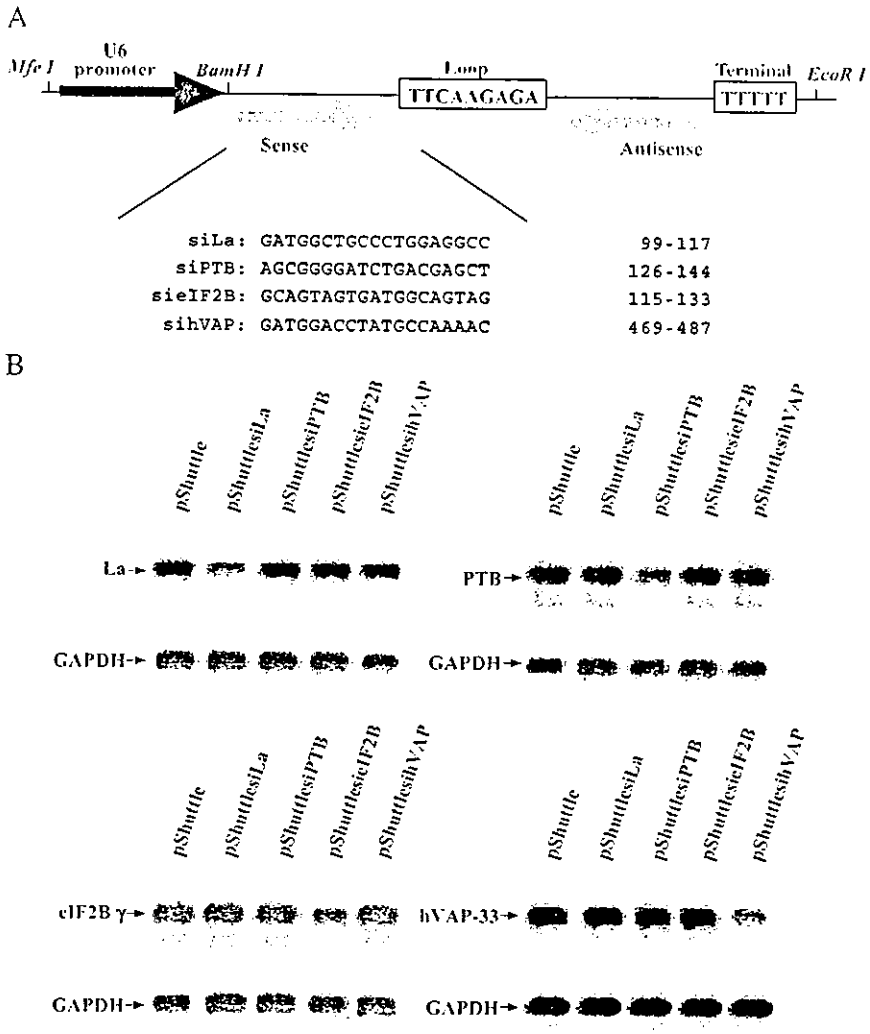

Fig. 1. (A) Schematic diagram of the siRNA expression cassette. The short hairpin form of siRNA is expressed under the control of a human U6 promoter. The five thymidines serve as a terminal signal. The sense and antisense siRNA strands are separated by a loop of 9 nt. Numbers indicate the positions of each target cDNA sequence. (B) Reduction of endogenous gene expression in Huh-7 cells transiently transfected by siRNA-expressing plasmids. Huh-7 cells were transfected with the indicated plasmids, and total RNAs were prepared at 48 h posttransfection. The mRNA level of La (upper left), PTB (upper right), eIF2Bγ (lower left), and hVAP-33 (lower right) was analyzed by Northern blot with each specific probe. GAPDH served as a loading control.

PTB as cofactors for HCV IRES activity, which consequently suggests the utility of these host factors-targeting siRNAs in combating HCV infection. Simultaneously, the data presented here also suggest a functional relevance of hVAP-33 in both HCV and EMCV IRES-controlled translation initiation, which has not been reported previously. The mechanism underlying this observation and whether hVAP-33 is a universal internal initiation factor are to be further investigated.

Enhanced siRNA effect by adenoviral-mediated gene delivery

One important issue in utilizing siRNAs as therapeutic agents for human disease is how to deliver them to cells of action. Among the gene delivery vehicles currently used in gene therapy approach, adenovirus is an attractive candidate for delivering siRNA against HCV, because it elicits long-lived transgene expression and allows targeting to the liver in vivo. Thus, we next cloned each siRNA expression cassette into recombinant adenovirus vector. Huh-NNRZ cells stably replicating the HCV subgenomic replicon were transduced with each siRNA-expressing adenovirus at the multiplicity of infection (MOI) of 80, 30, and 10, cells were harvested at day 3 postinfection. Target mRNAs and proteins were analyzed by Northern and Western blot analysis in parallel. Transduction of the siRNA-expressing adenoviruses dose-dependently reduced the expression of their respective target genes, and the mRNAs or proteins of endogenous genes were markedly diminished by infec-

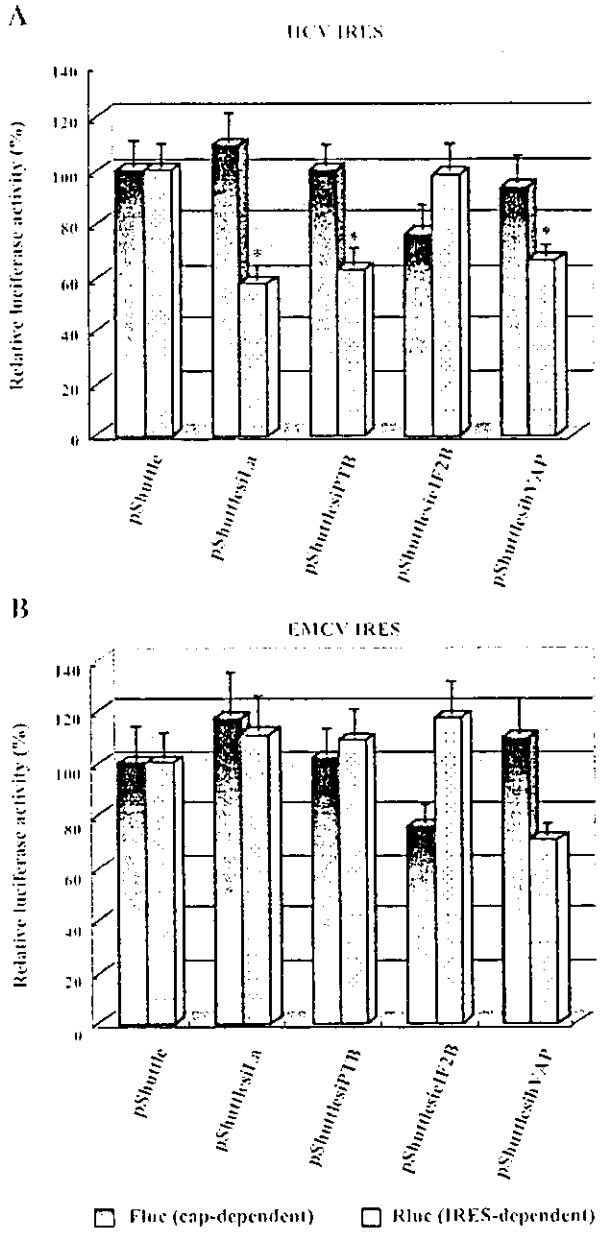

Fig. 2. Effect of endogenous genes silencing on HCV (A) or EMCV (B) IRES-dependent translation. HepT cells stably expressing T7 RNA polymerase were transfected with the reporter vector pNC371RL (A) or pEMCVRL (B), pGL3-Control vector, and the indicated siRNA-expressing plasmid. The cap-dependent firefly (Fluc) (shaded columns) and the IRES-dependent Renilla (Rluc) (dotted columns) luciferase activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. *$P < 0.05$ compared with pShuttle.

tion of AdsiRNAs at the MOI ≥ 30 (Figs. 3A and B). While infection with the irrelevant AdsiLuc expressing siRNA against luciferase gene, even at the MOI of 80, had no effect on the expression of the target genes. Thus, adenovirally expressed siRNA elicits much more potent RNAi activity when comparing with transient transfection of plasmids.

Effects of the endogenous gene silencing on HCV replication

To test the therapeutic potential of these siRNAs, we next investigate the effect of the endogenous gene silencing on HCV replication. Because HCV does not grow in cultured cells, we evaluated antiviral efficacy of the siRNAs in Huh-7 cells harboring an autonomously replicating HCV subgenome (Huh-NNRZ cells) (Kishine et al., 2002). Structurally, the subgenomic replicon in Huh-NNRZ cells contains the 5′-UTR, the first 36 nucleotides of the core region fused directly with the neomycin phosphotransferase gene, IRES element from EMCV, a nonstructural protein (NS) region from NS3 to NS5B and the 3′-UTR of HCV genome. The G418 resistance of Huh-NNRZ cells is conferred by persistent expression of neomycin phosphotransferase from replicating HCV RNAs. Thus, the ability of Huh-NNRZ cells to grow in G418-containing medium is an indirect measure of HCV replication. Huh-NNRZ cells were infected with AdsiLa, AdsiPTB, AdsieIF2B, AdsihVAP, or AdsiLuc at the MOI of 80, 30, and 10, and cultured in the absence or presence of G418 selection. After 10 days, the viable cells that were resistant to G418 because of the propagation of replicon were quantified with Cell Proliferation Reagent WST-1. In the absence of G418, viability of the cells transduced with each AdsiRNA was comparable to that of mock-infected cells (Fig. 4), except a moderate decrease in cells infected at the MOI of 80, probably reflecting some cellular toxicity or impaired survival due to higher degree of endogeneus gene silencing. Following a 10-day exposure to G418, cells transduced with AdsiLa, AdsiPTB, and AdsihVAP showed a substantial and dose-dependent reduction in viable cell count, decreasing viable cell count by 99% and 97% at the MOI of 80 and 30, respectively. Significantly, even a modest reduction of La and PTB expression by infection of AdsiRNA at the MOI of 10 (Fig. 3) also resulted in substantial inhibition of HCV replication, leading to a 95% and 90% reduction in viable cell count, respectively. These results indicated that G418 resistance in most cells was lost due to the down-regulation of replicating HCV RNA by siRNA-mediated endogenous gene silencing. Consistent with the results from reporter assay, transduction with AdsieIF2B (Fig. 4) or irrelevant AdsiLuc (data not shown) had no significant effect on cell growth in the presence of G418. Because synthesis of the nonstructural proteins from the replicon is directed by EMCV IRES, silencing of cellular cofactor for EMCV IRES may also block the replication of HCV replicon. However, EMCV IRES-mediated translation was not affected by siLa or siPTB under the conditions in our study (Fig. 2B), so we conclude that significant loss of G418 resistance in replicon cell by depletion of La or PTB reflected a net interference effect of siLa and siPTB on HCV infection. In addition to