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Expression studies of the HCV-1a core+1 open reading frame in mammalian cells

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Abstract

The hepatitis C virus (HCV) genome possesses an open reading frame overlapping the core gene in the +1 frame (core+1 ORF). Initial studies, mainly in rabbit reticulocyte lysates, indicated that the HCV-1 core+1 ORF is expressed by a −2/+1 frameshift at codons 8–11 during translation elongation of the viral polyprotein, resulting in a protein known as alternative reading frame protein (ARFP), frameshift (F), or core+1. However, subsequent investigation, based on reporter constructs carrying portions of the core+1 ORF, suggested the function of alternative mechanisms for core+1 expression in mammalian cells, including translation initiation from internal codons 85/87 or 26. Because results from these studies have been variable, we sought to re-evaluate expression of the core+1 ORF using constructs carrying the complete core+1 coding sequence fused to GFP or LUC. We showed here that codons 85/87 serve as the predominant initiation sites for internal translation initiation of core+1 ORF in Huh-7 and Huh-7/T7 mammalian cells, which support nuclear or cytoplasmic transcription, respectively. We also showed that internal translation initiation can occur concomitantly with the expression of the core+1/F protein that is produced artificially in Huh-7 or naturally in Huh-7/T7 cells. Furthermore, translation of core+1 ORF is not significantly affected by the presence of the HCV IRES element. The core+1/S–GFP protein is cytoplasmic and exhibits an ER distribution similar to that of the core+1/F–GFP protein.
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Keywords: Hepatitis C; Core+1 open reading frame; Core+1/S protein; Core+1/F protein; Internal translation initiation; Frameshift

1. Introduction

The hepatitis C virus (HCV) is a major etiological agent of chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Hoofnagle, 2002; Zoulim et al., 2003). A vaccine against the virus is not available at present and therapeutic approaches are still limited (Feld and Hoofnagle, 2005; Houghton and Abrignani, 2005). HCV is classified into the genus Hepacivirus of the family Flaviviridae (Murphy et al., 1995). The small single-stranded, positive-sense HCV RNA genome (about 9.6 kb) is flanked at both termini by conserved, highly structured non-translated regions (NTRs) and encodes a polyprotein precursor (about 3000 aa) (Lindenbach and Rice, 2005; Penin et al., 2004). This polyprotein is co- and post-translationally processed by host and viral proteases to produce three structural (core, E1 and E2) and at least six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. Initiation of translation of the viral polyprotein is controlled by an internal ribosome entry site (IRES) located mainly within the 5′-NTR of the viral RNA (Rijnbrand and Lemon, 2000).

Interestingly, an additional HCV protein that is synthesized by a coding sequence that overlaps the HCV core protein coding sequence in the +1 reading frame (core+1 ORF) has been recently identified (Branch et al., 2005). This protein is known as ARFP (for alternative reading frame protein) (Walewski et al., 2001), F (for frameshift) (Xu et al., 2001), or core+1 (to indicate the position of the new open reading frame) (Varaklioti et al., 2002, 2000). Evidence for the expression of the core+1 ORF during natural HCV infection is based on cumulative data from several laboratories showing the presence of specific antibodies in HCV-infected patients (Branch et al., 2003; Komurian-Pradel et al., 2004; Varaklioti et al., 2002; Walewski et al., 2001; Xu et

Abbreviations: HCV, hepatitis C virus; ORF, open reading frame; ARFP, alternative reading frame protein; core+1/F, core+1 protein expressed by translational frameshift; core+1/S, short form of the core+1 protein expressed by internal translation initiation; nt, nucleotide; IRES, internal ribosome entry site; LUC, luciferase; GFP, green fluorescent protein.

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al., 2001). Also, T-cell mediated immune responses against the ARFP/F/core+1 protein have been reported (Bain et al., 2004). On the other hand, results related to the mechanism that controls the expression of the core+1 ORF and the nature of the resulting protein are currently variable. Initial expression studies from Xu et al. (2001) and our laboratory (Varaklioti et al., 2002, 2000) mainly performed *in vitro*, in rabbit reticulocyte lysates, showed that the core+1 protein from HCV-1a is produced by a ribosomal frameshift which takes place within an A-rich region of the core coding sequence (codons 8–11) of the HCV-1 isolate. This produces a chimeric protein with ~10 amino acids derived from the core protein coding sequence at the amino-terminus, known as core+1/F or F or ARFP protein. However, subsequent studies from our laboratory based on transient transfection assays of core+1–luciferase (core+1–LUC) constructs carrying the first 95 out of 160 amino acid residues of core/core+1 ORF from genotype 1a in liver cells showed that the main mechanism responsible for the core+1 ORF expression is based on internal translation initiation at AUG codons located at the positions 85 and 87 rather than a +1 ribosomal frameshift. This results in the synthesis of another form of the core+1 protein that is shorter than that produced by the +1 frameshift mechanism in rabbit reticulocyte lysates and is known as core+1/S (short) (Vassilaki and Mavromara, 2003).

Consistent with our data, most recently, Baril et al. used a similar approach to show that the synthesis of the HCV-1a core+1 protein is mediated by an internal translation initiation mechanism rather than a +1 ribosomal frameshift in cultured cells, but they identified codon 26 as the location of the core+1 translation initiation site (Baril and Brakier-Gingras, 2005). Inasmuch as Baril et al. used core+1–LUC constructs that carry no more than the first 55 core/core+1 codons, it remains an open question whether or not codon 26 could serve as a non-canonical internal translation initiation site for the core+1 ORF in the presence of core+1 codons 85 and 87. Furthermore, in light of the extensive RNA secondary structure predicted within the core/core+1 coding region of the HCV genome (Smith and Simmonds, 1997; Tuplin et al., 2004, 2002; Walewski et al., 2002) that may control translation initiation of the core+1 ORF, it becomes very important to analyze the expression of core+1 ORF in the context of the complete core+1 coding sequence.

In order to clarify the results outlined above, in the present study we analyzed the expression of the core+1 ORF using chimeric constructs carrying the complete core+1 coding sequence fused to the GFP or the LUC gene. The synthesis of the core+1 protein was assessed in transiently transfected mammalian cells supporting nuclear (Huh-7) or cytoplasmic (Huh-7/T7) transcription, by Western blot analysis or enzymatic activity quantification, respectively, combined with *in vitro* site-directed mutagenesis. We showed that in Huh-7 cells when the complete core+1 ORF is present, only a single form of the tagged core+1 protein is synthesized at detectable levels, corresponding to the tagged core+1/S, indicating that the core+1 codons 85/87 serve as the main site for internal translation initiation of the core+1 ORF. We also showed that translation initiation at codons 85/87 is functional when core+1/F protein is artificially produced from the expression plasmids and is not influenced by the presence of the HCV IRES element. Furthermore, codons 85/87 appear to serve as the main internal translation initiation site in Huh-7/T7 cells, a mammalian expression system that supports cytoplasmic transcription due to the presence of the T7 polymerase and that is closer to that supporting the expression of the viral RNA during natural HCV infection. Notably, in agreement to our most recent data (accepted in JGV), Huh-7/T7-based expression system, in contrast to Huh-7 cells, supports also the expression of core+1/F when 10 consecutive adenine residues are present within codons 9–11. Finally, immunofluorescence staining indicated that the GFP-tagged short form of core+1 protein (core+1/S–GFP) is cytoplasmic, shows ER-like distribution and also localizes to the cell periphery, like the frameshift form (core+1/F). Interestingly, however, an artificially produced core+1/F protein lacking the first 10 amino acids of the core protein coding sequence was found to be localized to the mitochondria.

2. Materials and methods

2.1. Chemicals

The protease inhibitor cocktail for mammalian extracts (containing AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-64) was obtained from Sigma.

2.2. Plasmid construction and site-directed mutagenesis

Cloning was performed following standard protocols (Sambrook et al., 1989). Site-directed mutagenesis was carried out using the Quikchange^{TM} kit (Stratagene). All mutations were confirmed by sequencing. The basic characteristics of the different plasmids used in this study are summarized in Table 1.

The HCV-1 core/core+1 coding sequence was fused to the green fluorescent protein (GFP) gene in the +1 frame, under the control of the human cytomegalovirus (HCMV) immediate early promoter in pEGFP-N3 (Clontech). In all plasmids the initiator codon of the GFP coding region was changed to a glycine codon (ATG → GGG) by site-directed mutagenesis, using as template pEGFP-N3 and the primer pair N238-N239 (Table 2). Plasmid pHPI-1427 contains nts 342–825 of the HCV-1 core/core+1 coding sequence (Fig. 1A). The corresponding sequence was amplified by Vent DNA polymerase (New England Biolabs) in PCR using as template the plasmid pHPI-755 (Varaklioti et al., 2002), which contains nts 342–920 of the HCV-1 core/core+1 sequence, and the primer pair C53-C203 (Table 2). First, the C53-C203 PCR product was digested with EcoRI and inserted into the EcoRI cloning site of the pCI vector (Promega) to yield pHPI-1352. Subsequently, the NheI-XbaI fragment from pHPI-1352, containing the core/core+1 sequence (nts 342–825), was cloned into the NheI site of pEGFP-N3 to yield pHPI-1427. Plasmid pHPI-1429 contains nts 9–825 of the HCV-1 IRES-core/core+1 sequence (Fig. 1A). pHPI-1429 was also constructed by two-step cloning. First, pHPI-790 was derived by cloning of the IRES-core (nts 9–630)–LUC sequence of pHPI-768 (Varaklioti et al., 2002), after HindIII–SalI digestion, into the HindIII and SalI cloning

Table 1
Summarized information for the core+1–GFP expressing plasmids

Plasmid (paternal vector)	ength of the HCV-1 sequence (nt)	Mutation	Primers	Forms of core+1 protein detected	Elements mediating core+1 translation: start codons (GCA_{346} defined as codon 1) and frameshift site	Expected size (kDa)	Core co-expression
pHPI-1427 (pEGFP-N3)	342–825	–	C53, C203	Core+1/S (Short)–GFP	ATG core+1 85, 87 (internal initiation)	38	+
pHPI-1429 (pEGFP-N3)	9–825	–		Core+1/S–GFP	ATG core+1 85, 87 (internal initiation)	38	+
pHPI-1801 (pEGFP-N3)	9–582	–		–	–		+
pHPI-1447 (pEGFP-N3)	342–825	Deletion of 1 A at core codons 8–11	N246, N247	Core+1/F (Frameshift) –GFP	ATG polyprotein/9As at codons 8–11	47.5	+
				Core+1/S–GFP	ATG core+1 85, 87 (internal initiation)	38	(by −1/+2 frameshift at codons 8–11)
pHPI-1428 (pEGFP-N3)	385–825	–	C189, C203	Core+1/Fdel–GFP	artificial ATG in core+1 ORF, at nt 385	46	–
				Core+1/S–GFP	ATG core+1 85, 87 (internal initiation)	38	
pHPI-1553 (pEGFP-N3)	385–825	N1 ($G^{473} \rightarrow A$, $Trp^{43} \rightarrow$ stop in core+1 ORF)		Core+1/S–GFP	ATG core+1 85, 87 (internal initiation)	38	–
pHPI-1554 (pEGFP-N3)	385–825	N21 ($GGT^{580} \rightarrow TAG$, $Gly^{79} \rightarrow$ stop in core+1 ORF)		Core+1/S–GFP	ATG core+1 85, 87 (internal initiation)	38	–
pHPI-1555 (pEGFP-N3)	385–825	N22 ($AT^{604} \rightarrow TA$, $Met^{87} \rightarrow$ stop in core+1 ORF)		–	–		–
pHPI-1450 (pEGFP-C2)	591–828	–	N221, N222	GFP–core+1/S	ATG GFP	35	–

Table 2
List of priming oligonucleotides used in PCR

Primer name	Primer sequence	Primer pair/Annealing temperature
N238 (sense) N239 (antisense)	TCCATCGCCACCGGGGTGAGCAAGGGCGAGG CCTCGCCCTTGCTCACCCCGGTGGCGATGGA	N238–N239/65 °C
C53 (sense) C203 (antisense)	GTGCTTGCGAATTCCCCGGGA CTCGAATTCAGTTGACGCCGTCTTCCAGAACC	C53–C203/60 °C
C189 (sense)	CCGGAATTCGTAATGCCAACCGTCGCCCACAGGACGTCAAGTTCC	C189–C203/66 °C
N246 (sense) N247 (antisense)	CCTAAACCTCAAAAAAAAACAAACGTAACACC GGTGTTACGTTTGTTTTTTTTTGAGGTTTAGG	N246–N247/59 °C
N221 (sense) N222 (antisense)	CCGGAATTCCCCCTCTATGGCAATGAGGG CTCGAATTCAGTTCACGCCGTCTTCCAG	N221–N222/64 °C

Restriction sites included in the primer sequence are underlined.

sites of pEGFP-N3. Second, the KpnI fragment of pHPI-790, containing nts 585–630 of the core/core+1 sequence followed by the LUC gene, was replaced with the KpnI fragment containing nts 585–825 of the core/core+1 sequence and derived from pHPI-1428. Plasmid pHPI-1428 contains nts 385–825 of the HCV-1 coding sequence (Fig. 1B). An artificial initiator ATG codon was inserted in the core+1 frame at nt 385. The corresponding sequences were amplified using as template the plasmid pHPI-755 (Varaklioti et al., 2002) and the primer pair C189-C203 (Table 2). First, the C189-C203 PCR product was digested with EcoRI and inserted into the EcoRI cloning site of pCI to yield pHPI-1353. Subsequently, the NheI–XbaI fragment from pHPI-1353, containing nts 385–825 of the core/core+1 sequence, was cloned into the NheI site of pEGFP-N3. Mutation N6 (Vassilaki and Mavromara, 2003), inserted in pHPI-1427 and pHPI-1429, changed the 25th codon (CCG, Pro^{25}) of the core ORF (at nt 414) to a TAA stop codon, resulting in plasmids pHPI-1452 and pHPI-1453, respectively. Plasmid pHPI-1801, containing nts 342–582 of the HCV-1 core/core+1 sequence fused to the GFP gene at the 3-prime ($3'$) end, was constructed by deleting a fragment contained within nts 583–825 from pHPI-1429. Plasmid pHPI-1427 was mutated to contain 9 instead of 10 A residues within core codons 8–11, resulting in pHPI-1447 (Fig. 1B). The A residue was deleted by site-directed mutagenesis using the primer pair N246-N247 (Table 2). Mutations N1, N21 and N22 (Vassilaki and Mavromara, 2003), introduced

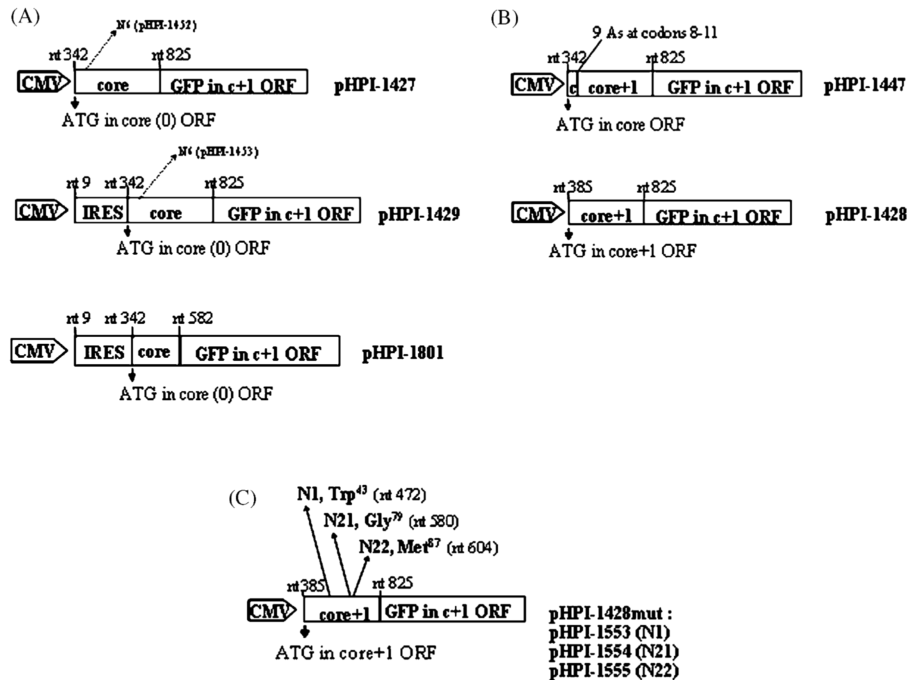


Fig. 1. Schematic representation of the GFP fusion constructs used in the transfection assays. (A) The HCV-1core/core+1 coding sequence, starting with the initiator ATG codon of the polyprotein, is fused to the GFP gene at the $3'$ end of the core+1 ORF (nt 825), in the absence (pHPI-1427) or presence of the viral IRES (pHPI-1429), or at nt 582 in the presence of IRES (pHPI-1801), under the control of the HCMV promoter. (B) Plasmids pHPI-1447 and pHPI-1428 encode artificial forms of the HCV-1 core+1–GFP fusion protein. In pHPI-1447, an adenine (A) deletion in the region of HCV-1 codons 8–11 fuses the core and core+1 ORFs, and in pHPI-1428, an initiator ATG codon is inserted in the core+1 frame at nt 385. (C) Three nonsense mutations have been separately inserted into the HCV-1 core+1 coding sequence (Vassilaki and Mavromara, 2003) of pHPI-1428, at nts 472, 580 and 604 (indicated by arrows), resulting in the mutant variants pHPI-1553 (N1), pHPI-1554 (N21) and pHPI-1555 (N22).

by site-directed mutagenesis into pHPI-1428, changed the 43rd (TGG, Trp^{43}), 79th (GGT, Gly^{79}) and 87th (ATG, Met^{87}) codons of the core+1 ORF (at nts 472, 580 and 604), respectively (arbitrarily defining the GCA alanine codon at nt 346 as the first codon of the core+1 ORF), to TAG termination codons, giving rise to pHPI-1553, pHPI-1554 and pHPI-1555 (Fig. 1C). Plasmid pHPI-1450, containing nts 591–828 of the HCV-1 core/core+1 sequence (Fig. 4A, a), was constructed by PCR using as template the plasmid pHPI-755 (Varaklioti et al., 2002) and the primer pair N221-N222 (Table 2). The PCR product after digestion with EcoRI was cloned into the EcoRI site of pEGFP-C2 (Clontech).

The dicistronic CAT-LUC plasmid pHPI-1700 contains the chloramphenicol acetyl transferase (CAT) gene as the first cistron followed by the IRES and the wild-type core/core+1 coding sequences included between nts 9 and 825 from the prototype HCV-1 isolate fused to the firefly luciferase (LUC) gene. The LUC gene is fused to the core+1 frame at its 3′ end. The dicistronic cassette is under the transcriptional control of both HCMV and T7 promoters. The plasmid was constructed by replacing the 203-bp NheI–XbaI fragment of the dicistronic pHPI-1046, which was previously described in (Psaridi et al., 1999) and contains nts 249–407 of the IRES-core sequence fused to part of the LUC gene (the first 50 nt) with the 630-bp NheI–XbaI fragment of pHPI-1684, carrying nts 249–825 of the IRES-core (IRES-$core^{825}$) sequence fused to the first 50 nts of the LUC gene. For the construction of pHPI-1684, the IRES-$core^{825}$ cassette was PCR amplified using the primer pair 888sense and N303. The PCR product was digested with BamHI and inserted into the BamHI cloning site of a mutated pGEM-luc (Promega) vector (pGEM-lucmut) that contains a glycine codon (GGG) in the place of the initiator ATG codon of the LUC gene. This mutation was inserted by site-directed mutagenesis using the primer pair N305 and N306. For the construction of pHPI-1802 and pHPI-1803 the mutation N25 alone or simultaneously with the mutation N19, respectively, were introduced in the core+1 coding sequence of pHPI-1700 by site-directed mutagenesis. Mutation N25 converted both 85th and 87th core+1 codons at nts 598 and 604 (ATG, Met^{85}, Met^{87}) to Gly codons (GGG) and mutation N19 introduced an A to G substitution and two A to C substitutions at nt 367, 369 and 373, respectively. The sequences of the priming oligonucleotides used for the insertion of mutations N19 and N25 in the HCV-1 sequence have been previously described (Vassilaki and Mavromara, 2003).

For the PCR amplification of the core/core+1 sequences, the following conditions were used: 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, annealing for 30 s, and 74 °C for 1 min, with a final extension at 74 °C for 10 min. For PCR site-directed mutagenesis the following conditions were used: 95 °C for 30 s followed by 18 cycles of 95 °C for 30 s, annealing for 1 min, and 68 °C for 10–12 min (2 min/bp), with a final extension at 68 °C for 10 min.

2.3. Cells and transfection experiments

Huh-7 (human hepatoma) and Huh-7/T7 were kindly provided by Dr. R. Bartenschlager (University Heidelberg, Germany). Cells were maintained in Dulbecco's modified Eagle medium (Biochrom KG) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), non-essential amino acids (1×; Biochrom KG), penicillin and streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively), 2 mM L-glutamine, and specifically for Huh-7/T7 cells with Zeocin (5 μg/ml) (Invitrogen). Cells seeded in six-well plates (Nunc), at a confluence of 60–70% for Huh-7 and 80–90% for Huh-7/T7 cells, were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol.

2.4. Flow cytometry

Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences) after suspending cells in 1× phosphate-buffered saline (PBS) supplemented with 3% FBS. Analysis of the flow cytometry data was performed with CELLQUEST software (BD Biosciences).

2.5. Quantification of LUC

Firefly LUC activity was quantified as previously described (Vassilaki and Mavromara, 2003). Luciferase activity was measured at 24 h post-transfection (p.t.) in Huh-7/T7 cells, when the T7-directed LUC expression normally peaks (data not shown), and at 48 h p.t. in Huh-7 cells.

2.6. Immunoblotting

Cell monolayers were harvested 48 h p.t., rinsed twice with ice-cold 1× PBS and lysed (20 min on ice) in triple detergent buffer (50 mM Tris–HCl [pH 8], 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 100 μg ml^{-1} phenylmethylsulfonyl fluoride, 1% NP-40, 0.5% sodium deoxycholate), in the presence of protease inhibitor cocktail for mammalian extracts (Sigma), as specified by the suppliers. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added to each sample, and the samples were boiled for 3 min before separation on 12% denaturing polyacrylamide gels, and transferred onto nitrocellulose. After blocking in PBS containing 0.02% (v/v) Tween 20 (PBST) and 5% (w/v) dried milk for 45 min at room temperature, the membranes were incubated with the primary antibodies in PBST-1% dried milk overnight at 4 °C. The membranes were then washed in PBST-1% dried milk three times for 30 min in total and incubated at room temperature for 2 h with a secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (DAKO), diluted in PBST-1% dried milk. After three washes with PBST-1% dried milk (total time 30 min) and three washes with PBS (total time 15 min), the membranes were soaked in enhanced chemiluminescence reagent (Pierce) and were exposed to film (Kodak) that was subsequently developed with the Kodak reagents.

2.7. Fluorescence microscopy

Huh-7 cells were cultured on 10-mm cover glasses (Mikroskopische-Deckgläser) a day before the transfection. At 48 h p.t. the cells were fixed with 3.7% (v/v) paraformaldehyde

for 30 min at RT directly, or after incubation (30 min, 37 °C) with the mitochondria-selective dye MitoTracker Orange CMTMRos (Molecular Probes), used at a final concentration of 1 μM. Following three washes with PBS, cells were finally mounted on glass slides (SuperFrost Plus; Menzel-Glaser, Germany) with Mowiol (10% w/v Mowiol, 25% v/v glycerol, 100 mM HCl, pH 8.5) (Sigma). Images were acquired with the 63× apochromat lense of a Leica TCS-SP1 four-channel confocal microscope equipped with argon ion laser and helium–neon laser.

2.8. Antibodies

For production of the polyclonal antibody against the core+1 ORF, the peptide NK1, consisting of amino acids TYRSSAPLLEALPGP(C) (core+1 aa 135–149), was chemically synthesized, conjugated to keyhole limpet hemocyanin (KLH) and used to immunize rabbits using a classical protocol for immunization (Harlow, 1988). The antisera were collected 2 weeks after the last boost and used in western blot analysis and enzyme-linked immunosorbent assays. The anti-core+1 polyclonal antibody was purified by a slightly modified affinity chromatography method based on CNBr-activated Sepharose 4B beads, as previously described (Harlow, 1988). The antibody was used in western blotting at a concentration of 1 μg/ml. The rabbit polyclonal antibody against GFP, purchased from Santa Cruz Biotechnology Inc., was used in western blotting at a dilution of 1:100. The mouse monoclonal antibody against core (amino acids 1–120) was obtained from Biogenesis and used at a dilution of 1:1000 in western blotting.

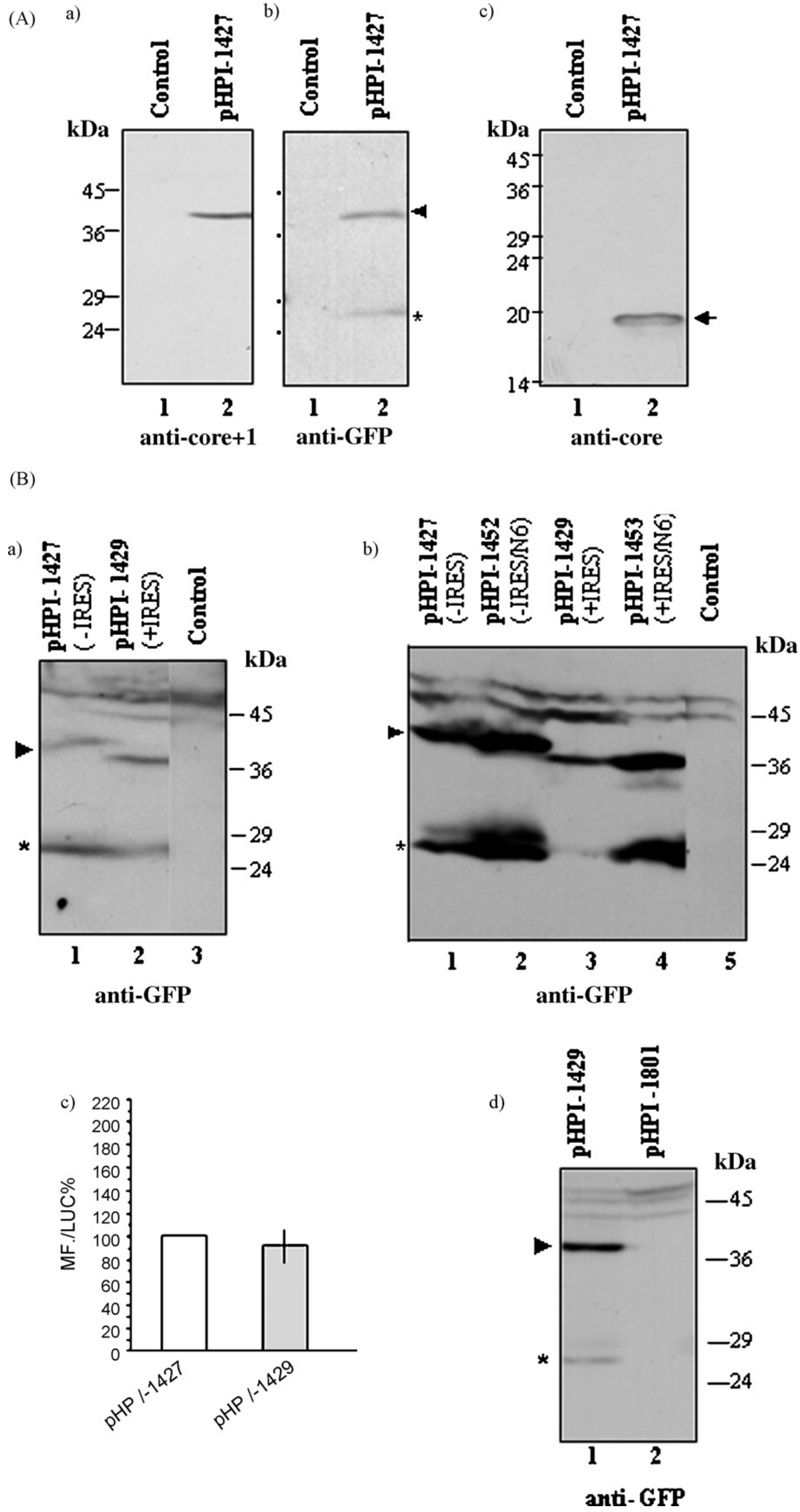
3. Results

3.1. Expression of the core+1–GFP protein in Huh-7 cells

To further analyze the expression of the HCV-1a core+1 ORF, we constructed plasmids pHPI-1427 and pHPI-1429, which contain the complete HCV-1 core+1 ORF fused in frame at the 3′ end to the GFP gene, under the control of the HCMV promoter (Fig. 1A). Plasmid pHPI-1429 carries the same core+1–GFP cassette as pHPI-1427 but also contains the HCV IRES element. The synthesis of GFP can be directly correlated to the expression of the core+1 ORF and was assessed in transiently transfected Huh-7 cells by western blotting. As shown in Fig. 2A, a single protein band of about 38 kDa was detected from the expression of pHPI-1427 with an antibody raised against a carboxy-terminal core+1 epitope (anti-NK1) (Fig. 2A, a; lane 2). The same band was also detected by an anti-GFP antibody (Fig. 2A, b; lane 2). Larger core+1–GFP forms would be expected if the proteins were to be synthesized by a +1 frameshift at the core codons 8–11 (around 47.5 kDa) or from internal initiation at the core+1 codon 26 (around 45 kDa) (Baril and Brakier-Gingras, 2005; Varaklioti et al., 2002; Xu et al., 2001). However, no additional larger forms of the core+1–GFP protein were produced at detectable levels. This result is consistent with our previous studies (Vassilaki and Mavromara, 2003) suggesting that the core+1 codons 85/87 serve as the main site for translation initiation of the core+1 ORF. The 38 kDa core+1–GFP protein will be referred to as core+1/S(short)–GFP. An anti-core antibody was used in these experiments to confirm the expression of core from pHPI-1427 (Fig. 2A, c; lane 2). Similar results were also obtained when the HCV IRES was present (pHPI-1429) (Fig. 2B, a; lane 2), indicating that the viral IRES has no effect on core+1 expression. The slight difference in the size of the core+1/S–GFP protein expressed from the pHPI-1427 and pHPI-1429 constructs is due to the presence of 20 additional amino acids, encoded by the polylinker of pEGFP-N3, only in the protein expressed from the pHPI-1427 construct (see Section 2). Each plasmid was co-transfected with the luciferase-expressing reporter plasmid pRL-CMV, to simultaneously assess transfection efficiency. Comparison of the core+1/S–GFP expression from the above plasmids, quantified by flow cytometry and adjusted according to the respective luciferase activity (M.F./LUC), indicated that the HCV IRES has no effect on the levels of core+1 expression (Fig. 2B, c).

The two protein bands that migrate at around 45 kDa are likely to be the result of non-specific reaction from the anti-GFP antibody, inasmuch as when present, they are also present in extracts of untransfected (control) cells (Fig. 2B, a; lane 3 and

Fig. 2. Expression of the chimeric HCV-1 core+1–GFP ORF in transiently transfected human cells. (A) Huh-7 cells were transiently transfected with 1 μg per well of the plasmid pHPI-1427 (a–c, lane 2), or left untransfected (control; a–c lane 1). Cell lysates were analyzed by Western blotting with the anti-core+1 rabbit polyclonal antibody produced in this study (a). The chimeric core+1/S–GFP protein expressed from pHPI-1427 was also detected with the anti-GFP antibody (b). An anti-core antibody was also used to test core co-expression from pHPI-1427 (c). The GFP-tagged core+1/S and the core proteins are indicated with a filled arrowhead and an arrow, respectively. The positions of the molecular mass markers are shown on the left. (B a and b) Huh-7 cells were either transfected, as described above, with the plasmid pHPI-1427 (a and b, lane 1), pHPI-1429 (a lane 2, b lane 3), pHPI-1452 (b lane 2) or pHPI-1453 (b lane 4) or left untransfected (control; a lane 3, b lane 5). Cell lysates were analyzed by western blotting with the anti-GFP antibody. The chimeric core+1/S–GFP protein is indicated with a filled arrowhead. The positions of the molecular mass markers are shown on the right. (B, c) Huh-7 cells were co-transfected with one of the core+1–GFP constructs pHPI-1427 or pHPI-1429, and the reporter plasmid pRL-CMV, expressing Renilla luciferase. Cell suspensions in 1× PBS + 3% (v/v) FBS were analyzed by flow cytometry at 48 h p.t. The ratio of the mean fluorescence intensity (M.F.) to LUC activity was determined and expressed as a percentage of the ratio derived from pHPI-1427. Bars represent the means obtained in two separate experiments. Error bars represent the standard deviation. (B, d) Huh-7 cells were either transfected with the plasmid pHPI-1429 (lane 1) or pHPI-1801 (lane 2, b lane 3). Cell lysates were analyzed by western blotting with the anti-GFP antibody. The chimeric core+1/S–GFP protein is indicated with a filled arrowhead. The positions of the molecular mass markers are shown on the right. (C) Huh-7 cells were either transfected, as described above, with the plasmid pHPI-1447 (a, b and e lane 2), the wild-type pHPI-1428 (a and e lane 3, c and d lane 2) or its corresponding core+1–GFP mutated variants (d lanes 3–5), or left untransfected (control; a–e lane 1). Cell lysates were analyzed by western blotting with the anti-GFP antibody (a and d). Lysates from cells transfected with pHPI-1447 and pHPI-1428 were also analyzed with the anti-core+1 (b and c, respectively) and anti-core (e) antibodies. The single and double open arrowheads indicate the chimeric core+1/F–GFP and core+1/Fdel–GFP proteins artificially expressed from plasmids pHPI-1447 and pHPI-1428, respectively. The filled arrowheads show the core+1/S–GFP fusion protein. Core protein produced from pHPI-1447 is indicated by an arrow. The positions of the molecular mass markers are shown on the left. The protein band marked with an asterisk corresponds to the molecular mass of free GFP protein.


(A)
a)
Control
pHPI-1427
kDa
45
36
29
24
1
2
anti-core+1
b)
Control
pHPI-1427
*
1
2
anti-GFP
c)
Control
pHPI-1427
kDa
45
36
29
24
20
14
1
2
anti-core
(B)
a)
pHPI-1427 (-IRES)
pHPI-1429 (+IRES)
Control
kDa
45
36
29
24
*
1
2
3
anti-GFP
b)
pHPI-1427 (-IRES)
pHPI-1452 (-IRES/N6)
pHPI-1429 (+IRES)
pHPI-1453 (+IRES/N6)
Control
kDa
45
36
29
24
*
1
2
3
4
5
anti-GFP
c)
MF./LUC%
220
200
180
160
140
120
100
80
60
40
20
0
pHP /-1427
pHP /-1429
d)
pHPI-1429
pHPI-1801
kDa
45
36
29
24
*
1
2
anti- GFP

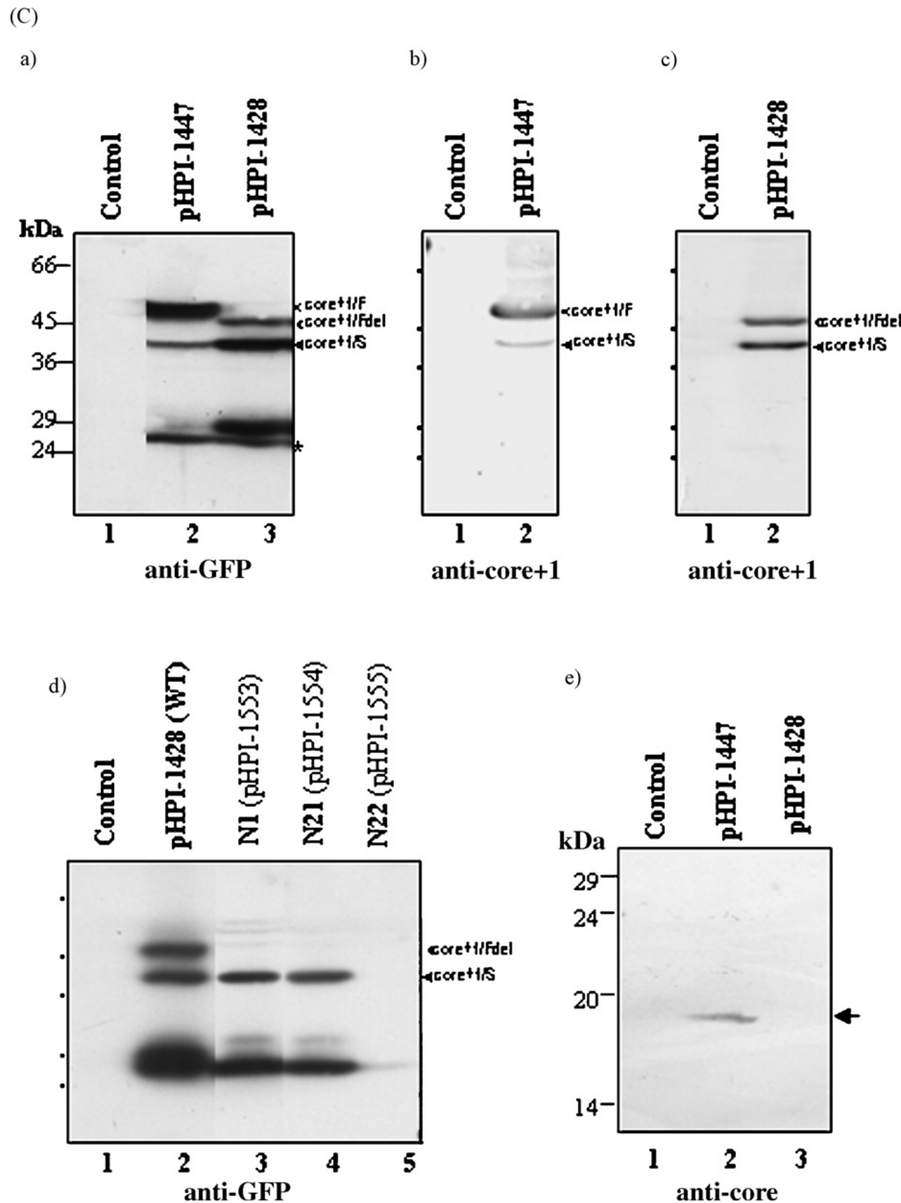


Fig. 2. (*Continued*).

B, b lane 5). Furthermore, the protein bands of around 27–29 kDa detected with the anti-GFP antibody from the core+1–GFP constructs are likely to be produced through cleavage of the chimeric core+1–GFP close to the N-terminus of GFP as the GFP translation initiation codon has been modified (see Section 2) to exclude the synthesis of the free GFP protein. Notably, the different levels of free GFP between pHPI-1427 and pHPI-1429 may be due to the different length of the polylinker.

Next, to improve the expression of core+1–GFP, we constructed plasmids pHPI-1452 and pHPI-1453 that carry a stop codon in the place of codon 25 of the core coding sequence (mutation N6) in the background of pHPI-1427 or pHPI-1429 (Fig. 1A). This is based on previous studies showing that blocking of core expression improves expression levels of core+1 (Vassilaki and Mavromara, 2003). As expected, significantly increased levels of the core+1/S–GFP protein with respect to the wild-type were observed both in the presence (pHPI-1453) and absence (pHPI-1452) of the IRES, but no additional bands specific for core+1–GFP were detectable (Fig. 2B, b; lanes 1–4).

Finally, to confirm that the 38 kDa core+1–GFP protein is translated by internal initiation at the codons 85/87, we examined core+1–GFP expression from plasmid pHPI-1801 that carries only the first 80 codons of core/core+1 sequence fused to the GFP gene under the control of the viral IRES (Fig. 1A). This construct contains all alternative translation initiation sites that have been previously described for core+1 expression (Baril and Brakier-Gingras, 2005; Varaklioti et al., 2002; Walewski et al., 2001; Xu et al., 2001) but lacks codons 85/87. As shown in Fig. 2Bd, no core+1–GFP protein was detected from pHPI-1801, suggesting that the core+1 codons 85/87 serve as the main site for translation initiation of the core+1 ORF, when the complete core/core+1 coding sequence is present.

Although only the 38 kDa core+1/S–GFP form of core+1 protein was detected under our experimental conditions, it is still

likely that other forms of the core+1 protein may be synthesized during natural infection (Varaklioti et al., 2002, 2000; Walewski et al., 2001; Xu et al., 2001). Thus, we sought to determine whether the core+1/S–GFP protein can be expressed concomitantly with the expression of larger forms of the core+1–GFP protein. For this purpose, we constructed plasmid pHPI-1447 (Fig. 1B), which carries an adenine deletion within the A-rich sequence at core codons 8–11. This would mimic the ribosomal shift to the core+1 frame after initiation of translation at the initiation codon of the polyprotein, resulting in the production of a chimeric GFP fusion protein containing the first 10 amino acids of the core protein attached to about 150 amino acids encoded by the core+1 ORF [core+1/F (Frameshift)–GFP]. Furthermore, plasmid pHPI-1428 (Fig. 1B) was made to direct core+1 expression from an artificial ATG codon inserted immediately downstream of the 10-As region, at codon 13 [core+1/Fdel–GFP]. As before, Western blot experiments were carried out with extracts from Huh-7 cells transfected with pHPI-1447 or pHPI-1428, using the anti-GFP (Fig. 2C, a) or anti-core+1 (Fig. 2C, b and c) rabbit polyclonal antibodies. As expected, a 47.5 kDa protein corresponding to the size of the core+1/F–GFP protein was produced from pHPI-1447 (Fig. 2C, a and b; lane2), and a 46 kDa protein corresponding to the size of the deleted form of the core+1/F–GFP protein was detected from pHPI-1428 (Fig. 2C, a; lane3 and C, c; lane 2). In addition to the larger forms of core+1–GFP, a protein band of about 38 kDa was detected with both the anti-GFP (Fig. 2C, a; lanes 2,3) and the anti-core+1 (Fig. 2C, b and c; lane 2) antibodies, indicating that the core+1/S–GFP protein was also produced from both plasmids. The levels of core+1/S–GFP were significantly lower when expressed from the plasmid pHPI-1447 in comparison to pHPI-1428, most likely due to the fact that the translation start site for the polyprotein and the artificially inserted core+1 initiator codon compete differently with the internal start codons of the core+1/S–GFP protein for the available 40S ribosomal subunits. Alternatively, expression of the core protein derived only from the pHPI-1447 plasmid and not from pHPI-1428 (Fig. 2C, e; lanes 2 and 3) could interfere with core+1 protein abundance (Vassilaki et al., 2007). Core protein is likely synthesized through a −1/+2 frameshift mechanism operating in the core codons 8–11 (Choi et al., 2003; Varaklioti et al., 2002). Also, the levels of free GFP protein vary between the two plasmids, which most likely is due to differences in the stability of the core+1/F–GFP and core+1/Fdel–GFP proteins. Taken together these results suggest that expression of the core+1/F protein does not exclude the expression of the short form of core+1 (core+1/S).

Finally, to confirm that the 38 kDa chimeric core+1–GFP protein expressed from pHPI-1428 is an internal translation initiation product, three nonsense mutations, N1, N21 and N22 (Vassilaki and Mavromara, 2003), were separately inserted into the core+1 coding sequence in pHPI-1428 (Fig. 1C). The N1 mutation introduced a TAG stop codon at nt 472 (Trp^{43}, TGG), resulting in pHPI-1553. Mutation N21 changed the 79th codon of the core+1 ORF at nt 580 (Gly^{79}, GGT) to a TAG stop codon, yielding pHPI-1554, and mutation N22 introduced a TAG termination codon eight codons downstream of mutation N21 at nt 604 (Met^{87}), giving rise to pHPI-1555. As shown by immunoblotting (Fig. 2C, d; lanes 2–4), mutations N1 and N21 abolished the expression of the 46 kDa core+1/Fdel–GFP protein from pHPI-1428, but they had no significant effect on the expression of the 38 kDa core+1/S–GFP protein. On the contrary, the N22 mutation abolished the expression of both GFP fusion proteins (Fig. 2C, d; lanes 2 and 5), indicating that the location of the translation initiation site for the 38 kDa core+1/S–GFP protein is between codons 80 and 87 (nts 583 and 606). To assure that the 46 kDa protein band corresponds only to the core+1/Fdel–GFP protein that is expressed by the artificial ATG codon inserted at codon 13 and not to other core+1–GFP protein forms translated by internal initiation, we converted this ATG into a TAG stop codon. As a result, the expression of the 46 kDa protein was abolished (data not shown/additional file).

Collectively, these results show that when the complete core/core+1 coding sequence is present the internal core+1 AUG codons at positions 85/87 serve as the main translation initiation site for the core+1 ORF in transfected Huh-7 cells.

3.2. Expression of the core+1–LUC in Huh-7/T7 cells

To investigate whether nuclear transcription may mask additional internal translation initiation events due to unexpected artifacts that can arise when viral genomes of naturally transcribed in the cytoplasm viruses are introduced in the nucleus, we also examined the expression of a full length tagged core+1 ORF in Huh-7/T7 cells. This cell line supports cytoplasmic transcription due to the presence of the T7 RNA polymerase (accepted in JGV).

For this purpose, the dicistronic plasmid pHPI-1700 was used that carries the CAT-IRES-core+1–LUC cassette under the control of the chimeric HCMV/T7 promoter (Fig. 3A, a) and expression was assessed at 24 h post-transfection, which is optimal for cytoplasmic transcription. Furthermore, to improve the detection of the core+1 products, we replaced the GFP gene with the LUC gene fused to the full-length core+1 ORF and we performed site directed mutagenesis. The T7-driven CAT–IRES-core+1–LUC bicistronic transcripts produced in the cytoplasm are uncapped and permit translation only of the core+1–LUC gene as this is under the control of the HCV IRES element. On the other hand, CAT that is produced from cap modified transcripts through nuclear transcription from the HCMV promoter is also detectable albeit at low levels serving as a control for transfection efficiency.

As shown in Fig. 3B, in Huh-7/T7 cells, mutation N25 that converts codons 85/87 from Met to Gly (Fig. 3A, b) reduces LUC activity by only 35%. This is in contrast to Huh7 cells and suggests alternative mechanisms for the core+1 ORF expression in the presence of cytoplasmic transcription. However, introducing the double mutation N25/N19 (N19 disrupts the A-rich slippery site within codons 8–11, Fig. 3A, b), which blocks frameshift and internal translation initiation, completely abrogates LUC expression in Huh-7/T7 cells suggesting that Huh7/T7 cells support the expression of both core+1/S–LUC and core+1/F–LUC proteins. However, no evidence for the expression of alternative forms of core+1–LUC protein could be detected. These results

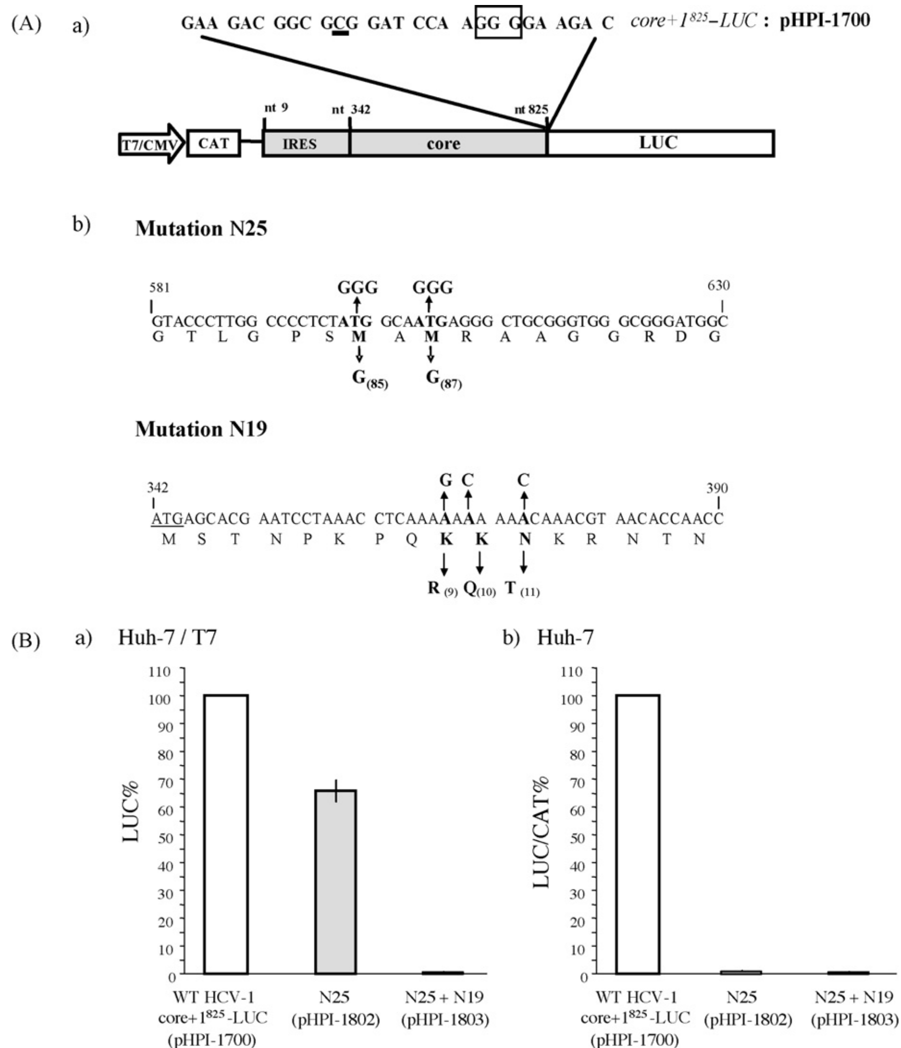


Fig. 3. Expression of the HCV-1 core+1 ORF in the cytoplasmic transcription system Huh-7/T7. (A, a) Schematic representation of the CAT–IRES-core–LUC bicistronic cassette cloned downstream of HCMV/T7 promoter in pHPI-1700. The LUC gene is fused to the 3′ end of the core+1 ORF (nt 825). The first codon of luciferase cistron, GGG, derived from the ATG initiator by site-directed mutagenesis, is boxed. The underlined nucleotide indicates an insertion of a thymidine residue. (A, b) Sequences of the HCV-1 core nt 581–630 and 342–390 depicting mutations N25 and N19, respectively, introduced in the CAT–IRES-core+1^{825}–LUC bicistronic cassette. (B) Cultures of Huh-7/T7 (B, a) and Huh-7 (B, b) cells were transiently transfected with 1 μg/well of either the WT core+1^{825}–LUC vector (pHPI-1700), or the corresponding mutants N25 (pHPI-1802) and N25/N19 (pHPI-1803). The LUC activity in Huh-7/T7 cells and the LUC/CAT ratio in Huh-7 cells for the WT core+1^{825}–LUC vector (pHPI-1700) were arbitrarily set at 100%. Bars represent the means obtained in two separate experiments in duplicate.

are in agreement with our recent studies in Huh7/T7 cells, based on the expression of LUC tagged core+1 protein carrying the first 95 aa residues driven by the HCMV/T7 or the T7 promoter alone (accepted JGV). Collectively these results indicate that internal translation initiation of core+1 ORF is mediated mainly from core+1 codons 85/87.

3.3. Subcellular localization of the core+1 protein

As a first step to address the biological importance and function of the core+1/S protein, we studied its subcellular localization in transiently transfected Huh-7 cells. For this purpose we used plasmid pHPI-1450 that carries the GFP gene fused to the 5-prime (5′) end of the core+1/S protein sequence (Fig. 4A, a). This was important in order to reduce the production of free GFP (Fig. 4A, b), which was found to mask the distribution of the core+1/S–GFP protein in the case of plasmids pHPI-1429 (+IRES) and pHPI-1427 (-IRES) (additional file). As indicated by confocal fluorescence microscopy, GFP–core+1/S is localized to the cytoplasm, it has a reticular, ER-like distribution (Fig. 4B, a), and is also detected perinuclearly in bright spots (Fig. 4B, b). The protein was also detected in the cell periphery (Fig. 4B, a and b). Similar intracellular distributions were observed (Fig. 4B, c and d) when the GFP-tagged core+1/S and core+1/F proteins were co-expressed from pHPI-1447 (Fig. 1B). In control experiments that were performed with the pEGFP-C2 plasmid that expresses GFP alone, staining was mainly nuclear (Fig. 4B, e). Thus, the different

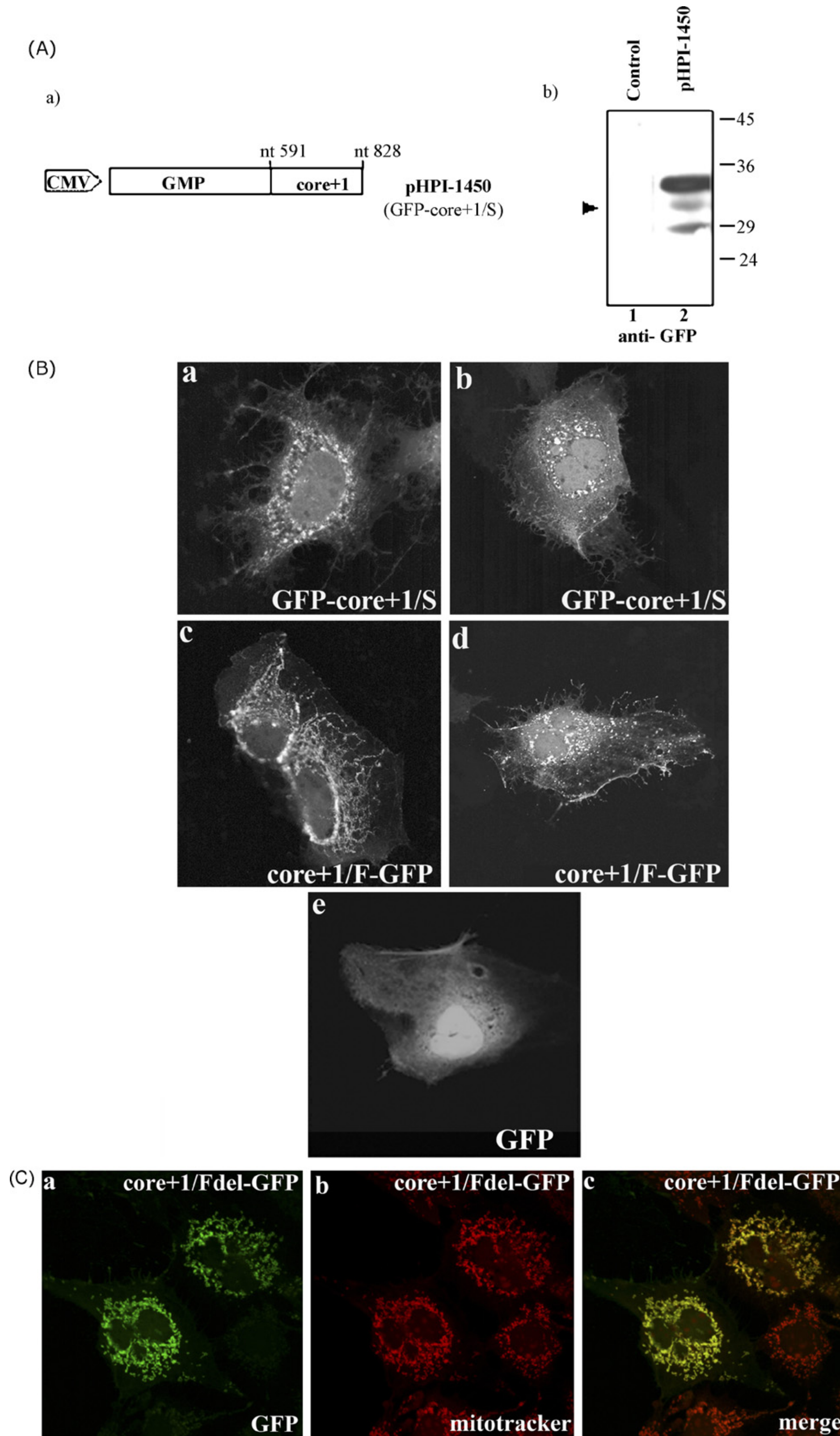


Fig. 4. Analysis of the subcellular localization of core+1 protein by confocal fluorescence microscopy. (A) Plasmid pHPI-1450 carries nts 591–828 the core+1 coding sequence fused to the 3′ end of the GFP gene (a), and in transfection experiments in Huh-7 cells, it produces a chimeric GFP–core+1/S protein detected by western blotting with the anti-GFP antibody at an apparent molecular weight of 35 kDa (b; indicated by the filled arrowhead). (B and C) Huh-7 cells were transfected with either the vector expressing GFP–core+1/S (pHPI-1450) (B, a and b), core+1/F–GFP (pHPI-1447) (B, c and d), core+1/Fdel–GFP (pHPI-1428) (C) or GFP alone (pEGFP-C2) (B, e). Co-localization studies were performed for the core+1/Fdel–GFP protein and the MitoTracker dye (C, a–c). Specifically, the green and red pseudocolors represent the GFP and the Mitotracker staining, respectively (C, a and b). The overlay of the two stainings is shown on the right panel (C, c).

localization pattern observed when plasmids pHPI-1450 and pHPI-1447 are used can be attributed to the expression of the core+1 protein. Interestingly, however, when core+1/S–GFP and core+1/Fdel–GFP were co-expressed from plasmid pHPI-1428 (Fig. 1B), GFP staining showed a mitochondria-like localization (Fig. 4C, a). This was confirmed in co-localization studies with the mitochondria-selective dye MitoTracker Orange (Fig. 4C, a–c). Analysis of the amino acid sequence of the core+1/Fdel protein, using the PSORT II algorithm for prediction of possible localization sites in the cell, resulted in a high probability for localisation of the protein to the mitochondria, confirming thus our data.

4. Discussion

We provide here strong evidence indicating that the main mechanism for core+1 ORF expression in mammalian cells is based on internal translation initiation. Our results from expression studies in Huh7 showed that, when the complete core+1 coding sequence is present, the core+1 codons 85/87 serve as the main site of the internal translation initiation event, suggesting that the initiation of translation at codons 85/87 is not subject to an effect of the secondary structure of the core/core+1 RNA (Smith and Simmonds, 1997; Tuplin et al., 2004, 2002; Walewski et al., 2002). These findings were confirmed in the Huh-7/T7 cells that support cytoplasmic transcription due to the presence of the T7 polymerase. This may be of importance as nuclear transcription may influence translation events of genomes that are solely transcribed in the cytoplasm. Notably, Huh-7/T7 cells support the expression of the core+1/F in addition to the core+1/S protein. However, there is accumulative evidence now indicating that the expression of the core+1/F is detected only in the presence of a stretch of 10 consecutive As constitute codons 8–11 (Baril and Brakier-Gingras, 2005; Vassilaki et al., JGV accepted). Taking into account that the sequence of 10 As at codons 8–11 is underrepresented in the majority of the reported HCV genomic sequences (2 out of 721 sequences available at the hepatitis virus database: http://www.s2as02.genes.nig.ac.jp/), whereas the AUG codons 85/87 are highly conserved, it is intriguing to speculate that internal translation initiation plays an important role for core+1 expression in mammalian cells.

Specifically, in agreement to our findings, Baril and Brakier-Gingras have recently reported that internal translation initiation rather than frameshift serves as the main mechanism for core+1 expression in cultured cells and have correlated the synthesis of core+1/F protein with the presence of the A-rich region within codons 8–11. However, they identified codon 26 as the main translation initiation site (Baril and Brakier-Gingras, 2005). Since their approach was based on the use of core+1–LUC fusion constructs carrying up to the first 35 codons, this study could not assess the effect of codons 85/87 or of the complete RNA sequence on the translation initiation at codon 26. The results reported in this study provide no evidence for an efficient initiation at codon 26.

The core+1/S protein can be expressed concomitantly with the core+1/F, indicating that the translation mechanisms directing the expression of the two forms of the core+1 protein are not mutually exclusive. However, it is interesting to note that core+1 is expressed independently of the HCV IRES element (Fig. 2B) or the synthesis of the HCV polyprotein (Vassilaki and Mavromara, 2003).

In immunofluorescence experiments we analyzed the subcellular localization of the core+1/S protein in comparison to the core+1/F form that was shown to be produced in previous studies (Varaklioti et al., 2002, 2000; Walewski et al., 2001; Xu et al., 2001). We showed that the GFP-tagged core+1/S protein in transfected mammalian cells is cytoplasmic with an ER-like and perinuclear distribution. It is also localized to the periphery of the cell. Similar localization was observed for the core+1/F protein. Interestingly, however, in the absence of the first 10 amino acids of the core coding sequence from the N-terminus of core+1/F–GFP the protein was localized in the mitochondria. This is important, as a difference in the localization and/or in the folding between core+1/F and core+1/Fdel may result in a difference in the function of the protein. This is of interest to note in light of previous studies where Fdel has been used as model protein to assess the functional importance of the core+1/F protein (Komurian-Pradel et al., 2004; Troesch et al., 2005).

Overall, the present study suggests that the short form of core+1 protein (core+1/S) derived from the core+1 codons 85/87 represents an important form of the core+1 protein in mammalian cells. The elucidation of the nature of the core+1 protein is an important step towards understanding the functional role of this protein.

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