Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Most infected subjects develop a chronic infection that may progress to steatosis, liver cirrhosis and HCC. Current treatment is based on the combination of interferon alpha and ribavirin, and leads to elimination of the virus in 50 to 80% of cases, depending on the genotype {Shepard, 2005}. The development of more effective treatments will require improvements in our understanding of the biology of HCV infection, but also in the nucleus in patients with hepatocarcinoma and in core-transgenic mice. HCV core contains nuclear localization signals (NLS), but no nuclear export signal (NES) has yet been identified. We show here that the aa(109–133) region directs the translocation of core from the nucleus to the cytoplasm by the CRM-1-mediated nuclear export pathway. Mutagenesis of the three hydrophobic residues (L119, I123 and L126) in the identified NES or in the sequence encoding the mature core aa(1–173) significantly enhanced the nuclear localisation of the corresponding proteins in transfected Huh7 cells. Both the NES and the adjacent hydrophobic sequence in domain II of core were required to maintain the core protein or its fragments in the cytoplasmic compartment. Electron microscopy studies of the JFH1 replication model demonstrated that core was translocated into the nucleus a few minutes after the virus entered the cell. The blockade of nucleocytoplasmic export by leptomycin B treatment early in infection led to the detection of core protein in the nucleus by confocal microscopy and coincided with a decrease in virus replication. Our data suggest that the functional NLS and NES direct HCV core protein shuttling between the cytoplasmic and nuclear compartments, with at least some core protein transported to the nucleus. These new properties of HCV core may be essential for virus multiplication and interaction with nuclear molecules, influence cell signaling and the pathogenesis of HCV infection.
When the entire polyprotein is synthesized in mammalian cells, core is found mostly at the ER membrane and on the surface of lipid droplets and mitochondria. HCV core may also be found in the nucleus, where it may act as a substrate for proteasomal degradation, particularly when C-terminally truncated forms of core are produced. These findings suggest that core is targeted away from the ER very soon after its synthesis. However, it remains unclear what determines the ultimate fate of core, whether it remains at the ER or is trafficked to other subcellular compartments, and the regulation of this process appears to be complex (see for review).

In addition to binding to the HCV RNA to form the virus nucleocapsid, core protein interacts with several cellular components, thereby influencing lipid metabolism, signal transmission, and the regulation of gene expression and transcription from several viral and cellular promoters. HCV core affects cell proliferation, apoptosis and host defense mechanisms, by interfering with both innate and adaptive immunity (see for review). Furthermore, studies in transgenic mice and observations in humans suggest that core protein has oncogenic potential. HCV core consistently interacts with several host factors, including the p53 tumor suppressor protein and p21, and LZIP, and with cellular shuttling proteins, such as p53, 14-3-3, DDX3, putative RNA helicase and proteasome activator PA28γ. These particular properties of core may be related to HCV-induced cell transformation (for review, see).

In liver biopsy specimens from HCV-infected individuals, HCV core is found mostly in the cytoplasmic compartment, but a nuclear localization of core has also been reported. In particular, tumor tissues from patients with HCC have been found to contain truncated forms of the HCV core protein within the nucleus. These studies suggested that core may localize to the nucleus at some stages of natural HCV infection, particularly in cancer patients. Similarly, in transgenic mice producing HCV core protein, a nuclear localization of core was associated with liver disease and HCC. The interaction of core protein with nuclear molecules may therefore play a key role in the development of HCC.

Some of the proteins of positive-strand RNA viruses, which replicate in the cytoplasm, may be located in the nucleus or the nucleolus at some point in the viral life cycle. Such proteins contain appropriate targeting signals, such as nuclear localization signals (NLS) and nuclear export signals (NES). The distribution of core protein may thus also be regulated by these signals. Indeed, three NLS have been identified in HCV core, in the aa(5–13), aa(38–43), and aa(58–71) sequences. These sequences constitute functional, at least bipartite NLS able to bind importin-α. However, no NES that could potentially direct the translocation of the protein from the nucleus to the cytoplasm has yet been reported in HCV core.

We demonstrate for the first time that core protein contains a functional NES (aa[109–133]) facilitating its export from the cell nucleus via the CRM-1/exportin pathway. In the HCV in vitro replication system (JFH1), HCV core was translocated to the nucleus early in infection. The presence of functional NLS and NES motifs raises the possibility of core protein shuttling between the nuclear and cytoplasmic compartments. These new properties of core may be important for virus multiplication and the pathogenesis of infection.

Materials and Methods

Cell culture

All cell lines were cultured at 37°C, in a humidified atmosphere containing 5% CO₂. The human hepatoma cell line Huh7, the rat hepatoma cell line ARL-6 and human embryonic kidney 293T cells were obtained from ATTC, immortalized human hepatocytes Fa2N-4 were purchased from XenoTech, Lenexa KS, USA, whereas the Huh 7.5 cell line was kindly provided by C. Rice. Cells were maintained in Dulbecco’s modified Eagle’s medium...
(DMEM; Gibco-Invitrogen) supplemented with 10% fetal calf serum, 1% non-essential amino acids and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). The CHO-K1 cell line (ATCC®#CCL-61) was obtained from LGP Prochem (Molsheim, France) and cultured in F12 medium (Invitrogen, Cergy-Pontoise, France) containing glutamine and supplemented with 10% fetal calf serum (FCS) antibiotics and anti-fungal compounds.

**Plasmid construction**

Plasmids were constructed by inserting core-encoding fragments of various lengths — aa(1–120), aa(1–140), aa(1–160), aa(1–173) — into pEGFPN1 (Clontech). These fragments were amplified by standard PCR, cleaved with EcoRI and BamHI and inserted into pEGFPN1.

We constructed several plasmids encoding enhanced green fluorescence protein (EGFP) or mCherry, to determine whether the NES and NLS sequences were functional. The reporter genes were fused with (i) the NLS of SV40 nuclear T antigen (EGFP-NLSSV40), (ii) the NLS of SV40 followed by the potential NES of the HCV core protein aa(109–133) (EGFP-NLSSV40-NESScore), or (iii) the NLS of SV40 followed by the functional NES of the HIV regulatory protein Rev (EGFP-NLSSV40-NERev). Three more plasmids, encoding core fragments aa(109–160), aa(144–160) and aa(134–143), were constructed. These fragments were fused with the EGFP or mCherry sequence and the NLS of SV40 (EGFP-NLSSV40-core(109–160); EGFP-NLSSV40-core(144–160); EGFP-NLSSV40-core(134–143)).

All viral coding sequences were amplified by standard PCR and introduced into the pDONR207 plasmid (Invitrogen) via a recombinational cloning strategy (Gateway; Invitrogen). Viral sequences were then transferred from pDONR207 into Gateway-compatible versions of pEGFP-C1 (kindly provided by Y. Jacob) or mCherry vectors (Clontech), for expression in mammalian cells with an N-terminal EGFP or mCherry tag.

The PCR primers used to amplify and clone viral ORFs had 20 to 30 specific nucleotides matching the ends of the ORF, giving a Tm close to 60°C. For the recombinational cloning of PCR products, the 5’ ends of both forward and reverse primers were fused to the attB1 5’-GGGGACCACTTTGTACAAGAAAGCTGGTTA-3’ recombinant sequence, whereas the reverse primers were fused to the attB2 5’-GGGGACCACTTTGTGACAGAGCACTTTA-3’ recombinant sequence. All the constructs used were amplified by transforming (by heat shock) Escherichia coli DH5α (Invitrogen).

**Cell transfection**

For overexpression, mammalian cells were transfected with pEGFP-C1 or mCherry vectors containing the viral ORFs, in the presence of the FuGene Transfection Reagent (Promega), according to the manufacturer’s instructions. Unless otherwise specified, we dispensed 5x10^4 cells per into each well of 24-well plates and, 24 to 48 h later, we transfected these cells with 500 ng of plasmid DNA per well.

**Mutations in the NES sequence**

Mutations (L119A, I123A, and L126A) were introduced into the NES sequence of HCV core protein with the QuikChange Lightning site-directed mutagenesis kit (Agilent-Stratagene). An EGFP expression plasmid (EGFP-NLSSV40-NESScore) encoding the putative HCV core export sequence aa(109–133) or EGFP-labeled core protein aa(1–173) was amplified with the 5’-phosphorylated mutagenic primers 5’-GGCGATACCGCTACATCGGCGCTTTCGGGACACGTAT and 5’-GAACCTTACCAGCATGAGCCACACCCGGGAT. Template DNA was then digested with DpnI and mutagenized plasmids were circularized by self-ligation with T4 DNA Ligase (New-England Biolabs). Mutant clones were selected and the sequences of their expression cassettes were checked.

**Sequence analyses**

Multiple sequence alignment was carried out with Clustal W. “Classical” NES were identified with NetNes software (www.expasy.org).

**HCVcc (JFH1) cell culture**

The plasmid corresponding to the genome of the JFH1 strain was kindly provided by T. Wakita and used to generate cell culture-produced virus (HCVcc). The virus was cultured as previously described. For cell infection, monolayers of Huh 7.5 cells were grown for 24 h in tissue culture plates. They were then inoculated by incubation with 25 µl of the virus preparation (containing approximately 10^6 IU of HCV RNA) for 2 h at 37°C, to allow infection. Cells were analyzed at various time points after infection, as indicated, by immunofluorescence studies, quantitative RT-PCR or electron microscopy.

**RNA interference**

For RNA interference-based knockdown experiments, a 25-nucleotide siRNA (5’-ccu cgu ugc uga agg ugg auc agg a-3’) targeting PA28γ was purchased from Invitrogen (Stealth Select RNA). Huh7.5 cells were transfected with 20 nM siRNA, in the presence of JetPRIME (Ozyme). Control ON-TARGET plus (Dharmacon) non targeting siRNA was used to confirm silencing specificity. For analyses of the influence of proteasome activator silencing on the subcellular distribution of core, cells were transfected with siRNA targeting PA28γ or with control siRNA 18 h before infection with HCVcc.

**LMB treatment**

We investigated the role of CRM-1-dependent transport in the subcellular localization of core, by treating cells with leptomycin B (LMB, Sigma). Huh7 cells were cultured and transfected as described above. After 22 to 48 h, the cells were treated for 2 to 4 h, at 37°C, with LMB at a concentration of 2 ng/ml or 10 ng/ml in DMEM. For studies in the JFH1 infection model, 10 ng/ml LMB was applied at various time points during or after infection, and cells were incubated in a medium containing LMB for the indicated times.

Cell viability after LMB treatment was determined by counting live and dead cells, after trypsin blue staining, in an automated cell counter (Countess; Invitrogen). We also quantified the ATP present in cultured cells, with the CellTiter-Glo® Assay (Promega), used according to the manufacturer’s instructions. Untreated cells and cells treated with 10% DMSO (to induce cell death) were used as negative and positive controls, respectively.

**Immunofluorescence and confocal microscopy**

We investigated PA28γ production in untransfected Huh 7.5 cells and in Huh 7.5 cells transfected with an siRNA targeting PA28γ (or control siRNA), with a rabbit anti-PA28γ antibody and Alexa Fluor 488-conjugated anti-rabbit IgG. The distribution of HCV core in infected cells was analyzed with a monoclonal anti-core antibody, ACAP27, followed by an Alexa Fluor 568-tagged anti-mouse IgG. Staining with rabbit anti-lamin B antibody core antibody, ACAP27, followed by an Alexa Fluor 568-tagged anti-mouse IgG. Staining with rabbit anti-lamin B antibody (or control siRNA), with a rabbit anti-PA28γ antibody and Alexa Fluor 488-conjugated anti-rabbit IgG was used to outline the cell nuclei. For fluorescence microscopy, infected or transfected cells were washed in PBS and fixed by incubation with 4% paraformalde-
Hyde (PFA) in PBS for 20 min at 4°C. The cells were washed several times in PBS and permeabilized with 0.5% Triton X-100. They were then incubated in a blocking buffer containing 1% gelatin and 0.1% Tween 20 in PBS. Primary antibodies were incubated with the cells for 2 h (mostly at a concentration of 1 μg/ml in blocking buffer). After subsequent washes with 0.1% Tween 20 in PBS, secondary antibodies were added and the cells were incubated for 1 h. The cells were washed again and mounted in Vectashield medium containing DAPI (Vector Laboratories, Abcys, France). Fluorescent fields were captured with a Widefield ApoTome AxioCam upright (66RC10P) microscope.

Relative fluorescence analyses

We analyzed the relative fluorescence intensity of the proteins in the cytoplasm and nucleus, by converting bright-field immunofluorescence images to grayscale images, with Image J software. Boundaries were applied to demarcate the nuclear and cytoplasmic compartments and the fluorescence intensity of each compartment was measured with a script created with Acapella 2.0 image software (Perkin Elmer). All measurements were normalized with respect to background fluorescence. A nonparametric one-way ANOVA assay was carried out to compare fluorescence between the cytoplasm and the nucleus of 1138, 712 and 573 cells transfected with plasmids (EGFP-NLSSV40), (EGFP-NLSSV40-NEScore) and (EGFP-NLSSV40-NEScore), respectively. Graphs showing the means and variances of the ratio of nuclear to cytoplasmic fluorescence intensities were plotted for the three constructs considered.

For analyses of the mutated NES, 153 cells transfected with the wild-type (EGFP-NLSSV40-NEScore) plasmid and 130 cells transfected with the mutated construct were analyzed, and the results are presented as a graph showing the means and variances of the ratio of nuclear to cytoplasmic fluorescence intensities for wild-type and mutated constructs. Similarly, 139 and 101 cells transfected with the wild-type and mutated core protein aa (1-173) constructs, respectively, were also considered. Graphs showing the means and variances of the ratio of nuclear to cytoplasmic fluorescence intensities were plotted for the wild-type and mutant proteins and a nonparametric t-test was used to evaluate the results obtained.

RT-quantitative PCR (RT-qPCR)

The HCV RNA associated with cells was quantified by one-step real-time RT-qPCR, with the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen). The 5′-AGYGTTGGGTYGC-GAAAAG-3′ and 5′-CACTCGCAAGCRCCCT-3′ primers were used to amplify HCV RNA from JFH clones, and 6-FAM-CCTTGTGGTACTGCCTGA-MGB (Applied Biosystems, Foster City, CA, USA) was used as an internal probe. Real-time detection of the PCR products was carried out with an ABI Prism 7000 machine. HCV RNA was quantified and standardized with respect to background fluorescence. A nonparametric t-test was used to evaluate the results obtained.

Electron microscopy

Supernatants collected from Huh7.5 cells producing JFH1 were concentrated by centrifugation through a 20% sucrose cushion for 4 h at 32,000 rpm, in an SW32Ti rotor. For electron microscopy analyses, Huh7.5 cells were grown in 3 cm dishes for 24 h, washed with cold serum-free DMEM and maintained for 10 min at 4°C. The medium was then replaced with 1 ml cold serum-free DMEM supplemented with a concentrated virus preparation (approx. 5 x 10^8 IU/ml HCV RNA) and the cells were incubated for 30 min at 4°C. At the end of this adsorption step, the cells were placed for 20 min at 37°C and examined by transmission electron microscopy (TEM). Briefly, cells were fixed with a 4% formaldehyde/glutaraldehyde solution in phosphate-buffered saline (PBS) pH 7.4, for 30 min at 37°C. They were washed with PBS and incubated for 10 min in blocking solution containing 0.5% v/v cold fish skin gelatin (FGS), 0.1% v/v saponin and 0.02 M glycine (all from Sigma Aldrich) in PBS pH 7.4, at room temperature. The cells were washed with 0.5% FSG in PBS (PBS/FSG) and incubated for 60 min with ACAP-27 monoclonal antibody, kindly provided by JF. Delagneau, diluted in PBS/FSG. The cells were again washed in PBS/FSG, incubated for 30 min with anti-mouse IgG conjugated with Nanogold particles (Yaphank, NY, USA) in PBS/FSG, washed in PBS/FSG and fixed by incubation with 1% v/v glutaraldehyde in PBS for 1 h at room temperature. The cells were washed in water and Nanogold® staining was enhanced by incubation for three minutes with the HQ silver enhancement kit (NanoProbe, Yaphank, NY, USA). Cells were then post-fixed by incubation with 1% v/v osmium tetroxide in PBS for 1 h and processed by dehydration in a series of ethanol solutions for embedding in epoxy resin. TEM examination was performed on a Philips CM120 microscope, and images were acquired with an ORIUS SC200D CCD camera (Gatan Inc.).

Results

Subcellular localization of core proteins

Several studies have shown that, when produced separately, the immature core protein aa(1-191) remains in the cytoplasm and the processed, mature core protein is present in both the nuclear and cytoplasmic compartments, whereas the shorter core proteins aa(1-120) or aa(1-140) are targeted to the nucleus.

Using plasmids encoding EGFP-labeled core proteins composed of aa(1-173), aa(1-160) or aa(1-140), we investigated the subcellular distribution of the protein in various cell lines. The short form of core aa(1-140) was found in the nucleus in all cell lines tested. By contrast, core proteins aa(1-160) and aa (1-173) were found in both the cytoplasmic and nuclear compartments in transfected Huh 7 cells, but solely in the nucleus of cells of non human origin, such as Chinese hamster ovary cells (CHO) (Figure 2) and rat hepatoma cells (ARL-6) (not shown). Results identical to those for Huh7 cells were obtained for other hepatic
cells (HepG2 hepatoma cell line and Fa2N4 immortalized human hepatocytes) in which HCV core proteins aa(1–160) and aa (1–173) were found in both the cytoplasmic and nuclear compartments. By contrast, in human non hepatic cells, such as embryonic kidney cells (HEK-293) these two core proteins were found exclusively in the nucleus (not shown), as for CHO and ARL-6 cells.

We performed Western Blot analyses of the EGFP-labeled core proteins generated, to check their integrity and to exclude the possibility of degradation of the constructs used for these experiments, which might affect the subcellular distribution of the corresponding proteins. These experiments (shown in Figure 3) confirmed that only one EGFP-labeled protein was produced in transfected Huh7 cells for each construct: a 47 kDa EGFP-labeled core protein for the plasmid encoding core aa(1–173), a 45 kDa EGFP-labeled protein for the plasmid encoding aa(1–160) and a 43 kDa EGFP-labeled protein for the plasmid encoding aa(1–140). There was therefore no degradation of the constructs and the distribution of core proteins was specific to the cells analyzed.

These results suggest that, in addition to the previously described NLS, there may be other signals in HCV core and interacting sequences in human hepatic cells, regulating the subcellular distribution of core protein. These mechanisms may be nonfunctional or differently regulated in non human and human cells of non hepatic origin.

Identification of a “classical” NES in the immature form of HCV core

The subcellular distribution of the HCV core protein, like that of other viral proteins, may be influenced by the presence of nuclear localization signals (NLS) and nuclear export signals (NES). Indeed, the tripartite NLS located in the N-terminal DI have been described and shown to be functional. These and other functional domains of the core protein are shown in Figure 1. However, no NES capable of directing the translocation of the protein from the cell nucleus to the cytoplasm has ever been reported in HCV core.

We therefore used NetNES software (www.expasy.org) to search for a “classical” NES in the core protein. Such “classical sequences” are relatively short linear oligopeptides enriched in leucine residues, usually consisting of the peptide sequence X-R(2-
4-X-R2-X-R-X, where X is leucine, isoleucine or valine and R is any amino acid. This analysis identified a “classical” leucine-rich nuclear export sequence (NES Classic) within the C-terminal part of core, between aa179 and 187 (Figure 4). Nevertheless, it is well established that sequential cleavages by signal peptidase and signal peptide peptidase remove the C-terminal hydrophobic DIII of core, to yield the mature form of core composed of aa(1–173), more recently defined as aa(1–177). Thus, the immature form core aa(1–191), attached to membranes, contains the classical NES, whereas the mature form of core does not (Figure 1).

Identification of a “non classical” NES in HCV core

As the mature core (aa(1–173)) was found in both the cytoplasm and the nucleus, another “non classical” or “atypical” NES is probably present in the mature form of core. Such sequences have been reported for several viral proteins, such as equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV) Rev.

We therefore searched for another, “non classical” NES in the mature form of core by generating multiple sequence alignments and comparing the core aa sequence with known viral sequences enriched in hydrophobic residues that can function as NES motifs, as described by Rowland et al. 2003 for the N-protein of porcine reproductive and respiratory syndrome virus (PRRSV). We found that the aa(109–133) region of core contained a sequence similar to that of other “atypical” viral NES. An analysis comparing the putative NES of core with other viral sequences is shown in Figure 5A.

Comparative analyses of consensus sequences available for the core proteins of different genotypes provided evidence that this region was well conserved in various HCV genotypes (Figure 5B).

The aa(109–133) sequence is a functional NES

We investigated whether the putative NES sequence (aa(109–133)) could direct the export of the protein from the nucleus to the cytoplasm, using an approach previously used to characterize the NES of PPRSV and HSV proteins. We constructed three plasmids (shown in Figure 5C). In the first, EGFP or mCherry was C-terminally tagged with the NLS of SV40 large T antigen, which targets proteins to the nucleus. The second plasmid encoded the SV40 NLS fused to the potential NES sequence of the HCV core protein aa(109–133). Finally, EGFP or mCherry fused to the SV40 NLS and the functional NES of the HIV Rev regulatory protein was used as a control for nuclear export. The Rev protein of HIV is a shuttling protein that promotes the nuclear export of mRNA encoding viral structural proteins in a CRM-1-dependent manner.

We transiently transfected Huh7 cells with these plasmids and, after 40 h, we determined the subcellular distribution of the encoded proteins by fluorescence and confocal microscopy (Figure 5D). Fluorescent proteins containing the SV40 NLS were found almost exclusively in the nucleus, whereas proteins containing both the SV40 NLS and the Rev NES were found in both the nucleus and the cytoplasm. Similarly, the protein containing the SV40 NLS and the putative core NES was found in both the nucleus and the cytoplasm, providing evidence that aa(109–133) of core is a functional NES capable of directing the chimeric protein from the nucleus to the cytoplasm. Similar results were obtained with plasmids encoding proteins tagged either with EGFP or with mCherry, excluding the possibility that the subcellular distribution of the encoded proteins was dependent on the fluorescent tag used.

We confirmed the subcellular distribution of the putative NES of core (aa(109–133)) and of the proteins encoded by control plasmids, in quantitative analyses (Figure 5E). These analyses provided evidence that the aa(109–133) core sequence acted as a functional NES, targeting the protein from the nucleus to the cytoplasm, like the HIV Rev protein NES.

Mutation of the NES sequence changes the subcellular distribution of HCV core

We hypothesized that the hydrophobic residues L(119), I(123) and L(126) in the core NES (Figure 5A, underlined within the frame) might be important for the nuclear export of this protein. We tested this hypothesis by carrying out comprehensive alanine-scanning mutagenesis and determining whether the replacement of these residues by alanine residues decreased the nuclear export of the protein, thereby resulting in accumulation of larger amounts of protein in the nucleus.

Cells were transfected with mutated constructs and the subcellular distributions of the resulting proteins were analyzed by immunofluorescence microscopy and compared with that of the non mutated control.

Transfection efficiency for the construct containing SV40 NLS fused to the mutated putative export sequence aa(109–133) of the core protein was similar to that for the wild-type construct, but the mutated protein had a different subcellular distribution. Indeed, whereas the wild-type chimeric protein with a putative export sequence aa(109–133) was found principally in the cytoplasm, the protein encoded by the mutated construct was mostly nuclear (Figure 6. A). Quantitative analyses confirmed that mutations affecting this core fragment significantly decreased the nuclear export of the protein produced and thus provided evidence that the aa(109–133) core sequence acted as a functional NES, targeting the protein from the nucleus to the cytoplasm.

The C-terminal aa(140–160) sequence contributes to the cytoplasmic localization of core

We have demonstrated above that aa(109–133) is a functional NES. Consistent with this finding, core protein aa(1–120), which does not contain this sequence but does contain a functional NLS in the N-terminus of core, is found exclusively in the nucleus of hepatoma cells. However, another core fragment, aa(1–140),
which includes the NES identified above, is also found exclusively in the nucleus, whereas larger core fragments, consisting of aa(1–160) or aa(1–173), were found in both the nucleus and the cytoplasm of hepatoma cells (Figure 2), as previously reported. We therefore conclude that, in addition to our NES, HCV core region aa(140–160) influences the subcellular distribution of the core protein.

The core protein sequence has been analyzed for determination of its secondary structure. Both aa(109–160) and aa(144–160) were found to be rich in α-helices. This fragment may therefore influence the subcellular distribution of HCV core protein. It is thus possible that either there is another NES in this region, or that this sequence affects the subcellular distribution of core due to interaction with membranes and/or lipid droplets.

We investigated the possibility of another NES being present in the aa(109–160) region of core, by constructing three new plasmids encoding protein fragments aa(109–160), aa(134–143), and aa(144–160) of core (Figure 7A). Huh7 cells were transfected with these plasmids and, 40 hours later, we determined the subcellular distribution of the encoded proteins by fluorescence microscopy.
The aa(109–160) core protein fragment was found predominantly in the cytoplasm, whereas aa(134–143) and aa(144–160) were found solely in the nucleus (Figure 7B). These experiments showed clearly that aa sequences (144–160) and (134–143) did not function as additional NES. Indeed, in the absence of the aa(109–133) sequence identified in this study, these core protein fragments were not exported from the nucleus. We therefore conclude that the cytoplasmic distribution of core requires a functional NES within aa(109–133). However, the presence of this sequence is not sufficient and an additional fragment aa(140–160) is also required to maintain the protein in the cytoplasm.

The hydrophobic C-terminal DII of HCV core, containing this sequence, is required for the binding of core protein to lipid droplets and membranes, and for core protein folding and stability. Indeed, residues of two amphipathic helices, aa(119–136) and aa(148–164), in DII are critical for this association, as shown by extensive mutational analyses. Therefore, the core fragment aa(109–160) is found mostly in the cytoplasm, because,
in addition to the NES, it mediates interactions of core with the cytoplasmic lipid droplets and/or membranes at specific sites in the aa(140–160) sequence.

The nuclear export mediated by the core NES is CRM-1-dependent

Proteins containing NES are recognized by the CRM-1 export receptor, a member of the karyopherin superfamily of importin-β nuclear transport receptors. CRM-1 exports cargo proteins containing an NES from the nucleus to the cytoplasm. Leptomycin B is a potent and specific nuclear export inhibitor that alkylates CRM-1, specifically inhibiting the CRM-1-dependent nuclear export of proteins such as HIV Rev.

We therefore investigated whether the nuclear export mediated by the NES in aa(109–133) was sensitive to LMB, and thus dependent on the CRM-1-dependent export pathway. Huh7 cells were transfected with a plasmid encoding this sequence and, 40 hours later, cells were treated with 2 or 10 ng/ml LMB. We found that, at both concentrations used, LMB inhibited the nuclear export mediated by the core aa(109–133) sequence (Figure 8). Nuclear export driven by the HIV Rev protein NES, used as a control, was inhibited by LMB to a similar extent, whereas aa sequences containing the SV40 NLS were always nuclear. The EGFP-tagged control protein was found in both the cytoplasm and nucleus, and this passive distribution was not sensitive to LMB treatment. The aa(109–133) region of the core protein may therefore be considered a functional NES, operating in a CRM-1-dependent manner.

Following the transfection of Huh7 cells with a plasmid encoding an EGFP-labeled core aa(1–173), the protein was found in both the nucleus and cytoplasm, unless translocation had been blocked by LMB. This treatment increased the proportion of core found in the nucleus although the protein was also present in the cytoplasm in several cells (data not shown). The subcellular distributions of core protein in the presence and absence of LMB were confirmed by confocal microscopy (Figure 9).

In addition, mutation of the hydrophobic residues L(119), I(123) and L(126) in the NES region of core protein aa(1–173) significantly increased the nuclear localization of this protein, to levels greater than observed for the wild-type protein (Figure 6B).

Thus, the mature core protein produced in transfected hepatoma cells is located in both the cytoplasmic and nuclear compartments. The translocation of the protein from the nucleus follows the CRM-1-dependent pathway, because it is inhibited by LMB. Similarly, mutations of the three hydrophobic amino acids of the NES region spanning aa(109–133) significantly increase the nuclear localization of the core protein.

Detection of core in the nucleus by electron microscopy

In a recent study of HCV infection, we detected HCV particles by immunoelectron microscopy during the entry of the virus into Huh 7.5 cells (P.Maillard, M.Walic et al. PloS One in press). In addition, core and some non structural proteins of other Flaviviruses are trafficked to the nucleus at very early phase of infection. These findings prompted us to investigate whether HCV core protein could also be directed to the nucleus at such early stages of infection. For electron microscopy analysis, virus particles produced in Huh 7.5 cells were concentrated by pelleting through the sucrose cushion and cells were infected with concentrated virus preparations as described in the Materials and Methods section. Virus core protein was stained with the ACAP27 monoclonal antibody, followed by an anti-mouse IgG secondary antibody labeled with colloidal gold. HCV core protein was observed in the cell nucleus 20 minutes after infection, where it was located close to the nuclear membrane or the nuclear pores, as shown by immunostaining with anti-core antibodies (Figure 10 A–C). Taking into account the time after infection, the intranuclear core protein was almost certainly derived from internalized virus particles rather than represented HCV core protein produced de novo in infected cells.

Quantitative analyses performed on HCV-infected and non infected cells after staining with anti-core antibodies confirmed the specificity of our observations and showed that a larger proportion of the gold-labeled HCV core was present in the cell nucleus than elsewhere in the cell at this stage of infection (Figure 10 D). No such staining was observed when unrelated monoclonal antibodies
were used. These observations suggest that, at very early stages of 
HCV infection, at least some of the HCV core protein is directed 
to the nucleus.

Detection of core in the nucleus by immunofluorescence 
analysis after LMB treatment

In most studies in the HCVcc in vitro replication system, HCV 
core protein has not been found in the nucleus. However, if 
PA28γ, an activator of the proteasome, which is responsible for 
ubiquitin-independent degradation in the nucleus, was inhibited or 
absent, very small amounts of core could be detected, by 
immunofluorescence microscopy, in the nuclei of HCV-infected 
Huh7 cells.

We investigated whether the NES identified was functional in 
the JFH1 HCVcc cell culture model. Immunofluorescence 
microscopy detected HCV core in the cytoplasm of infected cells, 
but no evidence of a nuclear localization of core was obtained at 
various times after infection, consistent with previous findings. We 
therefore used a small interfering RNA to silence the PA28γ gene, 
to determine whether this treatment increased the amount of core 
protein in the nucleus, as previously reported.

PA28γ expression was successfully transiently knocked down by 
a specific siRNA targeting PA28γ, but not by a control, unrelated 
siRNA (Figure 11A). Despite careful analysis, no core was 
detected, by immunofluorescence microscopy, in the nucleus of 
either siRNA-silenced cells or cells transfected with a control RNA 
(Figure 11B). Instead, HCV core protein was concentrated in the 
perinuclear area of the cell, in both silenced and untreated cells.

Nevertheless, the treatment of infected cells with LMB, which 
was maintained in the culture medium for several hours to block 
the nuclear export of core protein, resulted in the detection of core 
within the nucleus in several cells, distributed as single points at 
several sites in the nucleus (Figure 12). We detected HCV core in 
the nucleus only when LMB was added at early time points (2–4 h) 
after infection, but not when the drug was added 48 hours after 
infestation.

These findings clearly demonstrate that at least some core 
protein is targeted to the nucleus in the JFH1 HCVcc infection 
model, at early stages of infection. Core seems to be exported from 
the nucleus via the CRM-1-dependent pathway, because a nuclear 
distribution of this protein was observed only in LMB-treated cells.

Leptomycin B affects HCV production

As core was detected in the nucleus of HCV-infected cells 
treated with LMB, we investigated the possible effects of LMB 
treatment on virus multiplication. We infected Huh 7.5 cells with 
HCVcc in the presence or absence of 10 ng/ml LMB or added the 
drug to the cell supernatant at various time points after infection. 
Cells were then grown in the medium containing LMB until 
32 hours post infection, and intracellular HCV RNA was 
quantified by RT-qPCR. Incubation with the drug for eight hours 
was needed for efficient inhibition of infection (Figure 13 A). 
Infection levels were about 60% lower than control infection levels 
when the drug was added during the first six hours of infection. By 
contrast, the percentage inhibition was only 20% if LMB was 
added eight hours after infection (not shown) and 0% if added 
twenty-four hours after infection (Figure 11 C). Control experi-
ments excluded a cytotoxic effect of LMB or its solvent on Huh 7.5 
cells (Figure 13 B, D).

Overall, these data showed that early LMB treatment 
influenced HCV RNA production, suggesting that the early 
shuttling of core between the cytoplasm and the nucleus might be 
important for virus multiplication.

Discussion

We provide here the first demonstration that a functional NES 
is present in HCV core protein. We show that amino acids 109 to 
133 are responsible for the active export of the HCV core protein 
out of the nucleus, via a CRM-1-mediated nuclear export 
pathway. This NES was functional in transfected cells and in an 
in vitro model of HCV replication (HCVcc). The trafficking of core 
protein into the nucleus early in infection may help to establish
infection and facilitate the interaction of core with nuclear molecules, with potentially important pathological consequences.

HCV core protein is thought to be a major viral factor promoting liver disease during HCV infection and the malignant transformation of hepatocytes, leading to the development of HCC through interactions with several host cell factors involved in a wide range of cellular processes. Indeed, in various experimental systems, HCV core has been reported to affect transcription mediated by various gene promoters and apoptosis, thereby contributing to cell transformation. The oncogenic activity of core might be related to its nuclear localization. In liver biopsy samples from HCV-infected patients, HCV core has been found mostly in the cytoplasm, being only rarely detected in the nucleus of infected hepatocytes. Nevertheless, a nuclear location of truncated core proteins was detected in tumor tissues from patients with HCV-related hepatocarcinoma. Similarly, the nuclear accumulation of core has been observed in transgenic mice producing the HCV core protein and developing HCC.

Taking into account the role of HCV core as a viral factor of major pathological significance and understanding the mechanisms regulating its subcellular distribution and trafficking are of critical importance. Several studies on transfected cells have shown the HCV core protein to be located in the cytoplasm or nucleus, depending on its length. Consistent with this dual localization, many studies have reported interactions with molecules located in either the cytoplasm or the nucleus.

Our findings confirmed that, in an in vitro transfection system based on human Huh7 and HepG2 hepatoma cell lines or immortalized Fa2-N4 human hepatocytes, the aa[1–173] and aa[1–160] core proteins were found in both the nucleus and the cytoplasm. These proteins were found exclusively in the nuclei of non human cells (CHO, ARL-6) and in human cells of non hepatic origin (HEK-293). By contrast, the shorter core proteins aa[1–120] and aa[1–140] were found exclusively in the nucleus. Thus, the nuclear/cytoplasmic subcellular distribution of core proteins aa[1–173] and aa[1–160] was specific to human cells of hepatic origin. Our observations suggest that core protein may contain signals for specific transport mechanisms controlling its distribution between the nucleus and the cytoplasm that are functional in human hepatic cells.

The differences in the distribution of the protein between the nucleus and cytoplasm in the cell types tested may reflect the availability and/or functionality of the carrier proteins in these cells. Indeed, the subcellular distribution of a given protein may be controlled by the differential expression of carrier proteins in various tissues or host species, and may depend on the differentiation status of the cell.

The nucleocytoplasmic trafficking of various proteins and RNA is controlled by importins and exportins (also called karyopherins) from the importin-β superfamily of proteins. These proteins can therefore gain entry into the nucleus only if they possess the appropriate NLS recognized by nuclear importin receptors, or if they react directly with the nuclear pore complex. Consistent with the nuclear localization of core in several experimental systems, three NLS have been identified in the N-terminal domain of this protein. These signals consist of clusters of basic amino acids in the aa[5–13], aa[38–43], and aa[58–71] regions; they are functional and able to target core to the nucleus.

For reentry into the cytoplasm, proteins must contain the sequences required for interaction with export factors (exportins), enabling them to leave the nucleus via the nuclear pore. The nuclear export of proteins is mediated mostly by NES, leucine-rich aa sequences recognized by soluble export receptors, such as Exportin1 (CRM-1). No functional export signal, directing
translocation of the core protein from the nucleus to the cytoplasm has been identified in the core sequence to date, although the presence of such a signal has been suggested.

We used NetNES software to search for a classical leucine-rich NES in HCV core, and found such a signal in the C-terminal part of the immature form of core at aa(179–187). This protein is always found in cytoplasmic compartment and, thus, the role of this signal remains unclear. In addition, this NES sequence is removed when the mature core protein is generated by cellular peptidase processing.

In addition to this classical leucine-rich NES in the C-terminus of the immature core protein, we identified a second, “atypical” NES in the mature form of core, by sequence alignment, as previously reported by Rowland et al. for the identification of NES in the PPRSV nucleocapsid protein. Indeed, multiple sequence alignments of the HCV core with other viral NES, generated with CLUSTAL W, led to the identification of a candidate NES sequence at aa(109–133). Consistent with the putative biological role of this NES, this sequence contains a cluster of hydrophobic residues with a sequence similar to those of the NES of HSV ICP27, HIV Rev, equine infectious anemia virus Rev, feline immunodeficiency virus Rev and porcine reproductive and respiratory syndrome virus N protein. Moreover, this signal was well conserved in consensus sequences from various HCV genotypes.

We provide evidence that the NES identified at aa(109–133) is functional in transfected hepatoma cells and can export core protein fragments from the nucleus to the cytoplasm. First, a peptide corresponding to aa(109–133) of HCV core, like the NES of the HIV Rev protein, counteracted the nuclear translocation driven by the strong NLS of SV40 large T-antigen. The nuclear export of this NES, observed by fluorescence microscopy, was confirmed by quantitative analyses. LMB treatment inhibited nuclear export of the core NES, demonstrating that the aa(109–

![Figure 10. Nuclear localization of core protein in HCV infection, as demonstrated by electron microscopy.](https://www.plosone.org/figure10-g010)

Figure 10. Nuclear localization of core protein in HCV infection, as demonstrated by electron microscopy. For electron microscopy analysis, virus particles produced in Huh 7.5 cells were concentrated from the cell supernatant by centrifugation through a sucrose cushion for 4 h at 32,000 rpm in an SW 32 Ti rotor. Concentrated virus preparation was incubated with Huh 7.5 cells at 4°C. The cells were then transferred to 37°C and incubated for a further 20 min. Cells were washed, fixed with 4% PFA and stained with monoclonal anti-core antibody ACAP27, followed by secondary, colloidal gold-labeled anti-mouse IgG. Nanogold staining was enhanced by incubation with the HQ silver enhancement kit, and cells were post-fixed by incubation with 1% osmium tetroxide (for details, see the Materials and Methods section). Non infected cells were also incubated with anti-core antibodies. Panels A, B and C, show representative pictures of HCV core localization in HCVcc-infected cells. The presence of one silver-enhanced gold particle is indicated. LD, lipid droplet; M, mitochondrion; NP nuclear pore. (D) Quantitative evaluation of HCV core labeling (gold particles) in the perinuclear vs other areas of HCV-infected and non infected cells, from randomly selected intracellular zones of equivalent area. Thirty cells were considered for each analysis. doi:10.1371/journal.pone.0025854.g010
nuclear export signal functions in a CRM-1-dependent manner. Indeed, “atypical” NES sequences may also function in a CRM1-dependent manner.

Export signals essentially consist of several closely spaced leucine residues, but other hydrophobic amino acids, such as methionine, isoleucine, valine, phenylalanine and tryptophan, may replace leucine in the recognition motifs. Using comprehensive alanine-scan mutagenesis we showed that the replacement of the Leu(119), Ile(123) and Leu(126) residues by alanine modified the export capacity of our NES. Indeed, whereas the wild-type chimeric protein encoded by the plasmid (EGFP-NLSSV40-NEScore) carrying the intact aa(109–133) sequence was exported into the cytoplasm, the protein encoded by the mutated construct was mostly found in the nucleus. These observations, validated by quantitative analyses, confirmed that the aa(109–133) core sequence acted as a functional NES, targeting the protein from the nucleus to the cytoplasm.

The functionality of the NES at aa(109–133) was further analyzed in the context of mature HCV core protein. Firstly, LMB treatment also modified the subcellular distribution of core aa(1–173), increasing its accumulation in the nucleus. Secondly, mutations of the hydrophobic residues Leu(119), Ile(123) and Leu(126), with the replacement of these residues by alanine residues, modified the export capacity of the protein. Our quantitative studies confirmed that the proportions of the protein located in the nucleus (as opposed to the cytoplasm) were significantly higher for the mutated protein than for the wild-type protein. These data provide experimental evidence for a role of the identified NES in the CRM-1-dependent nuclear export of HCV core.

CRM-1 has been identified as the export receptor, the principal mediator of nuclear export, allowing the nuclear-cytoplasmic shuttling of proteins and RNA between cellular compartments. CRM-1-dependent transport is well conserved throughout eukaryotes and LMB is a recognized inhibitor of the active export of most molecules from the nucleus. Indeed, the “steady-state” localization of proteins does not always reflect the biological importance of their site of action. The use of LMB to block CRM-1-mediated nuclear export results in the detection of the NES-containing protein in the nuclear compartment, although its “steady-state” location appears to be exclusively or predominantly cytoplasmic, with the equilibrium of bidirectional transport favoring nuclear export. Nevertheless, CRM-1-mediated transport is a highly regulated process and this regulation includes the masking of NES, phosphorylation and heterodimerisation of the protein and the formation of disulfide bonds by an oxidative process. The availability of specific cofactors may also influence this regulation. The presence of such cofactors may contribute to the observed differences in the subcellular distribution of core proteins in various cell types (see above).

In our study, both the NES aa(109–133) and the adjacent hydrophobic sequence in domain II were required for a cytoplasmic distribution of core. Indeed, we found that only a relatively long core protein fragment, aa(109–160), was located in the cytoplasm, with shorter core fragments lacking either NES or this adjacent hydrophobic sequence being found in the nucleus. We showed that no other NES capable of enhancing core export from the nucleus was present in the adjacent (133–160)aa sequence. However this core fragment, in addition to the NES, was necessary for a cytoplasmic distribution of the protein. As this was necessary for a cytoplasmic distribution of the protein.
hydrophobic core fragment contains specific sites interacting with LDs and membranes, these interactions are probably required, in addition to our NES aa(109–133), for the maintenance of core protein in the cytoplasm. Consistent with this notion, the aa(120–150) core protein (containing a hydrophobic fragment interacting with LDs/membranes but not the NES) was found in the nucleus.

Figure 12. Intranuclear localization of core in HCV-infected cells treated with LMB. Huh 7.5 cells were infected with the JFH1 strain of HCVcc and, 2 h after infection, 10 ng/ml LMB was added, with maintenance of this concentration of LMB in the cell culture until 48 h post infection. (A) Detection of intranuclear core in LMB-treated cells. Several cells are shown in which core is present in the nucleus (designated a-i). These cells were analyzed further: 18 cross sections of images were taken to visualize the intranuclear localization of core, at different levels within the nucleus. (B) Cut view of Z stacks from the cells shown in A with the same designation (a–i). Images were captured with an Axioplan 2 microscope with Apotome (Zeiss) and Axiovision 4.6. Orthogonal views were obtained from Z-stack images, with a resolution of 0.33 μm. The nuclear membrane is stained with rabbit anti-lamin B antibody, followed by anti-rabbit IgG tagged with Alexa Fluor 488 (in green), for better visualization of the intranuclear localization of core. HCV core protein is stained with anti-core ACAP27 antibody, followed by Alexa-Fluor 568 labeled anti-mouse IgG (in red). Bar represents 10 μm. Negative controls for these studies (without LMB treatment) are shown in Figure 11, in which no core was detected in the nucleus, whether or not PA28γ was silenced.

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rather than the ER. Similarly, C-terminally truncated core proteins with NES, but lacking the hydrophobic fragment of DII, accumulated in the nucleus, thereby probably contributing to the development of HCC. Collectively, our observations indicate that both a nuclear export signal aa(109–133) and the adjacent hydrophobic sequence in domain II are required to target core protein to the cytoplasm and to keep it in this compartment. The identification of a NES that was functional in transfected Huh7 hepatoma cells raised questions about the role of this signal in HCV infection. In the context of the infectious cell culture model in Huh7.5 cells (HCVcc), HCV core protein was found to colocalize with lipid droplets and membranes, but was not detected in the nucleus in most studies. However, knockdown of the PA28γ proteasome activator gene, blocking ubiquitin-independent nuclear degradation, led to the detection of a very small quantity of HCV core in the nuclei of infected Huh7 cells on immunofluorescence analysis.

Our key findings for transfected cells are consistent with the data obtained in the HCVcc replication model. First, our immunoelectron microscopy studies based on staining with antiprotease antibodies provided evidence for a nuclear location of core as early as 20 minutes after the start of infection. This suggests that the nuclear trafficking of core takes place very early in the viral cycle, shortly after internalization of the virus. Second, the use of LMB to block CRM-1-dependent export resulted in the detection of HCV core protein in the nuclei of a number of JFH1-infected cells on confocal microscopy. Nuclear staining for HCV core was observed as several ‘spots’ when various nuclear images (virtual cross sections) were analyzed by confocal microscopy. In particular, core was detected within the nucleus only if LMB treatment was applied early in infection (2 h post infection), and no core was detected in the nucleus if LMB was applied late in infection (48 h post infection). Intranuclear core was also observed in Huh7.5 cells in which PA28γ expression was knocked down but only after LMB treatment. Early LMB treatment also decreased HCV RNA production, suggesting that the early shuttling of core between the cytoplasm and the nucleus may be important for virus multiplication. LMB
had no toxic effect on the cells tested, but we cannot rule out the possibility that LMB treatment also influences other cell processes, in addition to core protein shuttling.

We conclude that the NES identified in HCV core protein is functional in the HCVcc replication system. Our electron microscopy studies suggest that some HCV core, derived from invading virus particles, is transported into the nucleus at very early stages of the viral life cycle. In addition, a fraction of core protein can be detected by immunofluorescence, in the nucleus, a few hours after infection, subsequently being exported to the cytoplasm in a CRM-1-dependent manner, as this export is blocked by LMB, a drug widely used to dissect nuclear export pathways.

Examples of viral proteins known to shuttle through the nuclear pore complex and for which the CRM-1-dependent pathway is known to export the corresponding viral RNA include HIV Rev and T-cell leukemia virus type 1 Rex. Structural and nonstructural proteins of several members of the flavivirus family, such as Japanese encephalitis virus (JEV), Dengue virus (DENV), and Kunjin virus (KUN), have also been shown to be actively translocated to the nucleus or to the nucleoli of infected cells, even when these viruses multiply entirely in the cell cytoplasm. This phenomenon may affect virus infectivity or disease pathogenesis. Indeed, DENV NS5 RNA polymerase can be detected in the nucleus very shortly after infection, and this protein is exported from the nucleus in a CRM-1-dependent manner. Nuclear NS5 suppresses the production of IL-8, a cytokine playing an important role in the antiviral response.

DENV core protein also localizes to the nucleus (and nucleoli) at very early stages in the viral life cycle, due to its bipartite NES. The mechanisms of DENV nuclear export remain unknown, as this process is insensitive to LMB, suggesting that it does not require a functional NES [65]. Indeed, many other pathways exist for protein import and export, including the calreticulin pathway. Nevertheless, the nuclear localization of DENV core may regulate its replication cycle and apoptosis (see for review. Consequently, the construction of recombinant viruses based on viral proteins deficient in nuclear trafficking signals could potentially lead to attenuation of the virus.

Our findings are consistent with the hypothesis that at least some HCV core protein is trafficked between the cytoplasmic and nuclear compartments early in HCV infection. Recombinant viruses mutated in the NES region investigated here, showed impaired virus production, producing less than 1% of the wild type virus in the HCVcc in vitro model. These observations provided evidence that this sequence is of importance for virus life cycle. The absence of the NES identified in this study and/or of the hydrophobic fragment of domain II (which act together to keep the protein in the cytoplasm) may account for the nuclear localization of the C-terminally truncated core proteins in patients with HCV-induced HCC and contribute to the cell transformation.

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Author Contributions

Conceived and designed the experiments: AB PM RM AC P-OV. Performed the experiments: AB PM FR E-IP. Analyzed the data: AB PM AG P-OV E-IP FR. Contributed reagents/materials/analysis tools: P-OV E-IP FR. Conceived and designed the experiments: AB PM RM AC P-OV. Provided fellowship for AC: MP. Wrote the paper: AB AC P-OV. Contributed to the writing of the paper: AB AC P-OV. Provided assistance in the preparation of art work and S. Bocchetta for help in the editing of the manuscript.

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