part of the structural protein-coding region (Shimoike et al., 1999). In addition, the core protein has a high affinity for the stem-loop IIId domain of the 5′UTR (Fig. 1) and for (G)-rich nucleotides (Tanaka et al., 2000).

In addition, evidence regarding the importance of the interaction between HCV core protein and HCV RNA in regulating viral translation is accumulating. We previously reported that expression of the core protein down-regulates HCV translation through interaction(s) involving 5′ regions of the viral genome (Shimoike et al., 1999). Although some evidence suggesting inhibition of HCV translation through RNA–RNA interactions, rather than core–RNA interactions, exists (Wang et al., 2000; Kim et al., 2003), several studies indicate that the core protein modulates HCV translation. Specifically, regions of the core protein corresponding to aa 34–44 (Zhang et al., 2002) or aa 1–20 (Li et al., 2003) are important for inhibition of HCV translation. The core protein may down- or up-regulate HCV IRES activity in a dose-dependent manner (Boni et al., 2005).

The aim of the present study was (1) to clarify the nature of interaction between the HCV core protein and the viral IRES element and (2) to gain insight into the relationship between core protein-mediated inhibition of translation and core–IRES interactions using a combination of techniques, including an in vivo reporter assay and in vitro surface plasmon resonance (SPR) analysis.

Results

Effect of the core protein-coding sequence on HCV IRES-initiated translation

Since there is conflicting data regarding the effect of the core protein or the core protein sequence on HCV IRES-directed translation (Shimoike et al., 1999; Zhang et al., 2002; Li et al., 2003; Boni et al., 2005), we sought to determine whether the RNA sequence of the core-coding region inhibits HCV IRES activity in the present experiment. A single substitution replacing A with U at nt 357 was introduced to produce a stop codon near the 5′ end of the region encoding the core protein, as previously described (Wang et al., 2000). This mutant, known as pCAGFS, produces core protein RNA with a single substitution, resulting in a core peptide, five residues in length, encoded by the N-terminal. Western blot analysis was then used to confirm that the core protein is not expressed by HepG2 cells following transfection with pCAGFS (Fig. 2C). RNA molecules transcribed in vitro from two reporter plasmids, HCVLuc and RLuc, expressing firefly luciferase (FL) controlled by the IRES of HCV genotype 1b and *Renilla* luciferase (RL) controlled by a cap-dependent mechanism, respectively, were cotransfected into cells after 48 h of transfection with pCAGFS39 or core-expressing pCAGC191 (Suzuki et al., 2001). Cell lysate samples were prepared 6 h post-reporter transfection and assayed for expression of both luciferases. As shown in Fig. 2A, the translational activity of HCV IRES was reduced in cells expressing the core protein, but not in cells transfected with

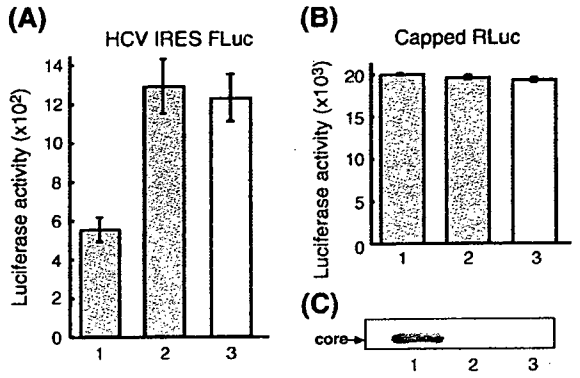

Fig. 2. Effect of the core protein-coding sequence on the translation initiated by HCV IRES. HepG2 cells transfected with pCAGC191 (lane 1), pCAGFS (lane 2), or pCAGGS (lane 3) were cotransfected with reporter RNAs of HCVLuc and the capped RLuc. The activities of both FL (A) and RL (B) were measured by a luminometer. The activities of both FL and RL were determined in at least three independent experiments, each of which conduced with triplicate samples. (C) Western blot analysis of the core protein expressed in the infected cells.

pCAGFS, indicating that the HCV core protein, but not the core-coding sequence, inhibits HCV IRES-directed translation. Transfection with neither core-expressing or non-expressing constructs modulated cap-dependent translation (Fig. 2B).

Effect of partial deletion of the HCV 5′UTR on inhibition of viral IRES-mediated translation by the core protein

In previous studies, we demonstrated that purified HCV core protein binds most efficiently and stably to the stem-loop IIId domain of the 5′UTR of HCV RNA followed by the stem-loop I domain and the region encoding nt 23–41 (Fig. 1; Tanaka et al., 2000). In addition, we revealed that the core protein expressed in HepG2 cells inhibits the IRES-dependent translation of HCV (Shimoike et al., 1999). It can be hypothesized that binding of the core protein to one or more regions of the 5′UTR might inhibit translation. To address this issue, we constructed three reporter plasmids: ΔIILuc, Δ23–41Luc, and ΔIIIdLuc, with deletions of domain I (Δ1–22), nt 23–41, and domain IIId (Δ254–278) of the HCV 5′UTR, respectively, also containing the FL gene (Fig. 3A). RNA molecules transcribed from these reporter plasmids in vitro were transfected into HepG2 cells, after which luciferase activity within the cell lysate samples was analyzed. Consistent with previous reports, deletions of domain I (ΔIIluc) (Luo et al., 2003; Friebe et al., 2001) or IIId (ΔIIIdLuc) (Jubin et al., 2000) profoundly impaired IRES activity, with a >95% reduction in activity (data not shown), thus demonstrating the importance of these loop structures for HCV translation. Therefore, in the following experiment, we adjusted the dose of each reporter transcript to ensure a consistent level of FL expression.

To investigate the effect of the core protein on translation mediated by wild-type or mutated HCV 5′UTR as described above, cells infected with a recombinant baculovirus carrying

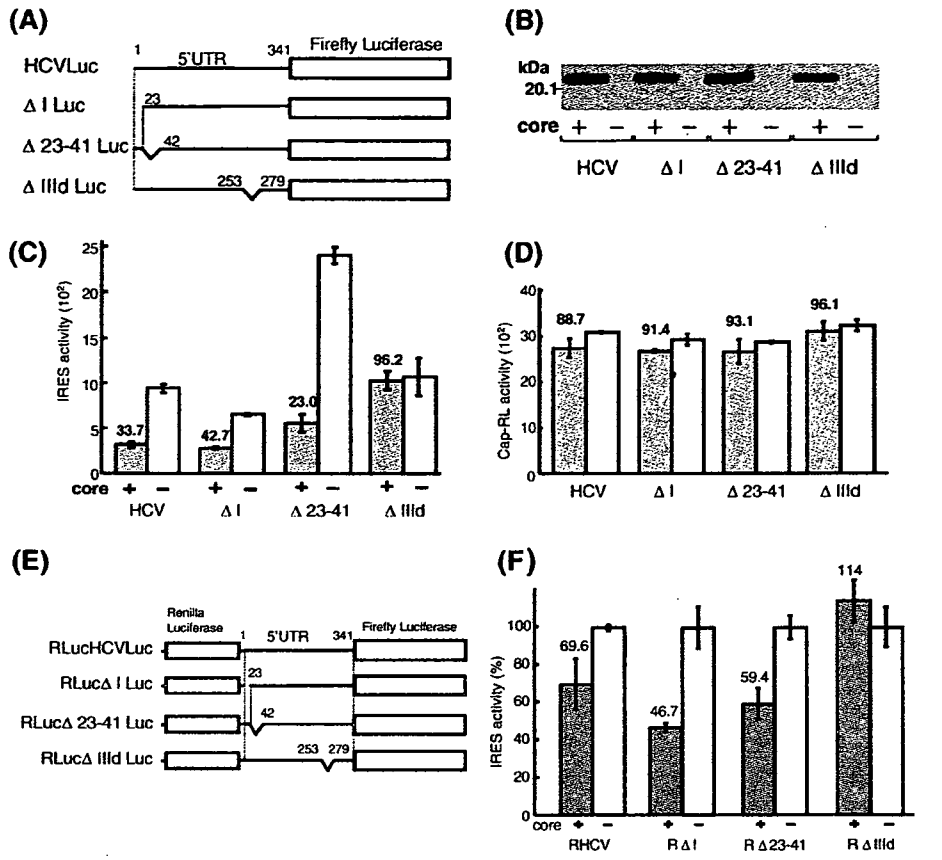

Fig. 3. Effect of deletion mutations in HCV 5'UTR on inhibition of the viral IRES-mediated translation by the core protein. HepG2 cells infected with AcCA39 or AcCAG at a multiplicity of infection of 20 were transfected with monocistronic wild-type (HCVLuc) or deletion mutants (ΔILuc, Δ23–41Luc, ΔIIIdLuc) of reporter RNAs together with the capped RLuc RNA or transfected with bicistronic wild-type (RLucHCVLuc) or deletion mutants (RLucΔILuc, RLucΔ23–41Luc, RLucΔIIIdLuc) of reporter RNAs. The activities of both FL and RL were determined in at least three independent experiments, each of which conduced with triplicate samples. Schematic representation of the monocistronic and bicistronic deletion mutants used in this study is shown in panels A and E, respectively. (B) Western blot analysis of the core protein in each cell lysate in which the luciferase activities were measured. (C) Relative luciferase activities were normalized with those of RLuc. (D) The activities of RLuc in cells cotransfected with RLuc and HCVLuc or deletion mutants are shown. (F) HCV IRES activity was determined by calculating the abundance of Fluc relative to RLuc, with that of each reporter in the absence of the core protein normalized to 100%. Mean values with standard deviations were indicated.

the entire HCV core gene (AcCA39; Shimoike et al., 1999) or an empty vector (AcCAG) were cultured for 2 days, followed by transfection with reporter transcripts, either wild-type HCVLuc (0.1 μg/well), ΔILuc (6 μg/well), Δ23–41Luc (0.2 μg/well), or ΔIIIdLuc (6 μg/well), together with capped RLuc RNA (0.08 μg/well). As indicated in Fig. 3C, expression of the core protein inhibited HCV IRES-mediated translation from ΔILuc and Δ23–41Luc, as well as from HCVLuc, by more than 50%. In contrast, inhibition of translation by the core protein was not observed in cells transfected with ΔIIIdLuc. As shown in Fig. 3D, the expression of neither the core protein nor any of the IRES-directed reporters influenced cap-directed translation. Thus, as previously demonstrated (Shimoike et al., 1999), RL activity was used as an internal control to normalize the efficiency of transfection in the following experiments (Figs. 4 and 6). Western blotting was used to confirm that core protein concentrations within the cell lysate of cells infected with AcCA39 were comparable in the presence of each of the reporter RNA molecules (Fig. 3B). We observed a similar effect of the core protein on HCV IRES activity when equal amounts (6 μg/well) of each HCVLuc, ΔILuc, Δ23–41Luc, or ΔIIIdLuc transcript were transfected (data not shown). These results eliminate the possibility that there is no translational inhibition because the core protein is destabilized in cells transfected with ΔIIIdLuc RNA. We also determined the effect of the core protein on HCV translation initiated from bicistronic reporters: RLucHCVLuc (wild-type), RLucΔILuc (deletion of domain I), RLucΔ23–41Luc (deletion of nt 23–41), and RLucΔIIIdLuc (deletion of domain IIId) (Fig. 3E). Consistent with results obtained from the monocistronic constructs, expression of the core protein showed an inhibitory effect on HCV translation mediated by RLucHCVLuc, RLucΔIluc, or RLucΔ23–41Luc, but not by RLucΔIIIdLuc (Fig. 3F). The capped RL activity from each reporter was similar and was not influenced by expression of the core protein (data not shown). These results suggest that the stem-loop IIId domain of the 5'UTR is important for inhibition of HCV translation by the core protein.

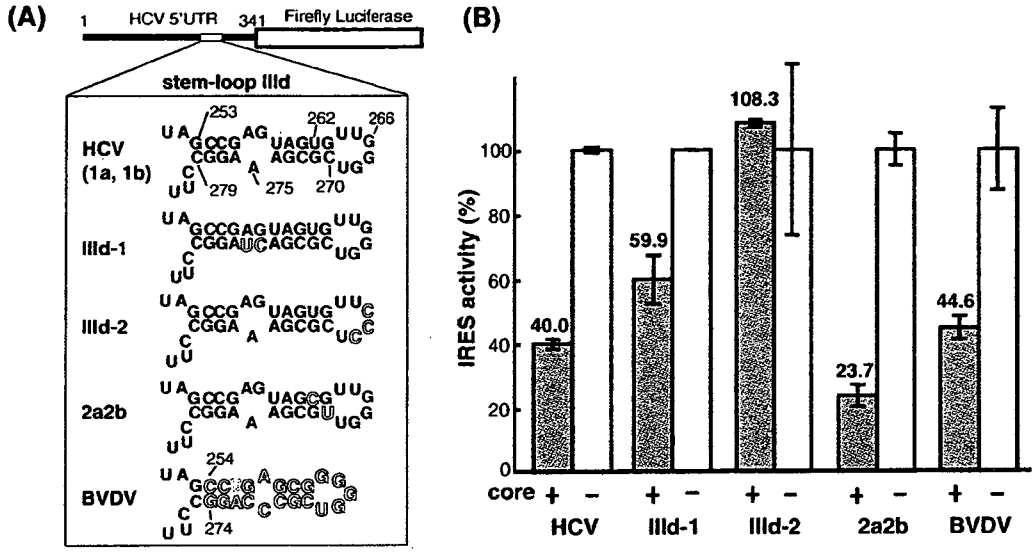

Fig. 4. Mutational analysis of the stem-loop IIId domain. (A) Schematic representation of the predicted secondary structures of the IIId domain of mutated reporters used in this study. (B) The core protein and luciferase reporters are expressed as described in the legend to Fig. 3, except reporters indicated. HCV IRES activity was determined and presented as described in the legend to Fig. 3C.

Mutational analysis of the stem-loop IIId domain

To further investigate the functional role of the stem-loop IIId domain (nt 253–279) in core protein-mediated inhibition of HCV translation, we engineered stem-loop IIId domains with the following four mutations (Fig. 4A): (1) IIId-1Luc, in which the A at nt 275 was changed to UC, thus forming a double-stranded structure instead of a bulge loop in the IIId, (2) IIId-2Luc, in which the GGG triplet (nt 266–268) was changed to a CCC triplet within the loop of stem-loop IIId, (3) 2a2bLuc, in which the U at nt 262 and the C at nt 270 were changed to C and U, respectively, thus changing the genotype to 2a/2b, and (4) BVDVLuc, in which the stem-loop IIId (nt 254–274) sequence was changed to that of bovine viral diarrhea virus (BVDV)-1. Cells that did or did not express the core protein were transfected with each of the above described reporter RNA transcripts, after which luciferase activity was measured. As shown in Fig. 4B, IIId-2Luc, containing a mutation of the GGG triplet of the apical loop, demonstrated no inhibition of HCV IRES-mediated translation by the core protein, whereas IIId-1Luc, containing a mutation within the bulge loop structure, showed only a marginally reduced inhibitory effect of the core protein. We previously demonstrated that HCV core protein binds most efficiently to (1) the stem-loop IIId domain, compared to other structural domains of the 5′UTR, and to (2) G octamer (G_8), as opposed to A_8, C_8, and U_8, using a quantitative SPR method (Tanaka et al., 2000). Thus, the results obtained here suggest that the apical loop is a critical recognition site for translational inhibition by the core protein. It is likely that the inhibitory activity of the core protein on HCV IRES-mediated translation is related to its efficiency of RNA binding.

We also observed the core protein to exert an inhibitory effect on translation directed by either 2a2bLuc or BVDVLuc, similar to that observed with wild-type HCVLuc, involving a 5′UTR sequence of genotype 1. Since the IIId domain sequence of HCVLuc is conserved among genotypes 1, 3, 4, and 5 and since that of 2a2bLuc is shared with genotype 6, it appears that inhibition of HCV translation by the core protein is independent of the viral genotype and occurs in most HCV isolates. Sequence alignment of HCV and various pestiviruses showed that, although the primary nucleotide sequence of the IIId domain exhibits considerable variability, the predicted secondary structure of the domain is highly conserved among these viruses as reviewed previously (Rijnbrand and Lemon, 1999). Furthermore, the GGG triplet followed by U at the apical loop and one bulge loop in the domain are well conserved among HCV and pestiviruses. These suggest that the nucleotide sequence of the apical loop, particularly the GGG triplet, is more important than the stem-structure sequence of the IIId domain for core protein-mediated translational inhibition.

Relationship between translational inhibition and ability of the core protein to bind to the IIId domain within the 5′ UTR

To investigate the relationship between inhibition of HCV translation by the core protein and ability of the core protein to bind to IIId RNA, we prepared two biotinylated oligo RNA molecules, IIId-1 and IIId-2 (nt 251–282), containing identical mutations in the bulge and apical loops of their IIId domains as the mutated reporters IIId-1Luc and IIId-2Luc, respectively (Fig. 4A). These mutant or wild-type oligo RNA (IIId-wt) molecules were then coupled to streptavidin-coated sensor chips and allowed to bind to purified recombinant core protein. The results of subsequent SPR analysis using a BIAcore biosensor are shown in Fig. 5. The core protein was observed to bind to IIId-1 RNA as efficiently as to IIId-wt RNA,

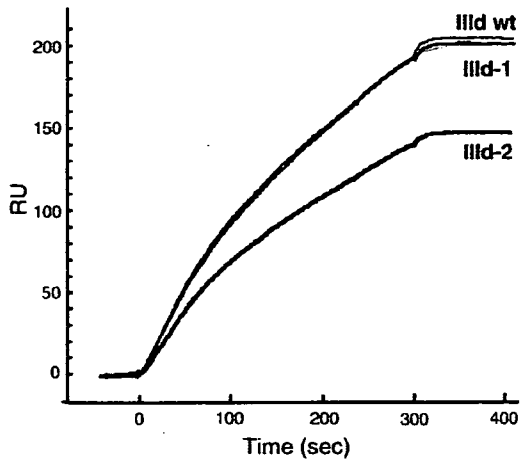

Fig. 5. Binding of the core protein to oligo RNAs corresponding to the mutated IIId domains. The real time binding between the core protein and wild-type (IIId wt) or mutants (IIId-1 and IIId-2) of the stem-loop IIId was examined. Biotinylated oligonucleotides were immobilized of the streptavidin pre-coated sensor chips followed by being exposed to 40 μl of the solution containing the core protein (4 μg/ml) with a flow rate of 8 μl/min. The sample flow was stopped, and the buffer washout began at 300 s. The amounts of immobilized synthetic oligonucleotides, IIId-wt, IIId-1, and IIId-2 were 211.0, 206.9, and 212.4 resonance units, respectively.

suggesting that RNA mutations disrupting the bulge loop structure have little or no effect on binding of the core protein. In contrast, a marked reduction in binding affinity of the core protein for mutant IIId-2 RNA was observed. As a negative control, we found that the core protein does not bind to oligo RNA corresponding to IIIe or IIIf domain (data not shown; Tanaka et al., 2000). It is likely that the apical loop sequence and/or the GGG triplet are important for RNA binding of the core protein, which is consistent with prior observations suggesting that the core protein binds to G-stretch sequence(s) with high affinity.

Combined with the data shown in Fig. 4B, the inhibitory effect of the core protein on HCV IRES activity correlates well with its ability to bind to wild-type and mutated IIId RNA. In light of the observation that the IIId domain is important for IRES activity and from suggestion that the domain IIId interacts with 40S (Otto et al., 2002; Jubin et al., 2000; Lukavsky et al., 2000; Spahn et al., 2001), the HCV core protein may inhibit viral IRES-dependent translation by preventing required interactions between RNA molecules and the 40S by binding to the IRES sequence including the apical loop of the IIId domain.

Role of basic-residue clusters within the core protein in inhibition of HCV translation

The amino-terminal portion of the core protein is able to bind to viral nucleic acids (Santolini et al., 1994). This region contains three clusters of arginine- and lysine-rich sequences (aa 5–13, 38–43, and 58–71). To investigate the role of these basic-residue clusters in inhibition of HCV translation by the core protein, we constructed a series of core mutants, in which lysine and arginine residues within one or more of the basic-residue clusters of the core protein were substituted with alanine residues, as depicted in Fig. 6A. Two days after transfection with either wild-type (pCAGC191) or core mutant (pCAGC191m1–m7) constructs, the cells were cotransfected with HCVLuc and capped-RLuc RNA. As indicated in Fig. 6B, core mutants containing alanine substitutions within one or two clusters (C191m1, m2, m3, m4, m5, and m6) retained the ability to inhibit HCV IRES-mediated translation, similar to the wild-type core protein. However, a core mutant with alanine substitutions involving all three clusters, C191m7, demonstrated little to no inhibition of translation. Expression of the core protein in each transfectant was determined by Western blotting (Fig. 6C), and none of the mutants influenced cap-dependent translation (data not shown). These results

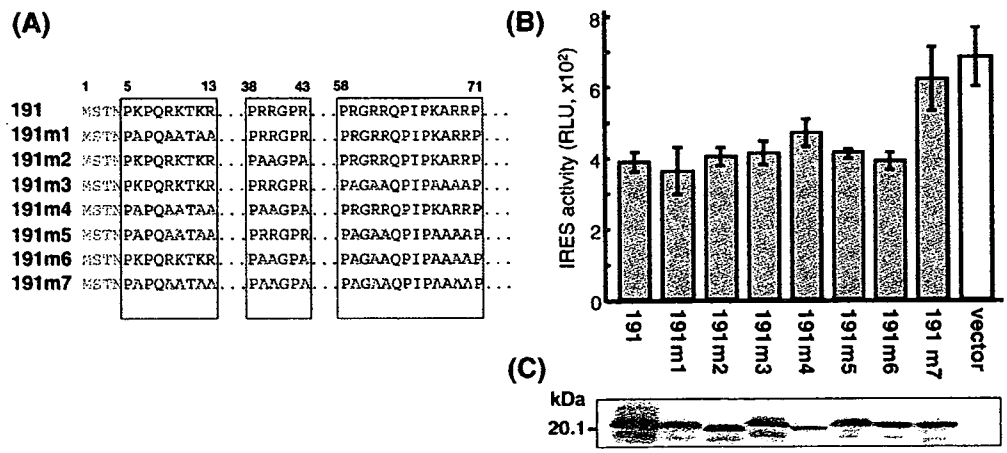

Fig. 6. A role of basic-residue clusters within the core protein in inhibition of the HCV translation. (A) Schematic representation of the mutated core proteins substituted in three basic aa clusters. Lysine or arginine residues substituted with alanine in the clusters are shown with outlined letters. (B) Two days after the transfection with either wild-type (191) or mutated (191m1–m7) core-expressing constructs, HepG2 cells were further cotransfected with HCVLuc and capped-RLuc RNAs. Relative luciferase activities (RLU) were determined as described in Materials and methods and the legend to Fig. 3. (C) The amounts of the wild-type and mutant core proteins expressed in HepG2 cells are shown by Western blotting.

suggest that all three basic-residue clusters of the core protein can mediate inhibition of HCV translation and that at least one cluster is required for inhibition.

Discussion

In this study, we investigated the mechanism by which the core protein modulates HCV IRES activity using an in vivo reporter assay and SPR technology. We demonstrated the importance of a stem-loop IIId domain, spanning nt 253–279, in core protein-mediated inhibition of HCV IRES-mediated translation. In a previous study, we demonstrated preferential binding of the core protein to domain IIId of the 5′UTR followed by domain I and a region spanning nt 23–41 (Fig. 1), upon examining 10 oligonucleotides corresponding to various structured domains of the viral 5′UTR (Tanaka et al., 2000). The core protein did not have an inhibitory effect on translation directed by mutated IRES lacking the IIId domain. However, translation initiated by IRES mutants with deletions of domain I or nt 23–41 was significantly inhibited by the core protein to a similar extent as wild-type IRES-mediated translation (Fig. 3B). Further mutational analysis was then used to determine whether specific IIId nucleotide sequences were important for inhibition of translation by the core protein. We determined that the GGG triplet (nt 266–268) within the IIId apical loop was most critical for core protein-mediated inhibition (Fig. 4B). Combined with the results of SPR analysis (Fig. 5), the data presented here suggest that inhibition of HCV IRES-directed translation by the core protein depends on the binding efficiency of the core protein for the viral IRES element.

Domain III, which is composed of six distinct regions containing stem-loop structures, forms the core of the HCV IRES and is essential for viral translation. Previous studies suggest that domain III plays a role in recruiting the 40S ribosomal subunit and eIF3 by direct interaction with stem-loops IIId/e/f and IIIb, respectively, even though the 40S subunit makes multiple interactions with the IRES and also binds to stem-loop II and the pseudoknot domain of the IRES element (Kieft et al., 2001; Kolupaeva et al., 2000; Sizova et al., 1998). Stem-loop IIId is a highly conserved region within domain III in most HCV isolates, consisting of two double-stranded helical elements separated by a 3-nt internal asymmetric loop with a 6-nt hairpin loop at the distal end of each helical region. IRES sequence deletions, including deletion of stem-loop IIId, as well as point mutations, inhibit binding of the 40S subunit and IRES function (Rijnbrand et al., 1995; Honda et al., 1996; Kieft et al., 1999). Specifically, substitution mutations of the GGG triplet within the IIId apical loop region produce significant loss of IRES activity, as well as alterations in RNA folding, indicating that the GGG triplet is a critical region for HCV translation (Kieft et al., 1999; Jubin et al., 2000). In addition, antisense 2′-*O*-methyloligonucleotides targeted to the IIId domain are known to compete with the 40S subunit for binding and to inhibit viral translation (Tallet-Lopez et al., 2003). Moreover, the secondary structure of the IIId domain is important for binding of the S9 ribosomal protein (Odreman-Macchioli et al., 2000). Consistent with these observations, we also observed that deletion of the IIId domain (ΔIIIdLuc), or a G-to-C substitution within the GGG triplet (IIId-2Luc), significantly reduced IRES activity.

Although the sequence of the IIId domain is highly conserved, sequence polymorphism of the helical region exists among the six major genotypes. With regard to nt 262 and nt 270 of the IIId domain, genotypes 1, 3, 4, and 5 of HCV encode U (nt 262) and C (nt 270), respectively. On the other hand, genotypes 2 and 6 encode C (nt 262) and U (nt 270), respectively. We observed that translation directed by the IRES sequence of genotypes 2 and 6 (2a2bLuc) was more efficient than that directed by the IRES sequence of genotypes 1, 3, 4, and 5 (HCVLuc) (Fig. 4B). Previous studies also demonstrated differences in the efficiency of IRES activity among different HCV genotypes and suggest that the 5′UTR of genotype 2(b) has the most marked IRES activity (Tsukiyama-Kohara et al., 1992; Kamoshita et al., 1997; Collier et al., 1998). Thus, sequence polymorphism involving the helical region of IIId might explain the observed variability in IRES activity when comparing the 5′UTR sequences of different HCV genotypes. Expression of the core protein inhibits HCV translation directed by 2a2bLuc to a similar or same extent as that directed by HCVLuc. This finding suggests that inhibition of viral translation by the core protein commonly occurs during the HCV life cycle and is not limited to certain genotypes. The deletion of the 5′-proximal stem-loop domain I (ΔILuc) significantly reduced IRES activity (data not shown), although the ability of the core protein to inhibit translation was retained (Fig. 3B). Published data regarding the role of domain I in inhibition of HCV translation are not consistent. Some researchers suggest that the 5′-proximal region containing domain I is not essential for HCV IRES activity (Honda et al., 1996; Kamoshita et al., 1997). However, other researchers suggest that this stem-loop element is required for optimal IRES-mediated HCV translation (Friebe et al., 2001; Fukushi et al., 1994; Luo et al., 2003). We compared HCV IRES activity mediated by monocistronic and bicistronic reporters with deletion of domain I and found that an inhibitory effect of the domain I deletion observed from the bicistronic reporter was less evident than that from the monocistronic one: the reduction in IRES activity caused by the deletion was 95% and 40% for the monocistronic and bicistronic constructs, respectively. Although similar trends were observed in the previous studies using cultured cells (Friebe et al., 2001; Luo et al., 2003; Kamoshita et al., 1997), in vitro transcription/translation studies demonstrated that the translational efficiency of the reporters deleted with domain I is higher than that of the wild-type (Honda et al., 1996; Kamoshita et al., 1997). It may be likely that differences in (1) gene constructs such as monocistronic and bicistronic reporters and (2) host cell conditions influence such inconsistent observations.

HCV core protein is highly basic, especially its N-terminal half, and it is thought to encapsulate the viral genome within a viral nucleocapsid. The RNA-binding domain of the core protein has been mapped to 75 aa residues within the N-terminal, in which three clusters of highly arginine/lysine-rich sequences are well conserved among HCV isolates (Santolini et

al., 1994). We previously observed preferential binding between the core protein and positive-stranded HCV RNA spanning the 5′UTR and part of the structural-protein coding region (nt 1–2327) (Shimoike et al., 1999). In this study, we demonstrated the importance of three basic aa residue clusters within the N-terminal region of the HCV core protein for its inhibitory effect on viral IRES activity. At least one cluster is required for inhibition of translation by the core protein. Previous studies with a series of deletion mutants suggest that aa 34–44 (Zhang et al., 2002) or aa 1–20 (Li et al., 2003) within the core protein are crucial for inhibition of translation initiated by HCV IRES. To investigate the contribution of these basic-residue-rich domains within the core protein to inhibition of viral translation, we employed substitution mutagenesis of the full-length core protein in order to reduce the occurrence of conformational changes in the core protein due to the introduction of mutations.

Although an increasing body of evidence shows involvement of the core protein in translational regulation, there are conflicting data regarding the exact mechanism by which this occurs. In contrast to studies describing direct inhibition of HCV translation by expression of the core protein (Shimoike et al., 1999; Zhang et al., 2002; Li et al., 2003), a recent report suggests that the core protein modulates HCV IRES function in a dose-dependent manner, with low amounts of the core protein producing up-regulation and greater amounts resulting in down-regulation (Boni et al., 2005). The core protein does not only inhibit translation initiated by the HCV IRES, but also cap-dependent translation and translation initiated by encephalomyocarditis virus (EMCV) IRES (Li et al., 2003). In an earlier study, neither cap- nor EMCV IRES-dependent translation were inhibited by expression of the core protein (Shimoike et al., 1999). Other studies suggest that the core protein-coding sequence, but not the core protein itself, modulates HCV IRES function, through a long-range RNA–RNA interaction (Wang et al., 2000; Kim et al., 2003). In the present experiment, however, down-regulation of HCV IRES-directed translation by the core protein-coding RNA sequences was eliminated by introducing a base-substitution mutation into the N-terminus of the core sequence in order to create a termination codon (Fig. 2). These contradictory findings might be due to different experimental conditions, such as the use of different reporter systems and host cells, as well as different levels of core protein in the assays used. To investigate the effect of the core protein on HCV IRES-dependent translation, we employed in vivo RNA transfection of monocistronic reporter constructs because HCV IRES is located at the 5′ end of the viral genome, and not internally, thus making it unnecessary to use a bicistronic reporter. Concerning bicistronic contexts, the possibility that the first cistronic sequence might influence IRES regulation directed by the second cistronic gene cannot be excluded. There is evidence to suggest that differences in translational regulation by the core protein might exist among different cell lines, including HepG2, Huh-7, and CV-1 cells (Wang et al., 2000; Li et al., 2003). We also observed differences between HepG2 and Huh-7 cells in terms of ability of the core protein to inhibit HCV IRES- and cap-dependent translation, which was not observed in Huh-7 cells (data not shown), as previously reported (Wang et al., 2000). Such cell-type specific effects might be related to differences in core protein expression since core protein expression by the recombinant baculovirus AcCA39 seems to be less abundant in Huh-7 cells, compared to HepG2 cells (data not shown). It is also possible that a cell-specific factor(s) are involved in translational regulation by the core protein. Thus, some interaction(s) between the highly ordered HCV IRES structure and/or the core protein and related host factors are likely cell-type-specific. Our previous report showed difference in the translation efficiency mediated by HCV IRES among human liver-derived cell lines, although the effect of the core protein on their translation was not determined (Aoki et al., 1998).

We performed the gel mobility shift assay to demonstrate the inhibition of the interaction between the HCV 5′UTR and the ribosome 40S subunit (40S). The complexes between purified 40S and the radiolabeled HCV 5′UTR (nt 1–330) were detected, and the amount of this band was decreased in the core protein-dose-dependent manner. In this condition, the core–5′UTR complex was competed with a non-labeled oligo RNA corresponding to IIId domain, but not with oligo RNAs of domain IV. However, the complex between the core protein and the 5′UTR was detected around the wells of the gel. To our knowledge, there has been no published data that in the gel mobility shift assay the core–5′UTR complex runs into the gel. Although these findings may support the idea that the core protein directly prevents binding of 40S to the HCV IRES, direct biochemical probing of the proposed interaction must wait for the advances in the protein chemistry of the HCV core protein.

Finally, based on the results of the present study and the existing literature, we propose a model of down-regulation of HCV translation mediated by the core protein (Fig. 7). In HCV-infected cells, the virus uncoats and releases its genomic RNA, which serves as a template for protein translation. Highly folded secondary and tertiary RNA elements in the 5′UTR function as *cis* signals for interaction with the 40S subunit and eIF3 during the initial process of HCV IRES-dependent translation. The high affinity interaction between HCV IRES and the 40S subunit is thought to be important for recruitment of the 43S particle to viral RNA, and the stem-loop IIId domain is a prerequisite for this interaction. Since the core protein binds most efficiently to the IIId domain in the HCV IRES element, it is relevant to note that the core protein may prevent an essential RNA–40S interaction by blocking the IIId domain, thereby reducing the viral translation efficiency. At an early stage of the HCV replication cycle, translation of the viral genome yields a polyprotein, which is subsequently processed to yield individual mature proteins. At a certain point, enough core protein is available to inhibit HCV translation by competing with the 40S subunit for IRES binding. Cells in which HCV translation is negatively controlled may have reduced levels of core protein due to its degradation by the ubiquitin/proteasome pathway (Suzuki et al., 2001; Moriishi et al., 2003), thereby decreasing the inhibitory effect of core protein. Thus, the core protein may

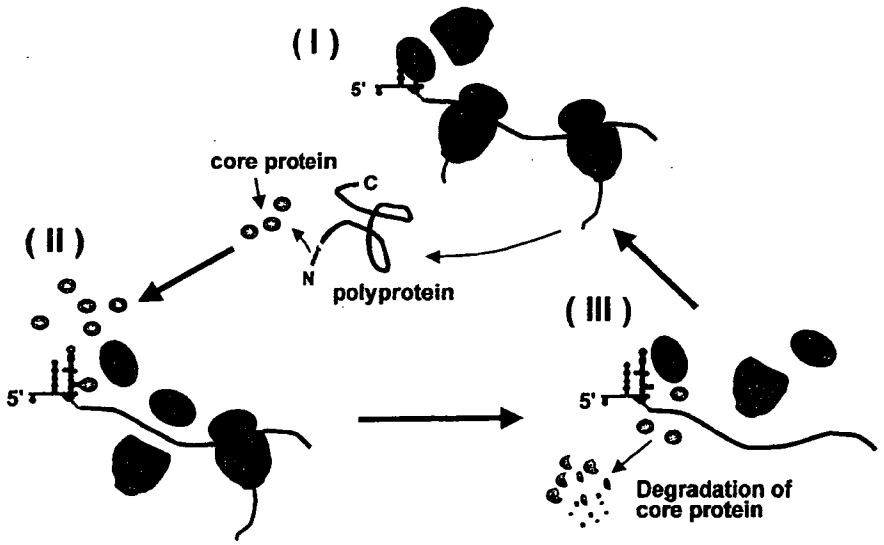

Fig. 7. Model for the regulation of HCV translation mediated by the core protein. Step 1: HCV translation is initiated through recognition of the 40S subunit and eIF3 by the IRES RNA tertiary structure. The viral polyprotein is expressed and processed into matured proteins, resulting in generation of the core protein. Step 2: The expressed core protein binds to the stem-loop IIId in the 5′UTR and inhibits the viral translation by competing with 40S subunit for binding to the IRES. Step 3: The reduced translational efficiency results in decreasing the levels of HCV proteins and replication. Degradation of the core protein through the ubiquitin/proteasome pathway may also contribute to reducing the amounts of the core protein in cells. A low concentration of the core protein possibly leads to recovery of the translational efficiency.

contribute, through its competitive interaction with the IRES IIId domain, to virus persistence by maintaining a low level of HCV replication.

Materials and methods

Plasmid construction

pT7ΔloopILuc (termed ΔILuc in this report), a 271-nt fragment containing a T7 promoter followed by nt 23–249 from the 5′ terminus of the HCV genome (clone NIHJ1; genotype 1b) (Aizaki et al., 1998), was amplified by PCR using pT7HCVLuc (HCVLuc) (Shimoike et al., 1999) as a template and primers *Hin*dIIIT7S (5′-CCCAAGCTTTAATACGACTCACTATACACTCCACCATAG-3′) and *Nhe*IAS (5′-CTAGCTAGCAGTCTCGCGCGGGG-3′). The PCR product was digested with both *Hin*dIII and *Nhe*I and ligated with a 5.3-kbp *Hin*dIII–*Nhe*I fragment of pT7HCVLuc. pT7Δ23–41Luc (Δ23–41Luc) was made using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in order to introduce a deletion of nt 23–41 from the 5′ terminus. The primers used for PCR were 23–41S (5′-CCTAGATTGGGGGCGACCCCTGTGAGGAAC-3′) and the 23–41 AS complement (5′-GTTCCTCACAGGGGTCGCCCCCAATCAGG-3′), and pT7HCVLuc was used as a template.

pT7ΔIIIdLuc (ΔIIIdLuc) was made by digestion of pT7HCVLuc with *Nhe*I and *Stu*I, thereby generating 30-bp (corresponding to the stem-loop IIId region), 1.8-kbp, and 3.8-kbp fragments. After this, the 1.8-kbp and 3.8-kbp fragments were isolated, blunt-ended, and then ligated.

pT72a2bLuc (2a2bLuc), pT7BVDVLuc (BVDVLuc), pT7IIId-1Luc (IIId-1Luc), and pT7IIId-2Luc (IIId-2Luc) were made as follows. pT7HCVLuc was partially digested with *Stu*I. A 5.6-kbp fragment was isolated and completely digested with *Nhe*I. The resulting fragment was ligated with annealed two partially complementary oligonucleotides with the following *Nhe*I and *Stu*I sites: 5′-CTAGCCGAGTAGTGTTGGGTCGCGACTAGG-3′ and 5′-CCTAGTCGCGACCCAACACTACTCGG-3′ for IIId-1,5′-CTAGCCGAGTAGTGTTCCCTCGCGAAAGG-3′ and 5′-CCTTTCGCGAGGGAACACTACTCGG-3′ for IIId-2, 5′-CTAGCCGAGTAGCGTTGGGTTGCGAAAGG-3′ and 5′-CCTTTCGCAACCCAACGCTACTCGG-3′ for 2a2b, and 5′-CTAGCCTGAGCGGGGGTCGCCCAGG-3′ and 5′-CCTGGGCGACCCCCGCTCAGG-3′ for BVDVLuc (the underlined nucleotides were substituted for the wild type nucleotides; see Fig. 4A).

pRLucHCVLuc, pRLucΔ23–41Luc, and pRLucΔIIIdLuc: 2.6-kb fragments were amplified by PCR using pT7HCVLuc, pT7Δ23–41Luc, and pT7ΔIIIdLuc as template DNAs, respectively, and primers *Xba*I 5′endS (5′-GCTCTAGAGCCAGCCCCCGATTGGGGGCGA) and *Xba*I 3′endAS (5′-GCTCTAGAACTAGTGGATCCGGAT). The PCR products were digested with *Xba*I and ligated with a 3.3-kb *Xba*I fragment of pRL-null Vector (Promega, Madison, WI).

pRLucΔILuc: 2.6-kb fragment was amplified by PCR using pT7ΔILuc as a template DNA and primers *Xba*IloopIS (5′-GCTCTAGACACTCCACCATAGATCACCCCC) and *Xba*I 3′endAS. The PCR product was digested with *Xba*I and ligated with a 3.3-kb *Xba*I fragment of pRL-null Vector.

pCAGC191 (Suzuki et al., 2001) carries nt 329–914, containing the entire HCV coding region of the core protein of clone HCV J1 (Aizaki et al., 1998), controlled by the CAG promoter. pCAGFS contains a frame shift mutation, involving substitution of A with T at nt 357, to make a stop codon (TAA)

(refer to Fig. 1). Only the first five residues (MSTNP) of the core protein are translated from this plasmid. To create a series of mutated core-expressing constructs: pCAGC191m1, -m2, -m3, -m4, -m5, -m6, and -m7, alanine substitutions were introduced into the basic-residue clusters of the core protein by PCR mutagenesis with primers containing base alterations, as described previously (Suzuki et al., 2005). The PCR products were then cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA) and verified by DNA sequencing. Individual cDNAs were excised and inserted separately into pCAGGS. The primer sequences used in these constructions are available from the authors upon request.

Cells

A human hepatocellular carcinoma cell line, HepG2, was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 50 μg/ml of Gentamycin (Biological Industries Ltd., Israel) and supplemented with 10% fetal calf serum.

RNA preparation

The reporter plasmids were linearized by digestion with adequate restriction enzymes, and the resulting DNA fragments were used as templates for in vitro transcription. HCVLuc and a series of HCVLuc mutants were linearized by digestion with *Xho*I. pRL-null (Promega, Madison WI) was linearized by *Xba*I digestion. pRLucHCVLuc and a series of pRLucHCVLuc mutants were linearized by *Bam*HI digestion. An in vitro transcription kit, MEGAscript (Ambion, Austin, TX), was used for RNA synthesis, during which reaction mixtures containing 1 μg of DNA template and 2 μl of T7 enzyme mix were incubated at 37 °C for 2 h. For capped RNA synthesis, linearized pRL-null, pRLucHCVLuc, and a series of pRLucHCVLuc mutants were used as templates, and 2 μl of each ATP, CTP, and UTP (7.5 mM), as well as 1 μl of GTP (7.5 mM) and 1 μl of cap homologue m7G (5′) ppp (5′) G (7.5 mM; Ambion), was used. The reaction mixtures were subsequently treated twice with 2 U of DNase I at 37 °C for 20 min followed by EDTA (25 mM) and lithium chloride (3.75 M) to terminate the reaction. Capped mRNA synthesized contained 11 nucleotides at 5′UTR and no poly(A) tail.

Transfection

For DNA transfection, 100 μl of Opti-MEM (Invitrogen Corp.) and 4 μl of TransIT-LT1 reagent (Mirus Corp., WI) were mixed and incubated at room temperature for 5 min followed by the addition of 2 μg of each plasmid expressing core protein, mutant core protein, or empty vector followed by incubation for a further 15 min. For RNA transfection, synthesized reporter RNA and 2.5 μl of Tfx-20 (Promega) were mixed in 100 μl of Opti-MEM and incubated for 15 min prior to transfection. One day prior to DNA transfection, cells (2.5×10^5) were seeded into a 12-well plate. The transfection mixture described above was added to the cells in 500 μl of Opti-MEM medium after the cells were washed twice with 500 μl of Opti-MEM.

Luciferase assay

The cells infected or transfected with a recombinant baculovirus or plasmid carrying the entire HCV core gene (AcCA39 or pCAGC191) or an empty vector (AcCAG or pCAGGS) were cultured for 2 days followed by transfection with reporter RNA, either HCVLuc (0.1 μg/well), ΔILuc (6.0 μg/well), Δ23–41Luc (0.2 μg/well), ΔIIIdLuc (6.0 μg/well), IIId-1 (0.1 μg/well), IIId-2 (6.0 μg/well), 2a2bLuc (0.1 μg/well), or BVDVLuc (0.1 μg/well), along with capped RL RNA (0.08 μg/well). After 6 h of incubation, FL and RL activities were determined using the Dual-Luciferase Reporter Assay System (Promega), as previously described (Aoki et al., 1998; Shimoike et al., 1999). Luminescent signals were measured with a TR717 luminometer (Applied Biosystems Japan Ltd., Tokyo, Japan).

Western blot analysis

Expression of HCV core protein was detected by Western blotting, as previously described (Shimoike et al., 1999). Briefly, protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Tokyo, Japan) after separation by SDS-PAGE. After blocking, the membranes were probed with a polyclonal antibody against glutathione-*S*-transferase core (aa 1–191) fusion protein, at a 1:100 dilution.

SPR experimental procedure

To prepare the core protein, insect Tn5 cells were infected with a recombinant baculovirus Ac39. The core protein was partially purified from the cell lysate, as previously described (Tanaka et al., 2000). Interactions between the core protein and synthetic RNA oligonucleotides were examined by SPR analyses with BIAcore 2000 (Biacore K.K., Tokyo, Japan). The SPR experimental procedure was as previously described (Tanaka et al., 2000). Briefly, a biotinylated oligonucleotide spanning nt 251–282 (IIId-wt) and mutant IIId domains (IIId-1 and IIId-2) (Fig. 4A) were synthesized followed by immobilization on streptavidin pre-coated sensor chips. Forty microliters of solution containing the core protein (4 μg/ml) was injected onto the sensor chip surface at a flow rate of 8 μl/min. The sample flow was stopped, and buffer washout started at 5 min post-injection.

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Molecular Determinants for Subcellular Localization of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is a putative nucleocapsid protein with a number of regulatory functions. In tissue culture cells, HCV core protein is mainly located at the endoplasmic reticulum as well as mitochondria and lipid droplets within the cytoplasm. However, it is also detected in the nucleus in some cells. To elucidate the mechanisms by which cellular trafficking of the protein is controlled, we performed subcellular fractionation experiments and used confocal microscopy to examine the distribution of heterologously expressed fusion proteins involving various deletions and point mutations of the HCV core combined with green fluorescent proteins. We demonstrated that a region spanning amino acids 112 to 152 can mediate association of the core protein not only with the ER but also with the mitochondrial outer membrane. This region contains an 18-amino-acid motif which is predicted to form an amphipathic α-helix structure. With regard to the nuclear targeting of the core protein, we identified a novel bipartite nuclear localization signal, which requires two out of three basic-residue clusters for efficient nuclear translocation, possibly by occupying binding sites on importin-α. Differences in the cellular trafficking of HCV core protein, achieved and maintained by multiple targeting functions as mentioned above, may in part regulate the diverse range of biological roles of the core protein.

Hepatitis C virus (HCV), the most important causative agent of posttransfusion and sporadic non-A, non-B hepatitis, is a positive-stranded RNA virus belonging to the family *Flaviviridae* (7). A precursor polyprotein of about 3,000 amino acids is encoded by a large open reading frame of the genome and undergoes cellular and viral protease-mediated posttranslational modification to produce a series of structural and nonstructural proteins (8, 13, 16).

HCV core protein, which is derived from the N terminus of the viral polyprotein, forms multimers and interacts physically with the viral RNA to constitute the nucleocapsid (28, 47, 50). Tissue transglutaminase is responsible for stabilizing the core protein by cross-linking it into a dimeric form (26). In addition, the core viral protein has properties which enable it to modulate a number of cellular processes, including transcription, inhibition or stimulation of apoptosis, and suppression of host immunity, as reviewed previously (21, 29, 51, 52). Several studies suggest that expression of the core protein affects mitochondrial function and lipid metabolism. The core protein increases the cellular production of reactive oxygen species with subsequent increases in lipid peroxidation (35, 39). The viral protein also colocalizes with human apolipoprotein AII, associates with lipid droplets, and has the capacity to influence metabolic events involving lipid storage (2, 17, 30, 36, 44). In addition, the core protein reduces microsomal triglyceride transfer, leading to defects in very low density lipoprotein assembly and secretion (40). Furthermore, the HCV core protein has transforming potential in some cells under certain conditions (5, 42). Transgenic mice expressing this protein in the liver develop hepatic steatosis due to increased oxidative stress in the absence of inflammation, with subsequent development of hepatocellular carcinoma (34, 36). These results suggest that the HCV core protein might play a pivotal role in the pathogenesis of hepatitis C in addition to its role as a structural component of the viral capsid.

The amino acid sequence of the core protein is well conserved among different HCV isolates and genotypes compared to other HCV proteins. The N-terminal domain of the HCV core protein is highly basic, while its C terminus is hydrophobic. Although several core proteins of various sizes exist (17 to 23 kDa) (15, 23, 25, 49, 56), two processing events result in the predominant production of a 21-kDa core protein. Both of these events utilize the endoplasmic reticulum (ER). The first one is to be cleaved from downstream envelope protein E1 at position 191, where the C-terminal hydrophobic domain serves as a putative signal peptide sequence. Subsequently, the signal sequence of 13 or 18 residues is processed by signal peptide peptidase (19, 23, 56).

The HCV core protein is found primarily within the membranes of cytoplasmic organelles, but it is also found in the nucleus (23, 48, 56). Immunofluorescence studies show a punc-

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tate pattern, consistent with ER localization, as well as perinuclear localization (15, 24, 32, 46, 56). Some studies suggest direct effects of the core protein on mitochondrial function. In fact, the core protein localizes to the mitochondria (34, 39). The N-terminal domain of the core protein contains three stretches of arginine- and lysine-rich sequences. Translocation of the core protein to the nucleus, mediated by these basic-residue stretches which function as nuclear localization signals (NLSs), is observed (6, 48). In addition, Moriishi et al. demonstrated that the N-terminal region of the core protein is also essential for nuclear retention through its interaction with the proteasome activator PA28γ (33).

In this study, we found a region that is important for localization of the mature core protein to the ER and to the mitochondrial outer membrane. We also identified a novel bipartite NLS responsible for nuclear targeting of the core protein, presumably via an importin-dependent pathway.

MATERIALS AND METHODS

Plasmid construction. The construction of a plasmid expressing the full-length core protein of 191 amino acids, pCAGC191, was described previously (49). pGFP, a construct expressing green fluorescent protein (GFP) with a C-terminal Myc epitope tag sequences, was prepared as follows. pCMV/Myc/mito/GFP (Invitrogen Corp., Carlsbad, Calif.) was digested with PmlI, followed by treatment with the Klenow fragment of DNA polymerase I. The resultant linear fragment was ligated to a PstI linker (GCTGCAGC) and digested with PstI to remove the mitochondrial targeting signal sequence, followed by self-ligation. A series of HCV core-GFP fusion constructs were made by amplifying the core gene fragments with PCR with primers containing Flag epitope tag sequences (sense) and a PstI site (both). After digestion with PstI, the segments were inserted into the PstI site of pGFP. A series of GFP-core-E1 fusion constructs were made by amplifying core and E1 gene fragments with PCR with primers containing a NotI site. After digestion with NotI, the segments were inserted into the NotI site of pGFP.

pGEX-4T-1 (Amersham Bioscience Corp., Piscataway, N.J.) was used to express core protein fused with glutathione *S*-transferase (GST) in *Escherichia coli*. Core cDNA fragments encoding amino acids 1 to 71 were inserted into the EcoRI site of pGEX-4T-1. Alanine substitutions were introduced into the core protein by PCR mutagenesis with primers containing base alterations. The PCR products were then cloned into pCR2.1 (Invitrogen Corp.) and verified by DNA sequencing. Individual cDNAs were excised and inserted separately into pGFP or pGEX-4T-1. The primer sequences used in this study are available from the authors upon request.

Plasmid pRSET-hSRP1α (54), containing importin-α cDNA under the control of a T7 promoter, was kindly provided by Karsten Weis (University of California, Berkeley). A cDNA clone of importin-α possessing 14 residues (MYPYDVP DYGGGGS), derived in part from the hemagglutinin (HA) tag at the N terminus, was constructed by PCR. The resultant linear fragment was inserted under the control of a CAG promoter of pCAGGS and designated pCAG-HA-imp.

Cell culture and transfection. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Monolayers of 293T cells were transfected with plasmid DNA in the presence of Lipofectamine (Gibco-BRL, Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions.

Confocal immunofluorescence microscopy. Transfected cells were grown on glass coverslips. Two days after transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. Intracellular localization of HCV core-GFP fusion proteins was visualized in cells transfected with a variety of GFP fusion constructs.

In order to detect the HCV core protein by immunofluorescence, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min at room temperature, followed by blocking with a nonfat milk solution (Block Ace; Snow Brand Milk Products Co., Sapporo, Japan). The cells were then incubated with anticore monoclonal antibody B2 (Anogen, Mississauga, Canada) for 60 min at room temperature, followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (IgG) (ICN Pharmaceuticals, Aurora, Ohio) for 45 min. To visualize mitochondria, MitoTracker Red CM-H_2XRos (Molecular Probes, Eugene, Oreg.) was added to the culture medium to a final concentration of 100 nM and incubated for 120 min at 37°C prior to fixation. To visualize the ER, goat anticalregulin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) and rhodamine-conjugated rabbit anti-goat IgG (ICN Pharmaceuticals) were used as the first and second antibodies, respectively. To visualize HA-importin-α, mouse anti-HA antibody (Roche Molecular Biochemicals, Indianapolis, Ind.) and rhodamine-conjugated goat anti-mouse IgG (ICN Pharmaceuticals) were used as the first and second antibodies, respectively. All specimens were examined with an LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron microscopy. Cells were transfected as described above. After 2 days, cells were fixed with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4). Free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. The cell pellets were embedded at progressively lower temperatures (down to −35°C) in Lowicryl k4M according to an established protocol (43). Ultrathin sections were prepared and mounted on carbon-coated nickel grids. To perform electron microscopy, Lowicryl k4M ultrathin sections, mounted on grids, were floated on a droplet of PBS containing 1% bovine serum albumin, 0.1% Triton X-100, and 0.1% Tween 20 for 10 min, after which they were exposed to droplets of mouse anticore monoclonal antibody (Anogen) diluted in PBS for 45 min. Following this, they were rinsed twice for 5 min each in PBS and incubated with anti-mouse IgG-coated 10-nm immunogold particles (British Biocell, Cardiff, United Kingdom) for 45 min. After rinsing with PBS and distilled water, the grids and embedded sections were air dried and exposed to uranyl and lead acetate contrast agents.

Subcellular fractionation. All steps were performed at 4°C in the presence of a protease inhibitor cocktail called Complete (Roche Molecular Biochemicals). To isolate the ER fraction, transfected cells were washed with PBS, lysed in homogenization buffer A (50 mM Tris-HCl [pH 8.0], 1 mM β-mercaptoethanol, 1 mM EDTA, and 0.32 M sucrose), and then centrifuged at 5,000 × *g* for 10 min. The supernatant was then collected and centrifuged at 105,000 × *g* for 1 h. The pellet was disrupted in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 10 mM sodium fluoride), after which it was centrifuged at 15,000 × *g* for 20 min. The resulting supernatant was used as the ER fraction.

To isolate the mitochondrial fraction, transfected cells were washed with PBS and homogenized in ice-cold homogenization buffer B (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl) at pH 7.4. The supernatant was then centrifuged at 1,000 × *g* for 10 min to remove large debris and nuclei. The resulting supernatant was then centrifuged at 20,000 × *g* for 20 min to obtain crude mitochondria. The crude mitochondria pellet was subfractionated in Nycodenz gradients for further purification of mitochondria. Nycodenz (Axis-Shield PoC AS, Oslo, Norway) solution at 50% (wt/vol) was prepared in buffer containing 5 mM Tris-HCl and 1 mM EDTA at pH 7.4. This stock solution was then diluted with buffer containing 0.25 M sucrose, 5 mM Tris-HCl, and 1 mM EDTA at pH 7.4 before use. The crude mitochondrial pellets was suspended in 4 ml of 25% Nycodenz solution and overlaid onto the following discontinuous Nycodenz gradients: 1 ml of 40%, 1 ml of 34%, and 2 ml of 30%. The samples were topped off with 2 ml of 23% Nycodenz solution after placement onto the discontinuous gradients. The tubes were then centrifuged at 52,000 × *g* for 90 min. The dense band seen after centrifugation at the 25 to 30% interface was recovered as the purified mitochondrial fraction.

To determine the submitochondrial localization pattern of the core protein, mitochondria were resuspended in SH buffer (0.6 M sorbitol and 20 mM HEPES-KOH [pH 7.2]) in the absence or presence of 30 μg of proteinase K per ml after purification by Nycodenz density gradient centrifugation. Samples were incubated for 30 min at 0°C, after which protease digestion was halted by the addition of *p*-aminophenyl methanesulfonyl fluoride hydrochloride (*p*-APMSF) (5 mM). Proteins lysed in sodium dodecyl sulfate (SDS) sample buffer were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted as described below.

Immunoblot analysis. The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Tokyo, Japan) after separation by SDS-PAGE. After blocking, the membranes were probed with monoclonal- or polyclonal-antibody against core protein (Anogen), prohibitin (Neo Markers, Fremont, Calif.), ribophorin I (Santa Cruz Biotechnology), translocase of the outer membrane (Tom) 20 (Santa Cruz Biotechnology), translocase of the inner membrane (Tim) 17 (Santa Cruz Biotechnology), or GFP (Santa Cruz Biotechnology). Immunoblots were developed as previously described (15).

GST pulldown assay. *Escherichia coli* BL21 cells were transformed with GST-core fusion plasmids and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 h. Bacteria were harvested, suspended in lysis buffer (1% Triton X-100 in PBS), and soni-

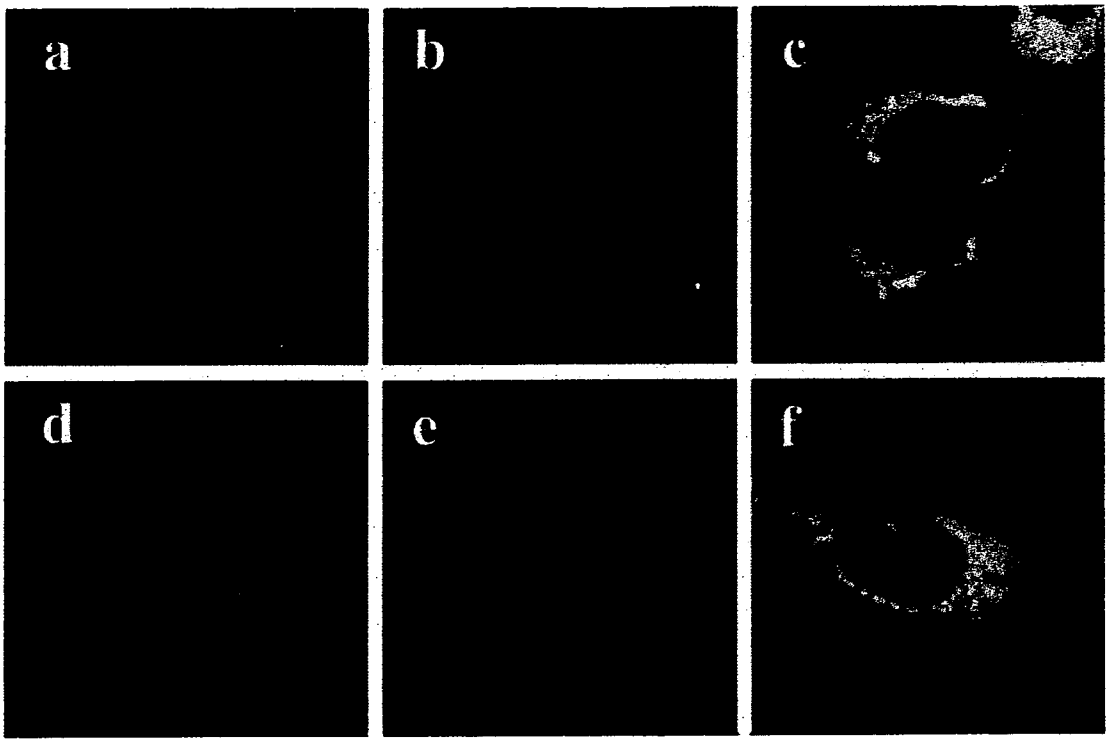

FIG. 1. Confocal analysis of double staining for HCV core protein and ER or mitochondria. 293T cells transfected with full-length HCV core expression plasmid, pCAGC191 were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c) or fixed after loading with Mitotracker (d to f). After permeabilization with Triton X-100, cells were subjected to immunofluorescence staining with a mouse anticore antibody. A goat anticalregulin antibody was used for ER staining. The green signals corresponding to the core were found with a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (a and d). The red signals corresponding to the ER were obtained with a rhodamine-conjugated rabbit anti-goat IgG secondary antibody (b). Mitochondria were stained with the mitochondrion-selective dye Mitotracker (e). Overlay resulted in yellow signals indicative of colocalization (c and f).

cated on ice. GST and GST fusion proteins were purified from bacterial lysates with glutathione-Sepharose beads (Amersham Bioscience Corp.). The beads were washed four times with lysis buffer. Approximately equal amounts of purified protein, as estimated by Coomassie brilliant blue staining, were used for the binding assays. For pulldown assays, in vitro transcription and translation of importin-α was done with pRSET-hSRP1α and the TNT-coupled reticulocyte lysate system (Promega Corp., Madison, Wis.) with T7 RNA polymerase. The reaction was carried out at 30°C for 4 h in the presence of [^{35}S]methionine/cysteine (ICN Pharmaceuticals). The translation product was then incubated with glutathione-Sepharose beads bound to GST fusion proteins in 1 ml of binding buffer (40 mM HEPES [pH 7.5], 100 mM KCl, 0.1% NP-40, and 20 mM 2-mercaptoethanol) at 4°C for 1 h. The beads were washed four times with binding buffer, and the pulldown complexes were separated by SDS-PAGE on 15% polyacrylamide gels. The gels were then fixed, dried, and analyzed with autoradiography.

RESULTS

Subcellular localization of HCV core protein. To assess the subcellular localization of HCV core protein, we first analyzed cells transfected with a full-length core-expressing construct by confocal microscopy. In accordance with previous observations (2, 15, 32, 45, 56), a granular cytoplasmic staining pattern of the core protein was observed in 293T (Fig. 1) and human hepatoblastoma HepG2 (data not shown) cells. Dual staining of transfected cells with antibody against the ER protein calregulin along with anticore antibody confirmed the ER localization of the core protein (Fig. 1a, b, and c show the core,

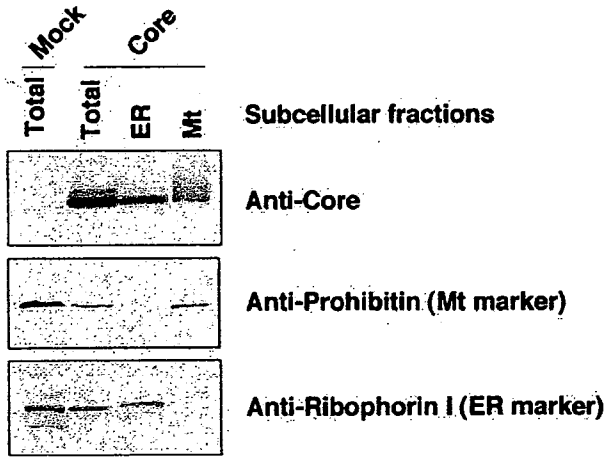

FIG. 2. Subcellular distribution of HCV core protein analyzed by immunoblotting. ER and mitochondrial (Mt) fractions were isolated from 293T cells expressing the full-length core protein (Core) or nontransfected cells (Mock) 2 days after transfection. Equal amounts of protein from each fraction as well as whole cell lysates (Total) were subjected to immunoblotting with a monoclonal antibody against either HCV core, prohibitin, or ribophorin I.

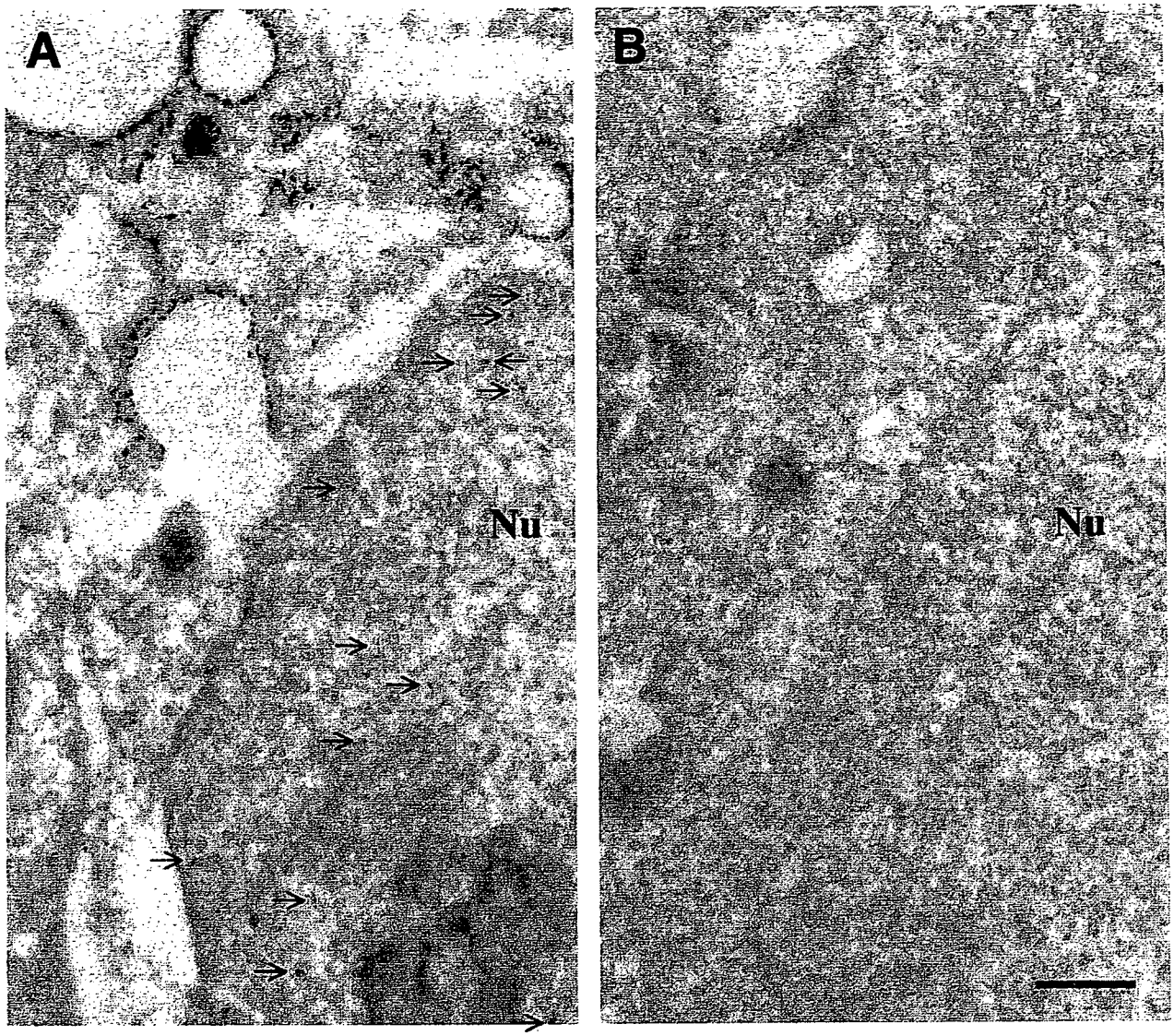

FIG. 3. Immunoelectron microscopy of HCV core protein. 293T cells expressing the full-length core protein (A) and nonexpressing cells (B) fixed 2 days after transfection. Immunoelectron microscopic analysis was performed with a mouse anticore antibody and a secondary anti-mouse IgG conjugated with gold particles. The arrows indicate the core protein localized in the nucleus (Nu). Bar, 500 nm.

calregulin, and a merged image, respectively). The pattern of subcellular localization of the core protein (Fig. 1d) was compared to the distribution of mitochondria, as revealed by MitoTracker staining (Fig. 1e). Although distribution of the core protein was not completely identical with that of the mitochondrion-selective dye, overlapping staining was observed, particularly in the perinuclear region (Fig. 1f).

Intracellular localization of the core protein was further examined in 293T cells by subcellular fractionation and Western blotting. The core protein was present in both the ER and mitochondrial fractions (Fig. 2), while it was not detected in the cytosol fraction (data not shown). The purity of the ER and mitochondrial fractions was confirmed with antibodies against ribophorin I as an ER marker and prohibitin as a mitochondrial marker.

It is generally difficult to identify the nuclear distribution of proteins of interest due to contamination of the nuclear preparation with unbroken, intact cells. Thus, to investigate whether the core protein localizes to the nucleus, we examined transfected cells by immunoelectron microscopy. Although gold particles were primarily observed within cytoplasmic membranes, perhaps highlighting the ER, immunoreactivity to anticore antibody was also observed in the nucleus (Fig. 3A, arrows). In contrast, no antibody labeling was observed in cells transfected with an empty vector (Fig. 3B).

Thus, HCV core protein predominates in the cytoplasm in a membrane-associated form(s) with ER and mitochondria, but nuclear localization is also observed.

Regions responsible for directing core protein to the ER and mitochondria. Given the tendency of the core protein to lo-

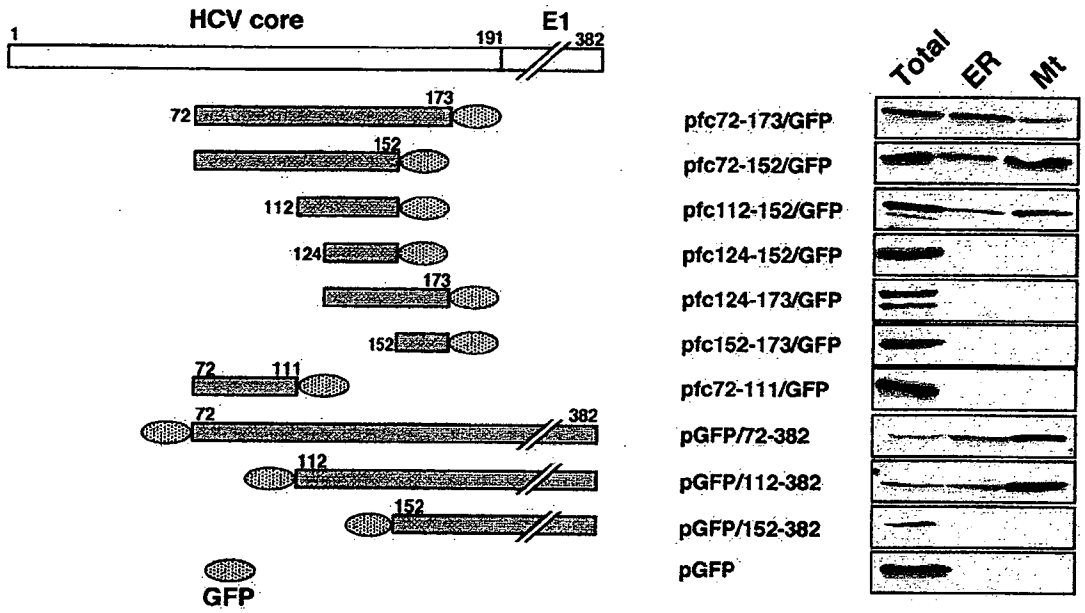

FIG. 4. Identification of segments that mediate association with ER and mitochondria in the core protein. Schematic diagram (left) and nomenclature (middle) of the core-GFP fusions are shown. Gray bars, expressed core and E1 regions. Subcellular distribution of fusion proteins is indicated on the right. ER and mitochondrial (Mt) fractions as well as whole-cell lysates (Total) were subjected to immunoblotting with an anti-GFP antibody.

calize to the ER and to mitochondria, we next investigated whether specific sequences might be responsible for transporting the core protein to these organelles. Fusion proteins between different regions of the core protein and GFP were developed, with specific emphasis on the region downstream of amino acid 72 because this region contains clusters of hydrophobic amino acids and the N-terminal 71 residues of the core are known to play a role in nuclear targeting (6, 48).

Western blotting of subcellular fractions with anti-GFP antibody revealed the localization of a core (72–173)-GFP fusion protein to the ER and to mitochondria (Fig. 4). Fusion proteins containing GFP and core proteins with N- or C-terminal deletions (72-152-GFP and 112-152-GFP) were likewise identified within the ER and mitochondrial fractions. In contrast, the ER and mitochondrial fractions did not contain GFP fusion proteins containing core protein amino acids 124 to 152, 124 to 173, 152 to 173, or 72 to 111. These fusion proteins demonstrated distribution profiles similar to that of GFP alone. We also tested GFP-core-E1 fusions, which are processed at the C terminus of the core by signal peptidase and signal peptide peptidase (19, 30). GFP-core fusions expressed from pGFP/72–382 and pGFP/112–382 were detected in the ER and mitochondrial fractions. The fusion expressed from pGFP/152–382 was not identified in these fractions.

We further analyzed subcellular localization of the fusion proteins by confocal immunofluorescence microscopy (Fig. 5). As expected, fusions of (72–173)-GFP and (112–152)-GFP exhibited localization to the ER and mitochondria. The patterns of subcellular localization of these fusions are indistinguishable from that of the full-length core protein, as shown in Fig. 1. Expression of (124–152)-GFP or (112–123)-GFP resulted in widespread diffusion of the fusion in the cell. Thus, these results indicate that the region spanning amino acids 112 to 152 can mediate association of the core protein not only to the ER but also to the mitochondria.

We subsequently examined the submitochondrial localization of the core protein with a protease protection assay. As shown in Fig. 6A, HCV core protein localized in the mitochondria was completely digested upon treatment with proteinase K for 30 min at 0°C. Under identical conditions, a marker specific for the mitochondrial outer membrane, Tom20, was also observed to disappear, whereas digestion of a mitochondrial inner membrane marker, Tim17, was not observed. These findings confirm that HCV core protein is localized to the mitochondrial outer membrane.

The predicted secondary structure of the region, amino acids 72 to 173, is shown in Fig. 6B. The presence of a long helical segment, lying between amino acids 116 and 134, and two short α-helices (amino acids 146 to 152 and amino acids 155 to 159) were predicted. The results of the cell fractionation assay and confocal microscopy with a series of deletion mutants shown in Fig. 4 and 5 suggest that an α-helix between amino acids 116 and 134 may be required for associating the core protein with the ER and the mitochondrial outer membrane. When amino acids 117 to 134 are portrayed as a helical wheel, we found an amphipathic structure with hydrophobic residues on one side and polar residues on the other side of the α-helix (Fig. 6C), which is often observed in membrane-associated proteins. This helical conformation might be important for directing the core protein to the ER and mitochondrial outer membranes.

Nuclear localization of the HCV core protein is mediated by a bipartite NLS, possibly via an importin-dependent pathway. Although HCV core protein is mainly localized within the cytoplasm, it is also found in the nucleus, as shown in Fig. 3.

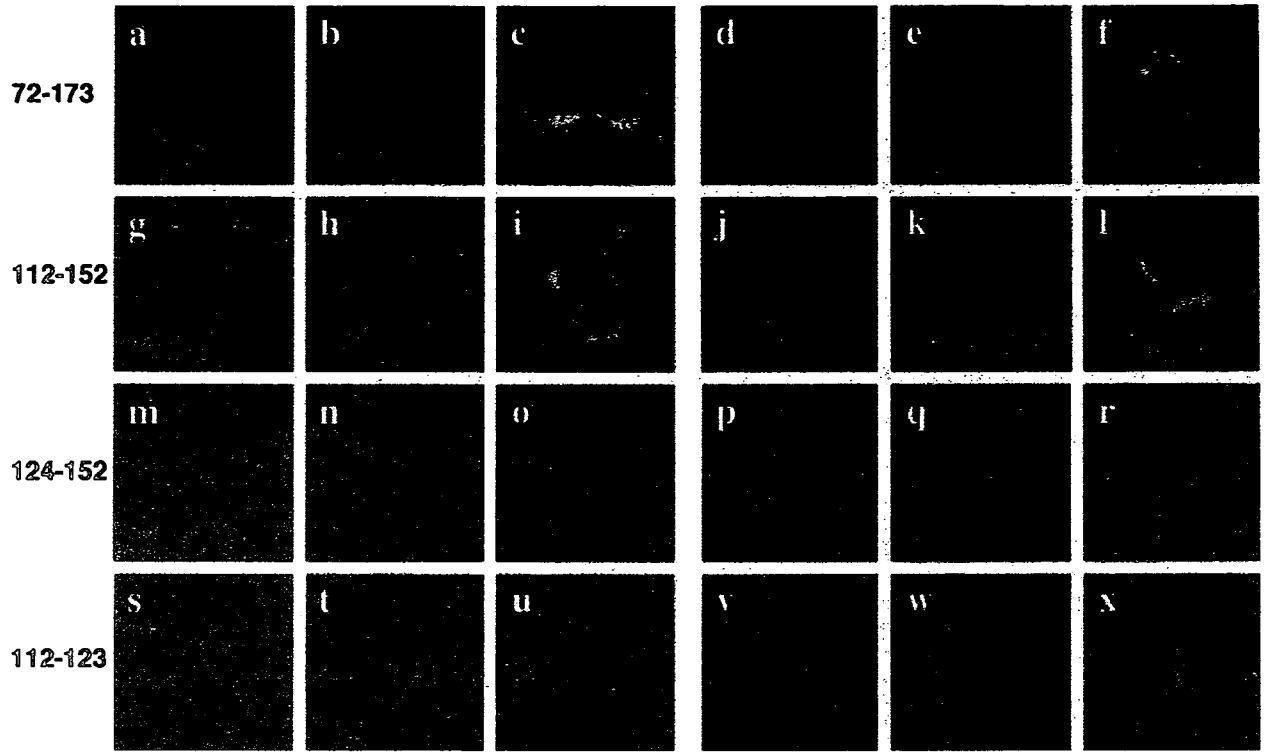

FIG. 5. Confocal analysis of double staining for core-GFP fusion protein and ER or mitochondria. 293T cells transfected with core-GFP expression plasmids (72–173, 112–152, 124–152, and 112–123) were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c, g to i, m to o, and s to u) or fixed after loading with Mitotracker (d to f, j to l, p to r, and v to x). After permeabilization with Triton X-100, a goat anticalregulin antibody was used for ER staining. The red signals corresponding to the ER were obtained with a rhodamine-conjugated rabbit anti-goat IgG secondary antibody (b, h, n, and t). Mitochondria were stained with the mitochondrion-selective dye Mitotracker (e, k, q, and w). Overlay resulted in yellow signals indicative of colocalization (c, f, i, l, o, r, u, and x).

The results of previous studies demonstrate that the N-terminal region of the core protein is responsible for nuclear targeting. It contains three clusters of basic amino acid residues that represent putative consensus motifs for NLS sequences PKPQRKTKR (amino acids 5 to 13), PRRGPR (amino acids 38 to 43), and PRGRRQPIPKARRP (amino acids 58 to 71) (6, 48). Nuclear targeting is generally governed by a family of transporters or cytosolic receptor proteins, known as importins or karyopherins, which function in concert with a guanine nucleotide-binding protein named Ran and other regulatory proteins such as NTF2/p10. Conventional NLS-dependent nuclear targeting occurs when importin-α recognizes the NLS sequence, mediating binding to importin-β1, after which the trimeric complex translocates to the nucleus (12).

In order to determine whether the putative NLS motifs identified within the core protein sequence are capable of binding to importin-α, we examined the in vitro interaction between bacterially expressed GST-fused core protein and ^{35}S-labeled importin-α with a GST pulldown assay. We then substituted lysine and arginine residues of one or more of the putative NLS motifs of the core protein (all contained within the first 71 amino acids of the N terminus) with alanine and fused the resultant constructs with GST, as shown schematically in Fig. 7A. As shown in Fig. 7B (upper panel), importin-α was pulled down by a GST fusion protein containing wild-type core (amino acids 1 to 71) protein but not with GST alone, suggesting that direct binding occurs between the core protein and importin-α. Importin-α was also pulled down by GST-core fusion proteins containing substitutions in one or two NLS motifs (NLS/m1, NLS/m2, NLS/m3, NLS/m4, NLS/m5, and NLS/m6). However, importin-α was not pulled down by GST-core fusion proteins containing alanine substitutions in all three NLS motifs (NLS/m7). It should be noted that similar amounts of GST fusion proteins were used for each of the in vitro pulldown assays, followed by SDS-PAGE and Coomassie brilliant blue staining (Fig. 7B, lower panel). These results demonstrated that all three putative NLS motifs of the N-terminal region of the core protein can mediate binding to importin-α, which suggests that nuclear translocation of the core protein occurs via an importin-dependent pathway (12).

The interaction between the core and importin-α was further analyzed by a colocalization assay (Fig. 7C). The GFP fusion containing the wild-type core (amino acids 1 to 71) was well colocalized with HA-importin-α; distribution of the two proteins showed similar nuclear staining patterns, confirming the presence of a functional NLS sequence(s) within the core protein. In contrast, NLS/m4, with substitutions in two NLS motifs, was partly colocalized with HA-importin-α near or around the nuclear membrane, suggesting that NLS motif double mutants bind to importin-α but their binding efficiency is lower than that of wild-type core protein.

Finally, we examined the subcellular localization of core

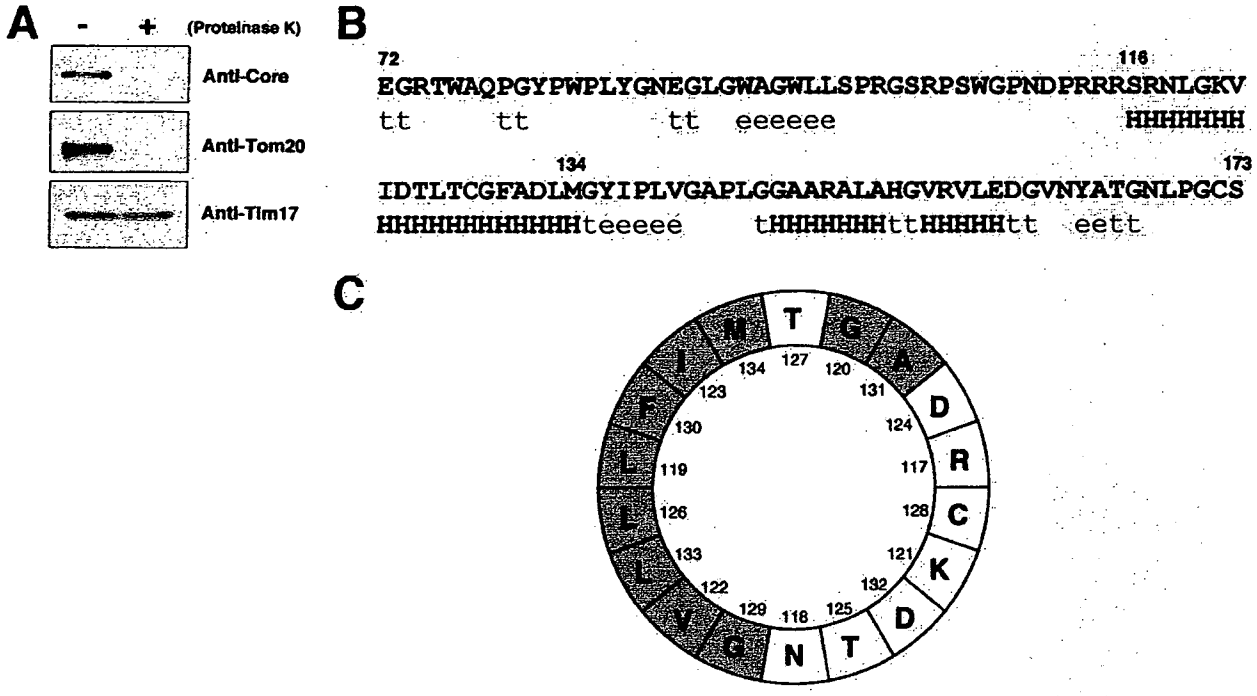

FIG. 6. (A) Protease protection assay. A mitochondrial fraction isolated from cells expressing the core protein was treated with proteinase K (+) as described in Materials and methods. The sample as well as the nontreated fraction (−) were subjected to immunoblotting with a monoclonal antibody against either HCV core, Tom20, or Tim17. (B) Protein sequence and predicted secondary structure of HCV core, amino acids 72 to 173. The secondary structure prediction was obtained with the self-optimized prediction method, a computer program on the internet (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa_sopm.html). H, α-helix; t, turn; e, extension. (C) α-Helical plot of amino acids 117 to 134 of the core protein. In the helical wheel plots, the gray shading represents apolar and hydrophobic residues; and the white represents polar residues.

protein expressed by the wild-type and NLS mutants (Fig. 7D). As expected, a fusion protein containing wild-type core protein (amino acids 1 to 71) and GFP was localized exclusively to the nucleus. Core proteins from three fusion proteins containing substitutions in each NLS motif (NLS/m1, NLS/m2, and NLS/m3) were detected primarily in the nucleus. Weak fluorescence was also observed in the cytoplasm, suggesting that these mutations caused a slight reduction in the efficiency of nuclear translocation. On the other hand, two or three NLS motif substitution mutations (NLS/m4, NLS/m5, NLS/m6, and NLS/m7) completely abolished nuclear translocation, resulting in a diffuse distribution of core protein, similar to that of GFP alone. Although it is likely that all three putative NLS motifs play a role, the above results suggest that at least two of the three putative NLS motifs are prerequisite for efficient nuclear translocation of the core protein.

DISCUSSION

HCV core protein is released from the viral polyprotein by a host protease(s) within the ER membrane at a signal peptide sequence lying between the core and envelope (E1) proteins (16, 41). Subsequently, the signal peptide is further processed by an intramembranous protease called signal peptide peptidase (38, 53). This mature form of the core protein is then released and undergoes subcellular trafficking (30, 53). The core protein localizes mainly to the ER, mitochondria, and lipid droplets. Some reports also describe localization of the core protein to the nuclei of hepatocytes in HCV-infected patients (10), transgenic mice (34), and cultured cells expressing viral polyproteins (56). Although it has been reported which sequence motifs are responsible for localization of the HCV core protein to lipid droplets and nuclei, it is uncertain which sequences target the core protein to the ER and to mitochondria. In this study, we identified sequences related to localization of the mature core protein to the ER and to mitochondria.

Through heterologous expression of core-GFP fusion proteins containing a series of deletions, we determined that a sequence extending from amino acids 112 to 152 of the core protein is required for its localization at the mitochondrial outer membrane. Translocation of nucleus-encoded mitochondrial proteins is usually dependent on N-terminal sequences, referred to as mitochondrial targeting sequences (37). However, it is also true that a significant proportion of mitochondrial proteins lack these N-terminal mitochondrial targeting sequences. Specifically, a number of outer membrane proteins do not have cleavable sequences at their N termini; rather, they are targeted to mitochondria by means of internal or C-terminal signals (31).

Since it has been reported that amino acid sequences required for targeting to the outer mitochondrial membrane form a highly hydrophobic α-helical wheel, as seen in A-kinase associated protein 84/12 (4) and NADH-cytochrome *b* reduc-

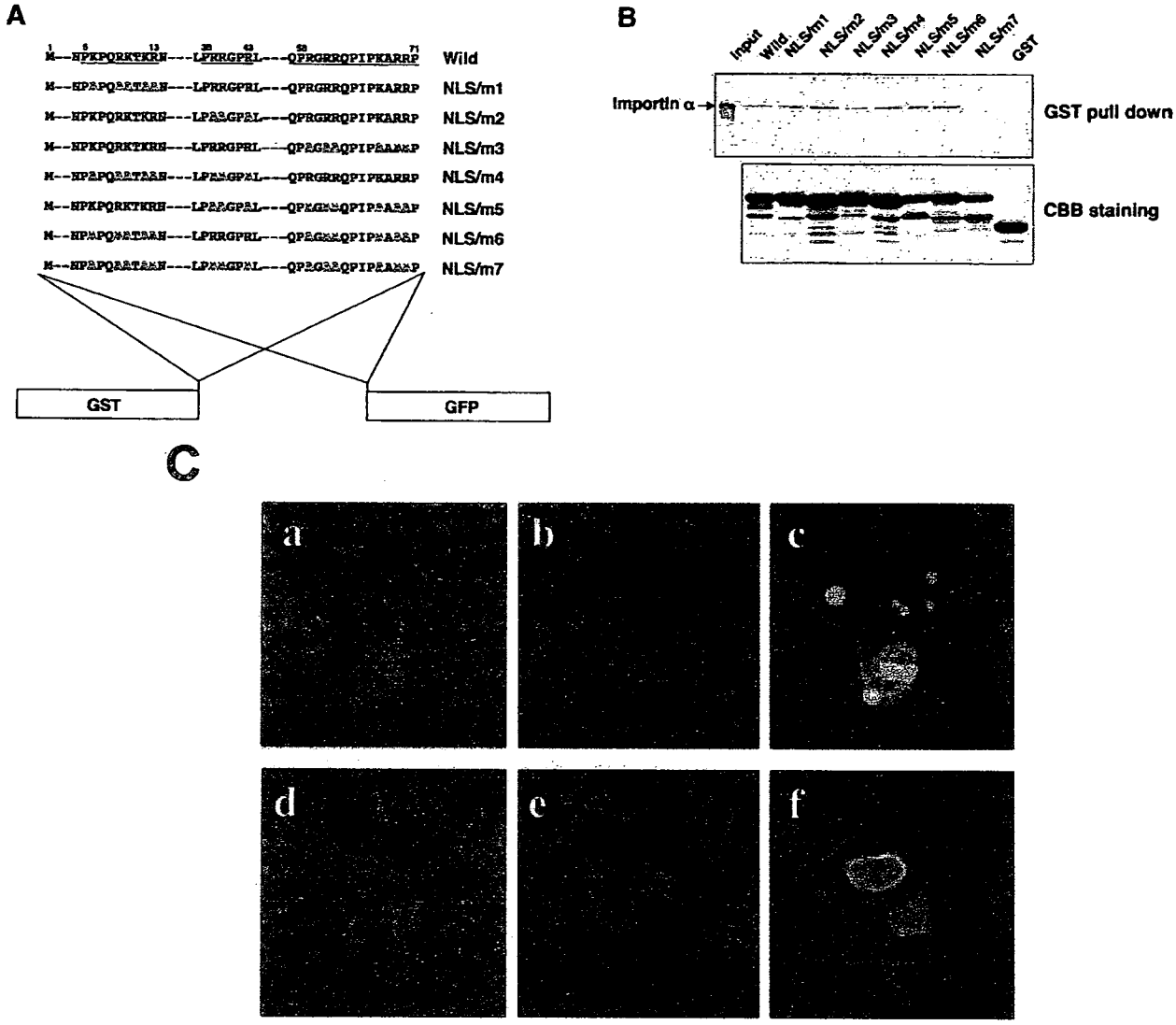

FIG. 7. Mutational analyses of NLS motifs in HCV core protein. (A) Schematic structures of fusion proteins and amino acid sequences corresponding to putative NLS motifs, three basic clusters (underlined) in the core protein. Two series of constructs fused with either GFP or GST were created. The mutated basic residues are indicated with outline letters. (B) GST pulldown assay. Equal amounts of GST fusions as described in A or GST alone was immobilized on glutathione-Sepharose 4B beads and incubated with in vitro-translated, [^{35}S]methionine-labeled importin-α. Bound material was separated by SDS-PAGE, and the amount of importin-α bound was detected by autoradiography. Direct electrophoretic separation of in vitro translation products served as a control (input). Coomassie brilliant blue staining of GST fusions and GST alone are shown in the bottom panel. (C) Confocal analysis of double staining for core-GFP fusion protein and HA-importin-α. 293T cells transfected with the wild-type core (1–71)-GFP (a to c) or NLS/m4 (d to f) expression plasmid and pCAG-HA-imp were allowed to express for 2 days. After the cells were fixed and permeabilized, they were incubated with a mouse anti-HA antibody. The red signals corresponding to HA-importin-α were obtained with a rhodamine-conjugated goat anti-mouse IgG secondary antibody (b and e). Overlay resulted in yellow signals indicative of colocalization (c and f). (D) Subcellular localization of GFP fusion proteins. GFP fusions with and without substitution mutations in the NLS motifs of the core protein as described in A were expressed in 293T cells. GFP images of the fixed cells were recorded.

tase (14), a predicted structure of an amphipathic α-helix present between amino acids 116 and 134 (Fig. 6B and C) possibly plays a role in directing the core protein to the mitochondrial outer membrane. Sequence comparisons demonstrate conservation of the amino acid sequence and secondary structure of the region, amino acids 112 to 152, among a variety of HCV isolates, including the infectious H77c clone (55), as well as a full-length adaptive replicon (3). To gain insight into the significance of the secondary structure of the region in targeting to the mitochondria, further structural and biochemical analyses are needed.

The association of HCV core protein with the mitochondrial membrane suggests that the core protein has the ability to modulate mitochondrial function, possibly by altering the permeability of the mitochondrial membrane. The core protein induces the production of cellular reactive oxygen species in

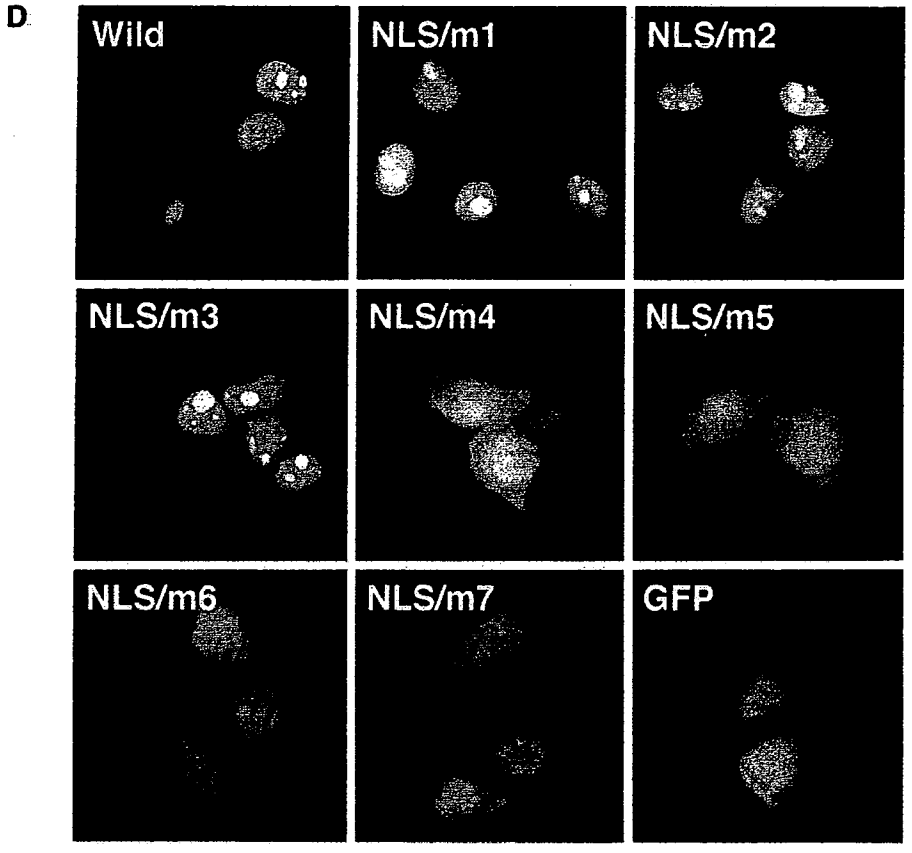

FIG. 7—*Continued.*

the livers of core-transgenic mice and in core-expressing cell lines (35). Reactive oxygen species, predominantly generated in mitochondria, induce genetic mutations and act as secondary messengers to regulate a variety of cellular functions, including gene expression and proliferation (1). Although the molecular mechanism by which core protein induces reactive oxygen species production is still unclear, HCV core protein is known to impair the mitochondrial electron transfer system (35). The core protein may also modulate apoptosis, since mitochondria play a major role in regulating programmed cell death. Expression of HCV proteins, including the core protein, suppresses the release of cytochrome *c* from mitochondria to the cytoplasm in HCV-transgenic mice, thus inhibiting Fas-mediated apoptosis (27).

Okamoto et al. recently reported that not only the C-terminal signal sequence but also amino acids 128 to 151 are required for ER retention of the core protein by using a series of N-terminally truncated core protein constructs (38). Here, in this study, we further showed that amino acids 112 to 152 mediate association of the core protein with the ER in the absence of the C-terminal signal sequence. Hope and McLauchlan demonstrated that the central domain of the core protein, amino acids 119 to 174, is important for association with lipid droplets (17). They also showed that this corresponding domain is shared with GB virus B, which is most closely related to HCV, but not with either pestiviruses or flaviviruses (18). It appears that the 41 residues identified as the sequence mediating association with the ER membrane in the present study are crucial for directing the core protein to lipid droplets, since the surface of lipid droplets must derive from the cytoplasmic side of the ER membrane.

The HCV core protein contains NLS sequences which are composed of three stretches of sequences rich in basic residues. These sequences were originally identified by experiments with fused forms of wild-type and mutated core proteins with β-galactosidase (6, 48). C-terminally truncated versions of the core protein localize exclusively to the nucleus (48). A fraction of the core protein is detected in the nucleus even when full-length HCV core gene is expressed (Fig. 2) and as described (34, 56). However, it is difficult to demonstrate clearly the nuclear localization of the core protein by immunofluorescence, presumably because of the instability of nuclearly localized core protein (49, 33). We only observed a nuclear staining pattern of the matured core protein after adding proteasome inhibitors to the culture (33).

Generally, NLS sequences fall into two categories; (i) monopartite NLSs, which contain a single cluster of basic residues, and (ii) bipartite NLSs, which contain two clusters of basic residues separated by an unconserved linker sequence of variable length (reviewed in reference 12). Nuclear translocation of an NLS-containing cargo protein is initiated when the soluble import receptor (importin) recognizes the NLS-contain-

ing protein within the cytoplasm. Importin-α contains an NLS-binding site(s), and importin-β docks importin-cargo complexes to the cytoplasmic filaments of a nuclear pore complex, after which translocation occurs through the nuclear pore. Thus, importin-α functions as an adaptor between the bona fide import receptor and the NLS-containing protein.

We further characterized the NLS of the core protein and found that each of the NLS motifs of the core protein is able to bind to importin-α and that at least two NLS motifs are required for efficient nuclear distribution of the core protein in cells. It appears that double mutations among three NLS motifs decrease the ability of the core protein to bind importin-α. These observations suggest that the binding of the double mutants with importin-α leads to no or little active translocation of the core protein into the nucleus. The double mutations may also block subsequent interactions with importin-β1, GTPase Ran, and/or NTF2/p10, which are required for translocation through the nuclear pore complexes.

The findings obtained in this study suggest that HCV core protein NLS motifs have a bipartite function. Crystallographic studies of monopartite (e.g., simian virus 40 large T antigen) and bipartite (e.g., nucleoplasmin) NLSs show that the basic residue clusters of bipartite NLSs occupy separate binding sites on importin-α. In contrast, while monopartite NLSs can bind to the same sites as bipartate NLSs on importin-α, they mainly bind to the N-terminal binding site, which is referred to as the major binding site on importin-α (9, 11). A recent report describes an importin-α variant with a mutation in the major site which results in decreased ability to bind both monopartite and bipartite NLSs. Another variant with a mutation in the minor site exhibits decreased binding only to bipartite NLS-containing proteins, making importin-α nonfunctional in vivo (22). Thus, we favor a model in which the core protein bipartite NLS, composed of any two of the three basic clusters, occupies both major and minor binding sites on importin-α, resulting in efficient nuclear translocation. Importin-α may be equally accessible to all clusters, given their close proximity to one another, as well as the distinct conformational flexibility of the ≈70-residue N-terminal region of the core protein.

With regard to the molecular mechanisms participating in nuclear localization of the core protein, Moriishi et al. found that PA28γ is involved in nuclear localization of the core protein. Interaction of the core protein with PA28γ plays an important role in retention of the core protein in the nucleus (33). Furthermore, in yeast cells, nuclear transport of the core protein requires the activity of the small GTPase Ran/Gsp1p and is mediated by Kap123p, but neither importin-α nor importin-β is involved (20). Differences in nucleocytoplasmic transport between yeast and mammalian cells might explain the inconsistencies observed in the present study. Further experiments are required to characterize the exact nature of the interaction between the core protein and components of the nuclear import machinery, particularly in cells where HCV is replicating.

In conclusion, the mature HCV core protein has an internal 41-amino-acid sequence mediating association of the viral protein with the ER and mitochondria. We also provide evidence for a novel class of bipartite NLS contained within the core protein, which comprises two of three basic motifs, thus enabling efficient nuclear targeting. Multiple functional domains influence the subcellular localization of the core protein, which ultimately depends on the balance of the respective signals.

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