

The replication competency in Sf9 cells of a gp64-null recombinant baculovirus could be rescued by incorporation of the VSVG gene (33, 34). We confirmed that the recombinant baculovirus deleted the gp64 gene and instead incorporated the VSVG gene under the control of the polyhedrin promoter, was replication competent in insect cells, and exhibited a high level of reporter gene transduction into 293T and BHK cells. The compatibility of VSVG in this system may result from similarities of the structural and functional characteristics of VSVG to those of gp64; both proteins are type I membrane glycoproteins, exist in trimeric complexes, and are capable of inducing membrane fusion at low pHs (6, 50, 54). Although the recombinant baculovirus in which gp64 is replaced with VSVG is able to replicate in insect cells and transduce foreign genes into a wide variety of mammalian cells, VSVG was suggested to recognize phosphatidylserine (58) or other ubiquitously expressed molecules other than phosphatidylserine (13) as a receptor(s), making it difficult to confer cell type specificity to gene delivery using VSVG pseudotype baculoviruses.

To establish ligand-directed gene delivery by pseudotype baculoviruses, it is necessary to propagate replication-deficient pseudotype baculoviruses possessing a ligand of interest in Sf9gp64, a replication-competent cell line stably expressing gp64. After three rounds of passage of the pseudotype virus in Sf9gp64 cells, however, replication-competent revertant viruses that had incorporated the gp64 gene were generated. Sf9gp64 cells were established by transfection of Sf9 with a plasmid encoding the gp64 gene lacking any flanking sequences. Southern blot analysis revealed that the gp64 gene was integrated into the genomes of the baculovirus revertants by nonhomologous recombination. Homologous recombination between the AcMNPV genome and either a transfer vector (32, 38), additional AcMNPVs (19), or *B. mori* NPV can occur in insect cells with high frequency (27, 28). In contrast, our data suggest that the revertant viruses were not generated by homologous recombination but by nonhomologous incorporation of the gp64 gene from the Sf9gp64 chromosome into the viral genome. Nonhomologous recombination between plasmid DNA and the baculovirus genome was previously reported upon cotransfection into insect cells (71). A gp64-null virus could propagate in Sf9^{Op1D} cell lines constitutively expressing OpNPV gp64 without incorporating the gp64 gene into the viral genome (33, 34). One possible explanation for our result may be the difference between the gp64 genes of OpNPV and AcMNPV. Although the Sf9gp64 cell line was established by sorting cells expressing a high level of gp64 on the cell surface, the expression level of gp64 might be lower than that of the Sf9^{Op1D} cell line, and the lower level of expression of gp64 might result in the selective amplification of revertants. To circumvent the high-frequency incorporation of foreign DNA into the baculoviral genome, we attempted the lipofection of recombinant bacmids into Sf9 cells instead of amplification of pseudotype baculoviruses in Sf9gp64. Although it is not possible to obtain a high pseudotype virus titer by this method, we can generate pure virus stocks without any contaminating replication-competent baculoviruses incorporating the gp64 gene. To prepare a high-titer stock of a replication-deficient pseudotype baculovirus carrying a foreign ligand, however, it is essential to propagate the pseudotype virus in Sf9 cells expressing gp64 without the transfection of plasmid DNA. Expression of gp64 by RNA trans-

fection, RNA viral vectors, or the RNA replicon system may be able to avoid the incorporation of gp64 DNA into the baculovirus genome.

We constructed a pseudotype baculovirus, Ac Δ 64/CD46/CALuc, bearing human CD46 in place of gp64 on viral particles. CD46 is a multifunctional protein involved in the infection of various microorganisms and the regulation of complement activation (10). CD46, also known as membrane cofactor protein, protects autologous cells from complement attack by serving as a cofactor for factor I-mediated inactivation of C3b and C4b, blocking the complement cascade at C3 activation (2). CD46 also serves as a receptor for human herpes virus 6 (56), group B adenovirus (17, 59, 62), bovine viral diarrhea virus (39), two bacterial strains (*Streptococcus pyogenes* and pathogenic *Neisseria*) (26, 46), and the Edmonston strain of measles virus (15, 42). In this study, we demonstrated that Ac Δ 64/CD46/CALuc exhibited specific reporter gene transfer to and expression in BHK cells expressing the measles virus H and F glycoproteins of the Edmonston strain but not those expressing the Ichinose strain glycoproteins that require SLAM as a receptor (67). Therefore, a CD46 pseudotype baculovirus bearing a suicide gene may be able to eliminate cells expressing pathogen ligands that utilize CD46 as a receptor. Furthermore, CD46 is frequently overexpressed on cancer cells, possibly serving as a mechanism to overcome lysis by complement (16). In support of the potential utility of this vector, the Edmonston strain of measles virus has a potent and selective oncolytic activity (52). CD46 pseudotype baculovirus may also be applicable for the clearance of tumor cells surviving oncolytic measles virus treatment.

Infections with Ac Δ 64/gp64/CALuc and Ac Δ 64/VSVG/CALuc, carrying gp64 and VSVG on the particles, respectively, could be decreased by treatment with ATPase inhibitors, chloroquine, or ammonium chloride, while Ac Δ 64/CD46/CALuc was resistant to treatment. This finding suggests the possibility of constructing a baculovirus vector capable of both targeting and modulating the viral entry pathway, as seen for VSV (7). This is different from influenza virus vectors, where acidic exposure within endosomes is critical for the dissociation of the matrix protein from the ribonucleocapsid (9, 37). In contrast to Ac Δ 64/CD46/CALuc, Ac Δ 64/SLAM/CALuc could not incorporate the full-length SLAM molecule into particles, preventing specific gene transduction. The inability to mediate gene delivery was not due to the absence of an interaction with specific targets, as Sf9 cells expressing SLAM induced membrane fusion with BHK cells expressing the H and F glycoproteins of either the Edmonston or Ichinose strain of measles virus (data not shown). The ligands incorporated efficiently into baculovirus particles, including gp64, VSVG, and CD46, all have relatively short cytoplasmic domains, measuring 7, 29, and 33 amino acids in length, respectively (49, 54, 60). In contrast, SLAM possesses a cytoplasmic tail of 77 amino acids (23). We therefore hypothesized that the length of the ligand cytoplasmic domain may be critical for efficient incorporation into baculovirus particles. Ac Δ 64/SLAM_{cyto7}/CALuc, possessing a mutant SLAM molecule with the C-terminal 70 amino acids of the cytoplasmic domain deleted, efficiently incorporated the mutant SLAM into particles and exhibited specific gene delivery to BHK cells expressing the H and F glycoproteins of both measles virus strains. Although the mechanism by which SLAM

incorporation into baculovirus particles is enhanced by C-terminal truncation of the cytoplasmic domain is not known, one possibility might be a change in SLAM localization. The gp64 protein localizes to the cell surface but is excluded from lipid raft microdomains (72). As VSVG and CD46 also do not associate with lipid rafts (35, 57), proteins localized to lipid raft microdomains may be excluded during virus assembly and budding. Although the cellular localization of SLAM is not known, further studies will be necessary to clarify the relationship between cell surface localization and incorporation into baculovirus particles and to test the involvement of lipid raft microdomains in this process.

Mangor et al. demonstrated that gp64-null baculoviruses pseudotyped with VSVG were not morphologically distinguishable from budded wild-type AcMNPV particles (34). We confirmed that Ac Δ 64/VSVG/CALuc, as well as Ac Δ 64/CD46/CALuc and Ac Δ 64/GFP/CALuc, exhibited a morphology similar to that of AcMNPV. These results indicate that expression of gp64 is not required for the morphogenesis of a rod-shaped structure for budded AcMNPV particles. Immunogold labeling of Ac Δ 64/VSVG/CALuc and Ac Δ 64/CD46/CALuc revealed that the VSVG and CD46 proteins were incorporated into and distributed throughout the whole viral surface. These results are consistent with previous observations that VSVG fusion proteins were distributed throughout the stalk and head domains of baculovirus particles, in contrast to gp64, which was primarily localized in the head domain (11, 44).

In this study, we have demonstrated the capability for ligand-directed gene delivery by pseudotype baculoviruses in vitro. For future in vivo applications of baculovirus vectors for gene targeting to specific organs or virus-infected cells as a method of treatment of inherited or infectious diseases, it is imperative to exhaustively study the transcription of baculoviral genes in mammalian cells for certification of safety. In addition, further studies are needed to establish replication-competent cell lines capable of supporting the propagation of pseudotype viruses without the possibility of replication-competent virus breakthrough by incorporation of gp64 and to optimize the conditions necessary for the efficient incorporation of ligands into recombinant baculovirus particles.

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Nuclear Localization of Japanese Encephalitis Virus Core Protein Enhances Viral Replication†

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Japanese encephalitis virus (JEV) core protein was detected in both the nucleoli and cytoplasm of mammalian and insect cell lines infected with JEV or transfected with the expression plasmid of the core protein. Mutation analysis revealed that Gly⁴² and Pro⁴³ in the core protein are essential for the nuclear and nucleolar localization. A mutant M4243 virus in which both Gly⁴² and Pro⁴³ were replaced by Ala was recovered by plasmid-based reverse genetics. In C6/36 mosquito cells, the M4243 virus exhibited RNA replication and protein synthesis comparable to wild-type JEV, whereas propagation in Vero cells was impaired. The mutant core protein was detected in the cytoplasm but not in the nucleus of either C6/36 or Vero cell lines infected with the M4243 virus. The impaired propagation of M4243 in mammalian cells was recovered by the expression of wild-type core protein *in trans* but not by that of the mutant core protein. Although M4243 mutant virus exhibited a high level of neurovirulence comparable to wild-type JEV in spite of the approximately 100-fold-lower viral propagation after intracerebral inoculation to 3-week-old mice of strain Jcl:ICR, no virus was recovered from the brain after intraperitoneal inoculation of the mutant. These results indicate that nuclear localization of JEV core protein plays crucial roles not only in the replication in mammalian cells *in vitro* but also in the pathogenesis of encephalitis induced by JEV *in vivo*.

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. Members of the genus *Flavivirus* are predominantly arthropodborne viruses and frequently cause significant morbidity and mortality in mammals and birds (6). JEV is distributed in the south and southeast regions of Asia and kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (6, 50, 57). JEV spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes and causes infection of the central nervous system, with a high mortality rate (6, 57). JEV has a single-stranded positive-strand RNA genome approximately 11 kb in length, which is capped at the 5' end but lacks modification of the 3' terminus by polyadenylation (34). The genomic RNA encodes a single large open reading frame, and a polyprotein translated from the genome is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the core, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (53). Although the core protein has very little amino acid homology to other flaviviruses—for example, the core protein of JEV has only 25% homology to that of tick-borne encephalitis virus (TBEV)—the structural properties, such as the hydrophobicity profile, abundances of basic amino acid residues, and second-

ary structures, are very similar (11, 20, 36). The flavivirus core proteins commonly contain two hydrophobic sequences in the center and a carboxyl-terminal end, and the carboxyl-terminal hydrophobic region serves as a signal sequence of prM. The signal-anchor sequence is cleaved off by the viral protease NS2B-3, and this cleavage is required for the subsequent liberation of the amino terminus of prM by the host signal peptidase (35, 52, 63). The mature core protein, released from the endoplasmic reticulum (ER) membrane, is believed to bind to the genomic RNA via the basic amino acid clusters at the amino and carboxyl termini and forms nucleocapsids (23). The central hydrophobic region of the core protein may be associated with the ER membrane, and this interaction is believed to facilitate the assembly of nucleocapsid and two membrane proteins, prM and E, and to bud into the ER lumen as virions (39). The removal of the central hydrophobic region of the TBEV core protein increased the production of the subviral particles that consist of (pr)M and E proteins but that lack a core protein and genomic RNA (26, 27).

In addition to their role as structural proteins, core proteins of dengue virus (DEN) and Kunjin virus (KUN) are localized not only in the cytoplasm but also in the nucleus, especially in the nucleoli of several infected cell lines (4, 38, 55, 59, 61). Transport from the cytoplasm to the nucleus occurs through nuclear pore complexes that penetrate the double lipid layers of the nuclear envelope. Small molecules up to 9 nm in diameter (<50 kDa) can freely diffuse through the nuclear pore complexes, while most macromolecules require an active transport process via nuclear import receptor proteins such as importin- α (37). In general, cargo proteins contain mono- or bi-

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† This study is dedicated to the memory of Ikuko Yanase.

partite cluster sequences of basic amino acids termed nuclear localization signals (NLSs) to bind to nuclear import receptor proteins (5, 21). As flavivirus core proteins are relatively small (approximately 14 kDa), they may diffuse into the nucleus. However, the successful translocation of DEN core protein fused with three copies of green fluorescent protein (GFP) (96 kDa in total) into the nucleus indicates that the DEN core protein is actively translocated into the nucleus by an energy-dependent pathway, and an NLS was assigned to the region of carboxyl-terminal residues from amino acids 85 to 100 (59). Despite the many studies investigating this matter, the biological significance of the nuclear localization of core proteins in the virus replication cycle remains unclear.

In this study, we showed that the JEV core protein is also localized in both the cytoplasm and the nucleus, particularly in the nucleolus, of mammalian and mosquito cell lines and determined that an NLS is present in the core protein. We generated a mutant JEV, replaced the NLS in the core protein with Ala, and confirmed the elimination of the nuclear localization of the mutant core protein in both mammalian and mosquito cells. The characterization of the mutant JEV indicates that the nuclear localization of the core protein plays important roles in the viral replication in mammalian cells and in the pathogenesis of encephalitis in vivo. Finally, we discuss the biological significance of the nuclear localization of the JEV core protein.

MATERIALS AND METHODS

Cells. The mammalian cell lines Vero (African green monkey kidney), 293T (human kidney), BHK (hamster kidney), HeLa (human cervix cancer), HepG2 (human hepatoma), SK-N-SH (human neuroblastoma), and N18 (mouse neuroblastoma) were maintained in Dulbecco's modified Eagle's minimal essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). A mosquito cell line, C6/36 (*Aedes albopictus*), was grown in Eagle's minimal essential medium supplemented with 10% FBS.

Plasmids. The mammalian expression vector pEGFP-C3 was purchased from Clontech (Palo Alto, Calif.). The plasmid pEGFP-JEVC105 was constructed by insertion of cDNA encoding the mature form of the JEV core protein without the C-terminal signal sequence (amino acids 2 to 105 of the AT31 strain) amplified by PCR into pEGFP-C3 as described previously (42). All of the expression vectors coding the enhanced GFP (EGFP)-fused mutant JEV core proteins were constructed based on pEGFP-JEVC105. Briefly, the gene encoding the JEV core protein with amino acids 38 to 44 deleted was amplified by splicing the overlapping extension (16, 17). For alanine scanning in putative NLS regions (amino acids 38 to 44 and 85 to 105), a series of point mutants of the JEV core protein were synthesized by PCR-based mutagenesis (14). All of the mutant genes were cloned into EcoRI and BamHI sites of pEGFP-C3. The plasmid that has a full-length cDNA of the JEV AT31 strain under the control of a T7 promoter was constructed and designated pMWJEATG1 (Z. Zhao, T. Date, Y. Li, T. Kato, M. Miyamoto, K. Yasui, and T. Wakita, submitted for publication). Guanine-to-cytosine and cytosine-to-guanine point mutations were introduced into pMWJEATG1 at nucleotides 220 and 222 of the JEV gene, respectively, by PCR-based mutagenesis to change Gly⁴² and Pro⁴³ of the core protein to Ala. The constructed plasmid was designated pMWJEAT/GP4243AA. For the mutant viral replication complementation experiments, the genes coding the C-terminal hemagglutinin (HA)-tagged core proteins derived from pMWJEATG1 and pMWJEAT/GP4243AA were cloned into pCAG-GS vector (43), and the resulting plasmids were designated pCAG-WC-HA and pCAG-MC-HA, respectively.

Antibodies. cDNA encoding the JEV core protein (amino acids 2 to 105) was inserted into pGEX-2TK (Amersham Biosciences, Piscataway, N.J.) and transformed into *Escherichia coli* strain DH5 α . The glutathione-S-transferase-fused JEV core protein expressed in the bacteria was purified with a column with glutathione Sepharose 4B (Amersham Biosciences) and intradermally injected five times into a Japanese white rabbit purchased from KITAYAMA LABES (Nagano, Japan). The collected antiserum was absorbed with glutathione-S-transferase-binding glutathione Sepharose 4B. Anti-JEV monoclonal antibodies

(MAb), anti-E 10B4 (E. Konishi, unpublished data) and anti-NS3 34B1 (K. Yasui, unpublished data), were used in immunostaining. Anti-nucleolin MAb, MS-3, and antiactin goat serum were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit antiserum to PA28 α was purchased from AFFINITY (Exeter, United Kingdom).

Transfection of plasmids. Plasmid vectors were transfected by Superfect (QIAGEN, Tokyo, Japan) for Vero cells or Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) for 293T, BHK, N18, HeLa, HepG2, and SK-N-SH cells. To examine the intracellular localization of the EGFP or EGFP-fused proteins, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS at 24 h after transfection. After treatment with 1 μ g of RNase A (QIAGEN)/ml, the nuclei were stained with 500 μ M propidium iodide (Molecular Probes, Eugene, Oreg.). Endogenous nucleolin, a major nucleolar protein (51), was immunostained by an anti-nucleolin monoclonal antibody and Alexa Fluor 564-conjugated anti-mouse immunoglobulin G (IgG) antiserum (Molecular Probes). All samples were visualized with a laser scanning confocal microscope (Bio-Rad, Hercules, Calif.).

Generation of JEV from plasmid. The wild-type and mutant (designated M4243) JEVs were generated from plasmids, pMWJEATG1, and pMWJEAT/GP4243AA, respectively, by previous methods (Zhao et al., submitted) with some modifications. Briefly, the plasmid DNAs digested by restriction enzyme KpnI were used as templates for RNA synthesis. Capped full-length JEV RNAs were synthesized in vitro by an mMESSAGE mMACHINE T7 kit (Ambion, Austin, Tex.), purified by precipitation with lithium chloride, and used for electroporation. Trypsinized Vero cells were washed with PBS and resuspended at 10⁷ cells/ml in PBS. RNA (10 μ g) was mixed with 500 μ l of cell suspension and transferred to an electroporation cuvette (Thermo Hybrid, Middlesex, United Kingdom). Cells were then pulsed at 190 V and 950 μ F by the use of a Gene Pulser II apparatus (Bio-Rad). Transfected cells were suspended in a culture medium and transferred to 10-cm-diameter culture dishes. After 3 or 4 days of incubation, the culture supernatants were collected as viral solutions. Due to a low viral yield, these viruses were amplified by a single passage in C6/36 cells. Viral infectivities were determined as focus-forming units (FFUs) by an immunostaining focus assay of Vero, C6/36, and 293T cells. Briefly, viruses were serially diluted and inoculated onto cell monolayers. After 1 h of adsorption, the cells were washed with serum free D-MEM three times and cultured in D-MEM containing 5% FBS and 1.25% methylcellulose 4000. At 2 or 3 days later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Infectious foci were stained with an anti-JEV E monoclonal antibody and visualized with a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, Calif.). Vero and C6/36 cells infected with wild-type or M4243 JEV were fixed with cold acetone at 48 h postinoculation and stained with the rabbit anti-JEV core protein antiserum and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) antibody. After treatment with 1 μ g of RNase A/ml, nuclei were stained with 500 μ M propidium iodide. Samples were examined with a laser scanning confocal microscope.

Subcellular fractionation. At 48 h postinoculation, 2 \times 10⁶ Vero cells were fractionated into cytoplasm and nucleus by using a Nuclear/Cytosol Fractionation kit (BioVision, Mountain View, Calif.) according to the manufacturer's instructions. Finally, 210 μ l of the cytoplasmic extracts and 100 μ l of the nuclear extracts were recovered and 10 μ l of each of the extracts was subjected to electrophoresis on an acrylamide gel. The JEV core protein was detected by Western blotting using the anti-JEV core protein rabbit polyclonal antibody. Endogenous PA28 α (3, 42) and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

Growth kinetics of mutant JEV in culture cells. Vero or C6/36 cells (2 \times 10⁵) in 24-well plates were infected with wild-type or M4243 virus at a multiplicity of infection (MOI) of 5 for 1 h at 4°C, washed three times with a medium to remove unbound viruses, and incubated with a medium supplemented with 5% FBS for a total duration of 30 h. The culture supernatants were used for titration of infectious virus, and cells were used for detection of viral proteins by Western blotting and for detection of negative-strand viral RNA by real-time reverse transcription-PCR (RT-PCR). Total RNAs were extracted from the cells by using an RNeasy Mini kit (QIAGEN) and quantified with a Gene Quant RNA/DNA calculator (Amersham Biosciences). RNA samples (5 μ l) were reverse transcribed at 52°C for 30 min with TaqMan reverse transcription reagents (Applied Biosystems, Foster, Calif.) by the use of a negative-strand-specific "tagged" primer corresponding to nucleotides (nt) 9307 to 9332 (5'-GCG TCA TGG TGG CGT ATT TAC CAG AAC TGA TTT AGA AAA TGA A-3'). The tagged sequence, which is underlined, had no correlation to JEV or other flaviviruses. The reverse transcripts were applied to a real-time PCR assay using a TaqMan PCR core reagents kit with sense (5'-GCG TCA TGG TGG CGT

ATT TA-3') and antisense (5'-TGG ACA GCG ATG TTC GTG AA-3') primers corresponding to the tagged sequence and nt 9519 to 9538 of the JEV AT31 strain, respectively. The kinetics of cDNA amplification were monitored with an ABI PRISM 7000 sequence detection system (Applied Biosystems) using a reporter probe corresponding to nt 9363 to 9380 of the JEV AT31 strain (5'-CAC CGC ATG CTC GCC CGA-3') conjugated with 6-carboxyfluorescein at the 5' terminal and 6-carboxy-tetramethylrhodamine at the 3' terminal. As references for the real-time RT-PCR, positive- and negative-strand RNAs were synthesized by *in vitro* transcription from plasmids containing nt 8907 to 9955 of JEV cDNA inserted in the forward and backward directions under the control of a T7 promoter.

Characterization of viral particles. Vero and C6/36 cells were inoculated with wild-type or M4243 viruses at an MOI of 0.1, and culture fluids harvested after 2 (Vero cells) or 3 (C6/36 cells) days postinoculation were clarified by centrifugation at $6,000 \times g$ for 30 min and precipitated with 10% polyethylene glycol (molecular mass, approximately 6,000 kDa). The precipitate was collected by centrifugation at $10,000 \times g$ for 45 min and resuspended in TN buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl). The infectious titers of the concentrated viral particles were determined on Vero cells. The hemagglutination (HA) titers were determined at pH 6.6 by the method of Clarke and Casals (9). The viral particles (400 HA units) were applied on 10 to 40% of sucrose gradients and were centrifuged at $147,000 \times g$ for 90 min. Fractions collected from the bottom were examined by the HA test.

Complementation of mutant virus replication in mammalian cells. pCAG-WC-HA, pCAG-MC-HA, or pCAG-GS (1 μ g) was transfected into 293T cells in a 24-well plate (5×10^4 cells). At 4 h after transfection, the cells were washed three times with a serum-free medium and infected with the wild-type or M4243 JEV at an MOI of 5. At 12, 18, and 24 h after inoculation, the culture supernatants were harvested and infectivity was determined on Vero cells. The infected cells were harvested, and expression levels of the core proteins and replication of viral RNA were determined by Western blotting and real-time RT-PCR, respectively.

Mouse experiments. Female ICR mice of strain Jcl:ICR (3 weeks old) were purchased from CLEA Japan (Osaka, Japan). All mice were kept in pathogen-free environments. Groups of mice ($n = 10$) were inoculated intracerebrally (ic) with 30 μ l of 10-fold-diluted solutions of wild-type or M4243 virus. The virus dilution solution (D-MEM) was administered to 10 mice as a control. The mice were observed for 2 weeks after inoculation to determine survival rates. The value of the 50% lethal dose (LD_{50}) for each virus was determined by the method by Reed and Muench (47). Groups of mice ($n = 10$ or 11) were inoculated intraperitoneally (ip) with 10^5 FFU (100 μ l) of the viruses. The mice were observed for 3 weeks after inoculation to determine survival rates. To examine viral growth in the brain, 100 FFU (ic) or 10^5 FFU (ip) of the viruses were administered to the mice. At 1 to 7 days after inoculation, the mice were euthanized, and the brains were collected. The infectious viral titers in the homogenates of the brains were determined in Vero cells as described above.

RESULTS

Determination of amino acids essential for nuclear or nucleolar localization of the JEV core protein. To examine the subcellular localization of the mature JEV core protein without the C-terminal signal sequence in mammalian cells, pEGFP-JEVC105 encoding the EGFP-fused core protein or parental vector, pEGFP-C3, was transfected into Vero cells. EGFP was diffusely distributed in both the cytoplasm and nucleus, while the EGFP-fused core protein exhibited a diffuse distribution in the cytoplasm but granular localization in the nucleus (Fig. 1A). The fusion JEV core protein in the nucleus was colocalized with nucleolin, a major nucleolar component, indicating that the core protein is accumulated at the nucleoli (Fig. 1B). A similar subcellular localization of the fusion core protein was observed in all of the cell lines examined, including neuronal (N18 and SK-N-SH) and nonneuronal (293T, BHK, HeLa, and HepG2) cells (data not shown). Wang et al. (59) reported that the DEN core protein possessed a bipartite NLS in residues 85 to 100 (RKEIGRMLNLRNRKR). A computer program, PSORTII (Institute of Medical Science, Tokyo Uni-

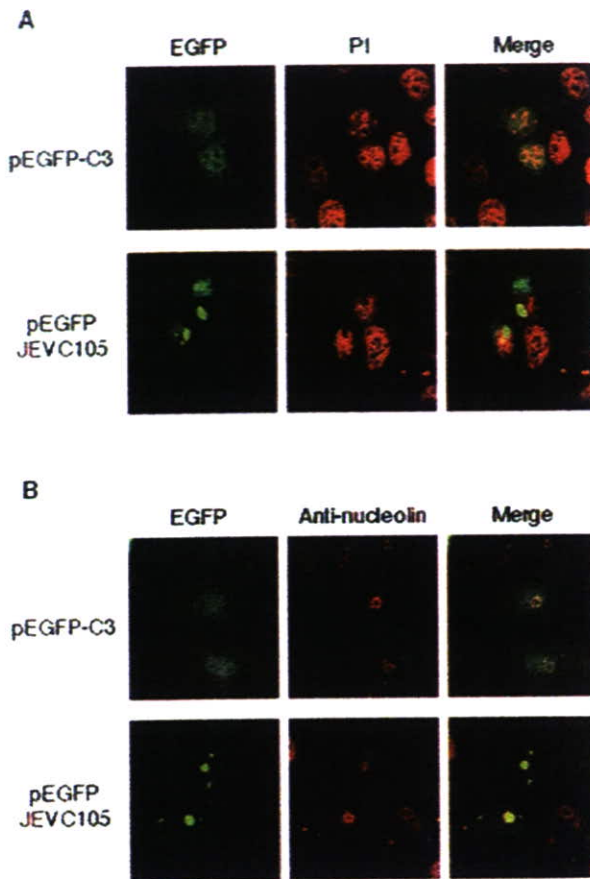


FIG. 1. Intracellular localization of EGFP-fused JEV core protein. Vero cells were transfected with expression plasmids encoding EGFP or EGFP-fused JEV core protein. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. (A) Nuclei were stained with propidium iodide. (B) A representative nucleolar protein, nucleolin, was stained with anti-nucleolin monoclonal antibody. All samples were observed with a confocal microscope.

versity[<http://psort.ims.u-tokyo.ac.jp/helpwww2.html>]), predicted that the JEV core protein also had an NLS at the corresponding region (residues 85 to 101 [KRELGLTIDAVNKRGRK]). To confirm whether the region functions as an NLS, an expression vector for the EGFP-fused mutant core protein in which all of the six basic amino acids (Arg and Lys) that were key amino acids in the NLS motifs were replaced by Ala (AAELGLTIDAVNAAGAA) was transfected into Vero cells. However, these mutations did not affect the nuclear or nucleolar localization of the JEV core protein (data not shown), suggesting that this region of the JEV core protein does not participate as an NLS.

Alternatively, we found another candidate for an NLS in the JEV core protein. The NLS of the core protein of hepatitis C virus (HCV), a member of the same family *Flaviviridae*, has been mapped to the amino acid residues 38 to 43 (54). This domain of the HCV core protein is found to be homologous with flaviviruses, including JEV, St. Louis encephalitis virus, KUN, West Nile virus (WNV), Murray Valley encephalitis virus, and DEN (type 1 to 4) (Fig. 2A). In particular, the two amino acids Gly and Pro are completely conserved among

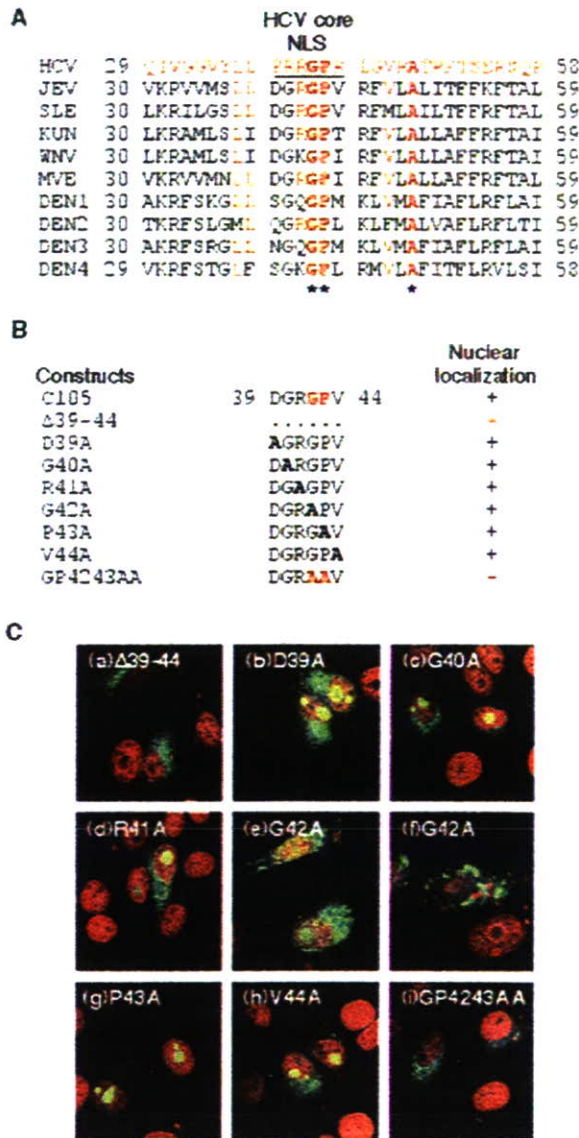


FIG. 2. Role of an HCV core protein NLS in nuclear localization of JEV core protein. (A) A partial alignment of amino acid sequences of core proteins of HCV (HCVJ1 strain, genotype 1b) and flaviviruses, including JEV, SLE (St. Louis encephalitis virus), KUN, WNV, MVE (Murray Valley encephalitis virus), and DENs. Amino acid sequences of the HCV core protein and identical amino acids in flaviviruses are indicated with red letters. Amino acids that were completely conserved among the viruses are indicated by asterisks and red boldface letters. The NLS of HCV core protein previously reported by Suzuki et al. (54) is underlined. (B) Expression plasmids encoding EGFP-fused JEV core proteins mutated in the NLS of the HCV core protein. The presence (+) or absence (-) of nuclear localizations of the EGFP-fused JEV core proteins is indicated. Dots indicate the deleted amino acids. Boldface letters indicate the substituted amino acids. (C) Intracellular localization of EGFP-fused JEV core proteins with deletion or substitution in the NLS region of HCV core protein in Vero cells. Panels (except for panel f) indicate merged images of EGFP and nuclear staining by propidium iodide. Panel f shows merged images of EGFP-fused JEV core protein with a G⁴²-to-Ala substitution and a major nucleolar protein nucleolin. All samples were observed with a confocal microscope.

these flaviviruses and HCV. Therefore, we next analyzed the effect of mutation in this region on the nuclear localization of the JEV core protein. The EGFP-fused JEV core protein with residues 39 to 44 deleted was localized only in the cytoplasm but not in the nucleus (Fig. 2B and panel a in Fig. 2C). To further identify the essential amino acids for the nuclear localization, a series of point mutants were constructed (Fig. 2B). No single-amino-acid substitution of the core proteins abolished nuclear localization except for a mutant of Gly⁴² in which the mutant core protein did not colocalize with nucleolin and was distributed as filamentous structures in the nuclei (Fig. 2C, panels e and f). However, double substitutions of the most conserved Gly⁴² and Pro⁴³ to Ala completely eliminated the nuclear localization of the JEV core protein (Fig. 2C, panel i). These results indicate that Gly⁴² and Pro⁴³, which are well conserved among flaviviruses and HCV (Fig. 2A), are important for nuclear and nucleolar localization of the JEV core protein.

Mutant JEV lacking the nuclear localization of core protein.

To generate a mutant JEV incapable of localizing the core protein in the nucleus, synthetic RNA transcribed from pMWJEAT/GP4243AA encoding a full-length cDNA of mutant JEV M4243 under the T7 promoter was electroporated into Vero cells. The wild type, which was similarly generated from pMWJEATG1, and M4243 viruses were amplified in C6/36 cells after recovery from Vero cells because of a low viral yield of M4243 virus in Vero cells after electroporation (2×10^3 FFU/ml at 3 days after transfection) and used in subsequent experiments. The entire genomic cDNAs of the recovered viruses were confirmed to be identical to those of the infectious clones by direct sequencing. Intracellular localization of core proteins of the wild-type and mutant JEVs was examined in Vero and C6/36 cells by an immunofluorescence assay. In both cell lines, the core protein of the wild-type virus was localized in both the cytoplasm and nuclei whereas the core protein of M4243 was detected only in the cytoplasm and not in nuclei in both cell lines, as we expected (Fig. 3A). To confirm the intracellular localization of the core proteins, cytoplasmic and nuclear fractions of Vero cells infected with the viruses were analyzed by Western blotting (Fig. 3B). The wild-type core protein was fractionated in both cytoplasmic and nuclear fractions, while the mutant core protein was detected in the cytoplasmic fraction but not in the nuclear fraction.

Growth properties of the mutant JEV in vitro. To examine the roles of the nuclear localization of the core protein in viral propagation, one-step growth kinetics of the viruses in Vero and C6/36 cells were determined after inoculation at an MOI of 5 (Fig. 4A). The M4243 virus exhibited impaired propagations, with the infectious titers being 773- and 31-fold lower than those of wild-type JEV at 30 h postinoculation in Vero and C6/36 cells, respectively. These results indicate that Gly⁴² and Pro⁴³ in the JEV core protein were important for viral propagation, especially in Vero cells. The size of infectious foci in Vero cells produced by the M4243 virus recovered from the culture supernatants at 1 day postinfection of Vero cells was markedly smaller than that of the wild-type virus (Fig. 4B, left and middle panels). However, supernatants of Vero cells recovered 3 days after infection with M4243 produced larger foci than those obtained after incubation for 1 day (Fig. 4B, right panel). This phenomenon was not observed in C6/36 cells. To

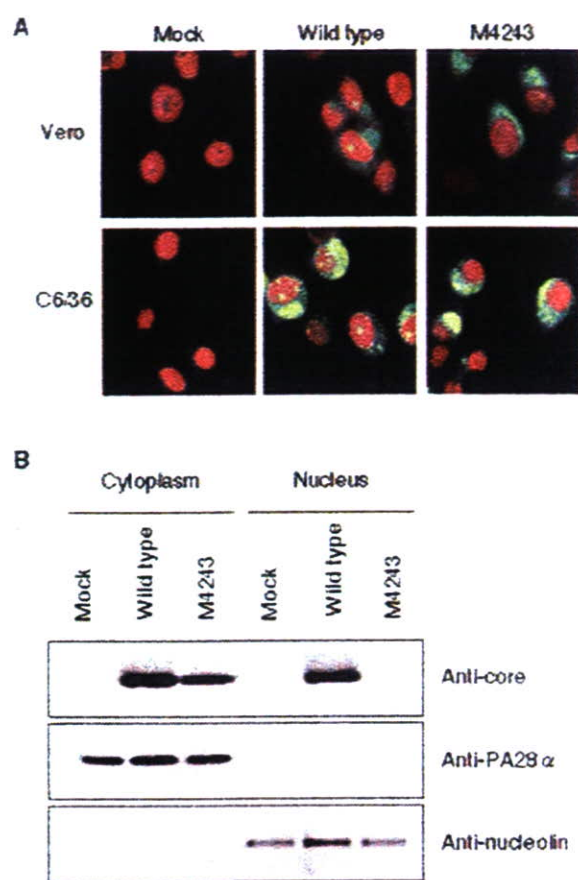


FIG. 3. Intracellular localization of core proteins in cells infected with wild-type or M4243 virus. (A) Intercellular localization of wild-type and mutant JEV. Vero or C6/36 cells were infected with wild-type or M4243 virus, fixed at 2 days postinoculation and immunostained with anti-JEV core rabbit serum. Nuclei were stained with propidium iodide. All samples were observed with a confocal microscope. (B) Intracellular fractionation of Vero cells infected with the viruses. The core proteins in the cytoplasmic and nuclear fractions were detected by Western blotting with the anti-JEV core rabbit serum. Endogenous proteins PA28 α and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

assess the possibility of the emergence of revertant viruses, the nucleotide sequences of two independent clones obtained at 3 days postinfection with M4243 in Vero cells were determined by direct sequencing. The majority of viruses carried a single-amino-acid reversion from Ala to Gly (GCG to GGG) at residue 42 in both clones. The single mutation of Pro⁴³ to Ala of the EGFP-fused JEV core protein did not abolish the nuclear or nucleolar localization, as shown in Fig. 2C (panel g). These results also support the idea that nuclear—especially nucleolar—localization of the JEV core protein is important for viral propagation in Vero cells.

Characterization of the released particles. It has been established that the flavivirus core protein is involved in the assembly and budding of infectious particles as a structural protein (34). Mutations in the core protein might possess the possibility to inhibit the release of infectious particles and, inversely, increase production of defective particles as described in previous reports (25, 26). Therefore, we determined the ratios between the infectivities and quantities of the par-

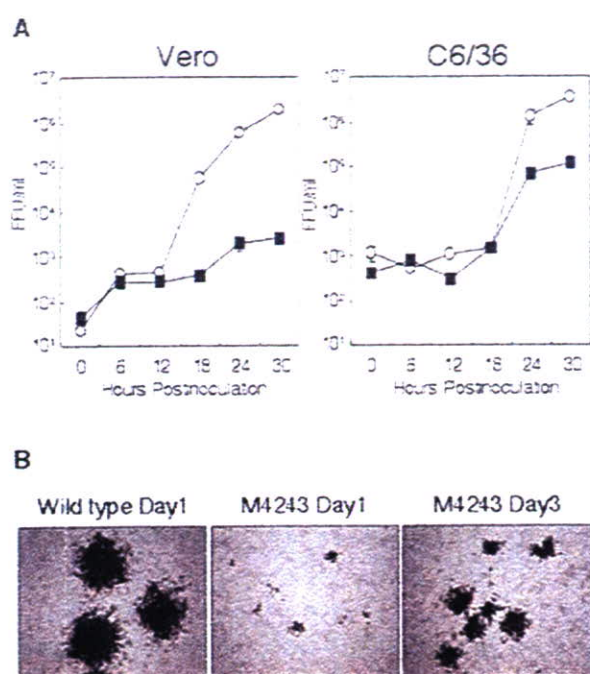


FIG. 4. Growth properties of wild-type and M4243 viruses. (A) Growth kinetics of the viruses in Vero and C6/36 cells. Both cell lines were infected with wild-type or M4243 virus at an MOI of 5. Culture supernatants were harvested at the indicated times postinoculation, and infectious titers were determined by focus-forming assays using Vero cells. Open circles and closed squares indicate the wild-type and M4243 viruses, respectively. Means of three experiments are indicated. (B) Infectious focus formation of the wild-type and M4243 viruses on Vero cells. Culture supernatants recovered at 1 or 3 days postinoculation in Vero cells were inoculated onto Vero cells and incubated for 3 days with methylcellulose overlay medium. The infectious foci were immunostained as described in Materials and Methods.

ticles released from Vero and C6/36 cells infected with the wild-type or M4243 JEV. The HA assay is able to detect viral particles irrespective of infectivity, because HA activity of the flavivirus is associated with E protein (28, 29). As shown in Table 1, the FFU/HA ratios of the wild-type JEV were significantly higher than those of the M4243 virus in both Vero and C6/36 cells, indicating that the M4243 virus produced a larger amount of defective particles than the wild-type virus. Although the ratios of defective particle production were equivalent between Vero and C6/36 cells, the mutant virus exhibited an HA titer comparable to that of the wild-type virus in C6/36 cells but significantly lower than that of the wild-type virus in Vero cells. In addition, the marked difference of infectious

TABLE 1. Infectious and HA titers of wild type and M4243 viruses derived from Vero and C6/36 cells

Cell	Virus ^a	FFU/ml	HA/ml	FFU/HA
Vero	Wild type	1.4×10^{10}	25600	5.5×10^5
	M4243	1.1×10^6	400	2.8×10^3
C6/36	Wild type	1.9×10^9	200	9.5×10^6
	M4243	4.0×10^7	800	5.0×10^4

^a Viruses were 100-fold concentrated by a polyethylene glycol precipitation and determined the infectivities and HA activities.

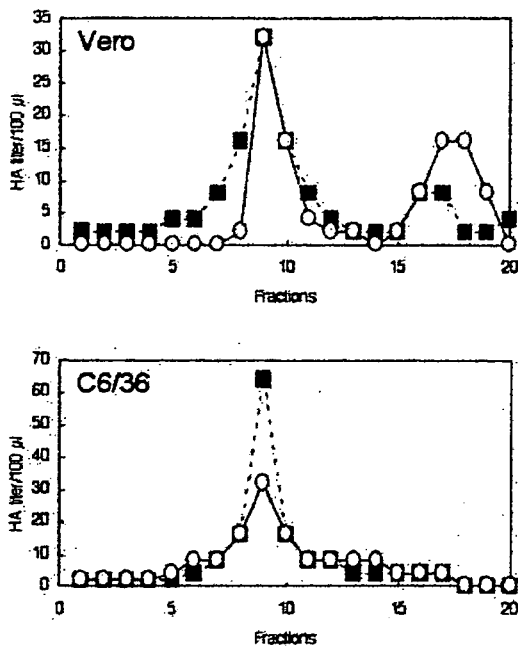


FIG. 5. Gradient fractionation of viral particles of the wild-type and M4243 viruses. The viral particles (400 HA units) derived from Vero or C6/36 cells were applied to 10 to 40% (wt/wt) sucrose gradient and centrifuged at $147,000 \times g$ for 90 min. Twenty fractions were collected from bottom to top and quantified by the HA test. Open circles and closed squares indicate the wild-type and M4243 viruses, respectively. The representative data from three experiments are indicated.

titers between the wild-type and M4243 viruses in Vero cells (Fig. 4A) indicates that there may be another mechanism(s) underlying the low-growth properties of the M4243 virus in Vero cells besides the increased production of defective particles. To examine the production of subviral particles in culture supernatants of cells infected with M4243 virus, we carried out gradient fractionations and quantifications of viral particles by HA assay. As indicated in Fig. 5, the patterns of the fractionations of the particles of the M4243 virus were similar to those of the wild-type virus in both Vero and C6/36 cells, and subviral particles were detected in the fractions (fractions 16 to 19) of the supernatants of Vero cells infected with the wild-type or M4243 JEV.

Effect of nuclear localization of core protein on RNA replication and protein synthesis. To clarify the reasons for the impaired growth of the M4243 virus in Vero cells, we measured viral RNA replication and protein synthesis in Vero and C6/36 cells infected with wild-type and M4243 viruses. It has been reported that the ratio of the positive strands to the negative strands of viral RNA in JEV-infected cells was 3:1 to 11.7:1 (58). Real-time RT-PCR specific for the negative-strand viral RNA used in this study is capable of detecting more than 10^2 copies/2 μ l of the negative-sense viral RNA in the absence of the positive-strand RNA (Fig. 6A). The amounts of negative-strand RNA in the presence of a 100- or 1,000-fold excess amount of the positive-strand RNA were less than 10-fold different compared with those determined in the absence of the positive-strand RNA (Fig. 6A), indicating that the PCR system is specific enough to measure the negative-strand viral

RNA levels in cells infected with JEV. We then measured the synthesis of the negative-strand viral RNAs in Vero and C6/36 cells infected with wild-type or mutant JEV at an MOI of 5 by RT-PCR. Although similar levels of negative-strand RNA synthesis were observed in C6/36 cells infected with either virus, M4243 exhibited 3- and 18-fold-lower RNA replication than the wild type in Vero cells at 18 and 30 h postinoculation, respectively (Fig. 6B). Metabolic labeling of the host proteins indicated that there were no significant differences between the viabilities of Vero cells infected with wild-type and M4243 virus (data not shown). To determine the level of impairment of RNA translation of M4243 in Vero cells, viral protein syn-

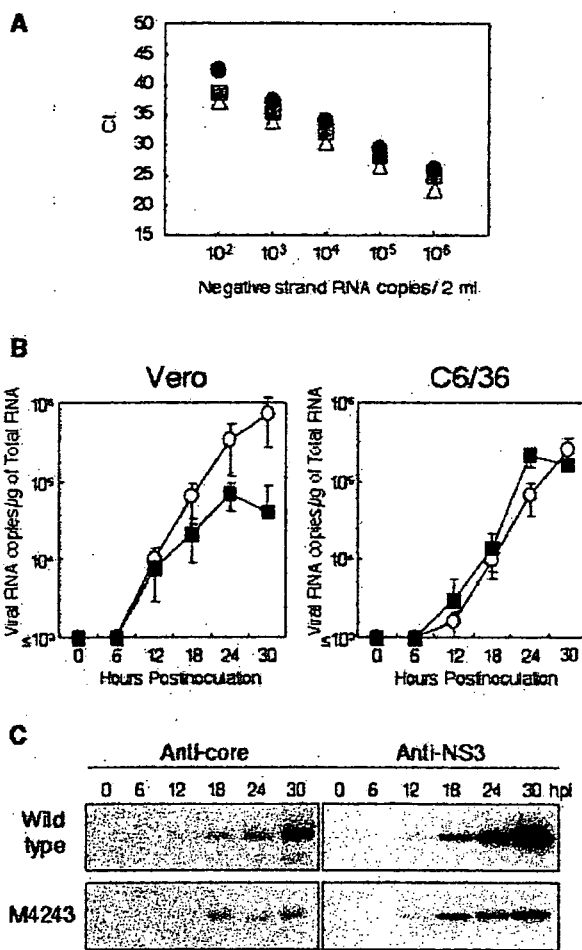


FIG. 6. Viral RNA and protein syntheses in Vero and C6/36 cells infected with wild-type or M4243 virus. (A) Establishment of negative-strand JEV RNA-specific real-time RT-PCR. A series of 10-fold dilutions of synthetic negative-strand RNA in the absence (closed circles) or presence of 100-fold (gray squares) or 1,000-fold (open triangles) synthetic positive-strand RNAs were applied to the real-time RT-PCR. The Ct value represents the first PCR cycle to detect the increase in signal associated with an exponential growth of PCR product. (B) Viral negative-strand RNAs were quantified in the infected cells by the real-time RT-PCR. Open circles and closed squares indicate wild-type and M4243 viruses, respectively. The detection limit was 10^3 copies of viral RNA/ μ g of total RNA. Means of three experiments are indicated. (C) Core and NS3 proteins were detected by Western blotting with anti-JEV core rabbit serum and anti-JEV NS3 MAb 34A1, respectively. A total of 4 μ g of each sample was loaded.

theses in Vero cells infected with the wild-type or M4243 virus were analyzed by Western blotting (Fig. 6C). Although comparable amounts of core and NS3 proteins were detected at an early phase of infection (12 and 18 h postinoculation) in Vero cells infected with either virus, saturation of protein syntheses by a mutant virus was observed at 24 h postinfection, in contrast to cells infected with the wild-type virus, in which protein synthesis increased until 30 h postinfection. The early saturation of viral protein synthesis of M4243 in Vero cells is quite consistent with that of RNA replication. These results suggest that nuclear localization of the core protein plays a crucial role in the maintenance of replication-translation of viral RNA in mammalian cells but not in mosquito cells, in which the mutant virus replicates at a rate similar to that of the wild-type JEV.

Complementation of mutant virus replication by expression of the wild-type core protein. We next examined the growth of a mutant virus in cells transiently expressing the wild-type or mutant core protein. The efficiency of gene transduction into Vero cells is very low, and we therefore selected 293T cells for their high efficiency of foreign-gene transduction and used them to transiently express the JEV core protein. HA-tagged wild-type and mutant core proteins (approximately 16 kDa) and the viral core protein derived from M4243 (approximately 14 kDa) were detected in 293T cells transfected with the expression plasmids and infected with M4243 virus (Fig. 7A). Expression of the wild-type core protein, but not that of the mutant core protein, drastically enhanced viral growth of the M4243 virus up to the level of wild-type virus growth (Fig. 7B). However, the expression of the core proteins did not affect the replication of the wild-type virus. Furthermore, the negative-strand RNA synthesis of the mutant virus was increased three-fold by the expression of the wild-type core protein, but not by that of the mutant protein, compared with mock-transfection results (Fig. 7C). These results indicate that the expression of the wild-type JEV core protein is able to compensate for the propagation of the M4243 virus.

Neurovirulence and neuroinvasiveness of M4243. To examine the neurovirulence characteristics of the wild-type and mutant viruses, we determined the LD₅₀ values by intracerebral inoculation of the viruses into 3-week-old ICR mice. The LD₅₀ values for the wild-type and the mutant viruses were 2.1 and 0.5 FFU, respectively. No significant differences in symptoms, mean duration period of diseases (wild versus mutant, 1.1 versus 0.9 days), and mean day of death (7.5 versus 7.6 days postinoculation) were observed between mice inoculated with 100 FFU of the wild-type virus and those inoculated with an equivalent dose of M4243 virus. To examine the growth kinetics of the viruses in the brain, 100 FFU of each virus was intracerebrally injected, and the viruses recovered from the brain homogenates were titrated. The growth of the M4243 virus was approximately 100 times lower than that of the wild-type virus (Fig. 8A, left panel), and revertant viruses exhibiting medium-sized plaques (Fig. 4B) were not recovered from the brains inoculated with the M4243 virus. The neuroinvasiveness of encephalitis flaviviruses is thought to be a reflection of their ability to grow in the peripheral organs, to breach the blood-brain barrier, and to infect central nervous systems following peripheral inoculation. To examine the neuroinvasiveness of wild-type and M4243 viruses, ICR mice were intraperitoneally

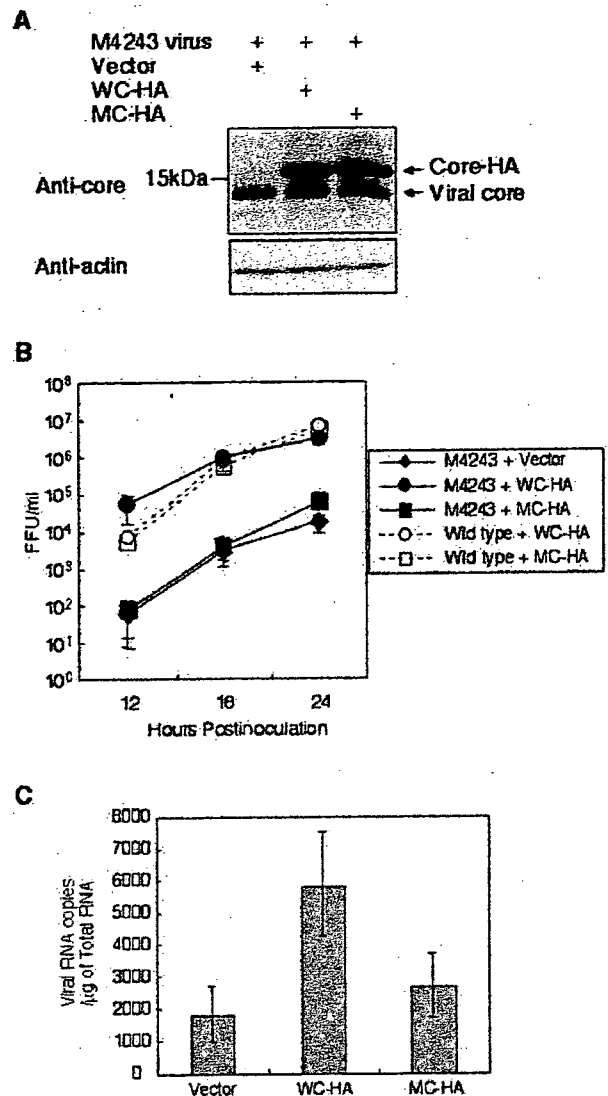


FIG. 7. Complementation of M4243 replication by expression of core proteins in 293T cells. At 4 h after transfection with pCAG-GS (Vector), pCAG-WC-HA (WC-HA), or pCAG-MC-HA (Gly⁴² and Pro⁴³ to Ala) (MC-HA), 293T cells were infected with wild-type or M4243 virus at an MOI of 5. At 12, 18, or 24 h postinoculation, culture supernatants and cells were harvested to apply to focus-forming assays and Western blotting or real-time RT-PCR, respectively. (A) Western blotting of 293T cells transfected with plasmids expressing HA-tagged wild-type or mutant core protein and infected with M4243 virus. Molecular mass marker was indicated in the left of the panel. (B) Growth of wild-type and M4243 viruses in 293T cells transfected with the plasmids. Viral titers were determined by focus-forming assays in Vero cells. (C) Complementation of M4243 in 293T cells transfected with the plasmids at 24 h postinoculation. Viral RNA levels were determined by the negative-strand-specific real-time RT-PCR. Means from three experiments are indicated.

inoculated with 10⁵ FFU of each virus. Only 1 of 11 mice inoculated with the M4243 virus had died by 9 days postinoculation, while on average 10 of the 11 mice inoculated with the wild-type virus had died by 9.6 days postinoculation (Fig. 8B). Over 5 days after inoculation, the viruses were recovered from the brain of mice inoculated with wild-type JEV but not from those inoculated with M4243 (Fig. 8A, right panel). These re-

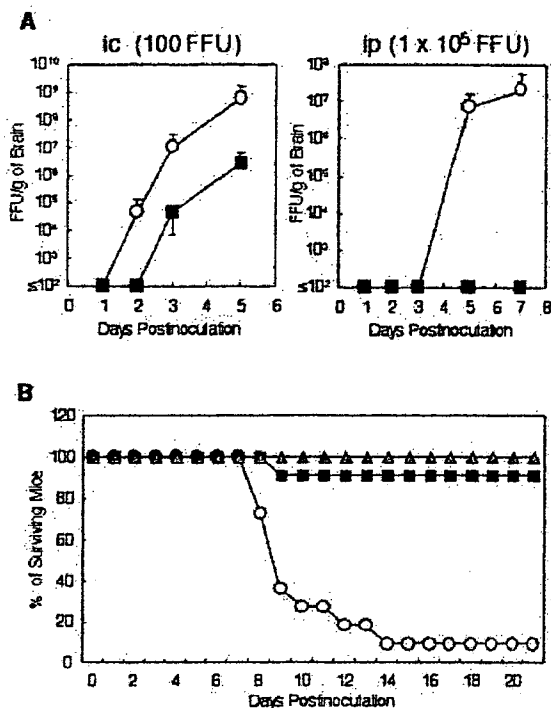


FIG. 8. Virulence of wild-type and M4243 viruses for ICR mice. (A) The infectious titers of wild-type and M4243 viruses in the brains of mice after inoculation with 100 FFU of the viruses intracerebrally (ic) or 10⁵ FFU intraperitoneally (ip). Open circles and closed squares indicate wild-type and M4243 viruses, respectively. The detection limit is 10² FFU/gram of brain. Means of titers from four mice are indicated. (B) Percentages of surviving mice (10 to 11 mice per group) inoculated with 10⁵ FFU of wild-type or M4243 viruses by an intraperitoneal route. Open circles, closed squares, and gray triangles indicate mice infected with the wild-type virus, infected with the M4243 virus, or mock infected, respectively.

sults indicated that the mutant virus exhibited a level of neurovirulence equivalent to that of the wild type but that its neuroinvasiveness was severely impaired in 3-week-old-ICR mice.

DISCUSSION

Like most animal RNA viruses, except for those of the families *Orthomyxoviridae*, *Bornaviridae*, and *Retroviridae*, members of the *Flaviviridae* replicate in the cytoplasm of host cells (34). However, it has been reported that the core proteins of DEN, KUN, and HCV are observed not only in the cytoplasm but also in the nucleus (4, 38, 55, 59, 61). In this study, we demonstrated that the JEV core protein was translocated into the nucleus and accumulated in the nucleolus of cells infected with JEV or transfected with an expression plasmid for the core protein. We revealed that Gly⁴² and Pro⁴³ were important for the nuclear localization and that Gly⁴² was essential for the nucleolar accumulation of the JEV core protein. The two amino acids Gly and Pro are well conserved not only among mosquito-borne flaviviruses such as JEV, KUN, WNV; and DEN but also among HCVs. According to the three-dimensional structures of KUN and DEN, the two amino acids are mapped to the unsheltered loop domain between α -helices 1 and 2 (11, 36). Substitutions of Gly⁴² and Pro⁴³ with Ala completely abolished the nuclear localization of the JEV core pro-

tein as well as that of the DEN core protein (data not shown). However, a previous study showed that deletion of the N-terminal 45 amino acids of the DEN core protein did not eliminate nuclear localization of the protein (59). Although the reason for this discrepancy is not presently clear, our data suggest that the well-conserved Gly and Pro are important for nuclear or nucleolar localization of the core protein of flaviviruses. The two amino acids and the flanking sequences exhibited no similarity to the well-known classical NLSs, such as a large T-antigen of simian virus 40 (PKKKRKV) (21), nucleoplasmin (KRPAATKKAGQAKKKK) (5), and nucleolar localization signals, such as the Rex protein of human T-cell leukemia virus type I (MPKTRRRPRRSQRKRPPTP) (44). This domain may be required for recognition of novel nuclear import receptor protein(s) and nucleolar component(s). Recently, Dokland et al. demonstrated that tetramers of the KUN core protein extended as filamentous ribbon structures (11). The change of Gly⁴² to Ala in the JEV core protein, therefore, may abolish the binding activity to nucleolar compartment(s) and exhibit a filamentous structure in the nuclei (Fig. 2C).

The recovery of the M4243 virus in which Ala was substituted for Gly⁴² and Pro⁴³ of the core protein suggests that nuclear localization of the core protein is not a necessary condition for viral propagation. However, replication of the mutant virus was impaired in mammalian cells but not in mosquito cells. The impairment of propagation of the M4243 virus in mammalian cells may be due to either of two phenomena: the decrease in production of infectious particles with a simultaneous increase of defective particles or the low efficiency of viral RNA replication. Since budding of flavivirus takes place through the interaction of prM and E proteins independently of the association with the core protein, disfunctions of core protein may reduce the production of infectious particles and enhance the production of subviral particles. In fact, mutations at the cleavage site of host signal peptidase in the core-prM junction of Murray Valley encephalitis virus caused an increase of subviral particle production (35). Furthermore, TBEV core proteins with deletions of the central hydrophobic region, containing Gly³⁶ corresponding to the Gly⁴² essential for nucleolar localization of the JEV core protein, increased the production of subviral particles due to the lack of association with the ER membrane, where budding of the flavivirus takes place (26, 27). In contrast, the mutations on the Gly⁴² and Pro⁴³ region of the JEV core protein caused the production of the defective particles different from subviral particles. Although the mechanisms underlying the production of the defective particles remain unclear, the mutations might affect the functions except for assembly and budding, such as maturation or uncoating. As far as we know, biological functions except for nuclear localization in the Gly⁴² and Pro⁴³ region of the flavivirus core proteins have not been studied. The mutations might collaterally disrupt the conformation of the core protein essential for their functions of JEV. Meanwhile, it might be feasible that the nuclear localization of the core protein directly participates in the viral infectivity. Tijms et al. (56) demonstrated that the capsid protein of equine arteritis virus mostly shuffled between the cytoplasm and nucleus prior to cytoplasmic viral assembly, suggesting that the nuclear localization is crucial for viral assembly. In any case, production of the defective particles by the mutant JEV was enhanced in both Vero and C6/36 cells, and

thus, a decrease of infectious particles cannot explain the impairment of M4243 in Vero cells.

It is believed that the core protein is not required for RNA replication, since the RNA replicon of flavivirus, which does not contain the whole core gene, has been shown to be capable of replicating (10, 13, 24). Therefore, it is noteworthy that RNA replication of M4243 was impaired in Vero cells but not in mosquito cells. Although a *cis*-acting nucleotide sequence element essential for RNA replication has been mapped to the flavivirus core genes (10, 22), the nucleotide changes were not involved in the impairment of replication of M4243 due to the compensation of RNA replication by the expression of wild-type core protein in *trans*. The kinetics study of viral RNA and protein syntheses suggested that the JEV core protein translocated at the early step of infection was translocated into the nucleus and enhanced RNA replication at the late phase of infection, although further studies are needed to clarify the precise mechanism. Earlier studies (30, 31) resulted in reports that flaviviruses, including JEV, but not alphaviruses failed to propagate and produce viral antigens in cells enucleated by cytochalasin B, suggesting the involvement of host nucleus factors in flavivirus replication. The DEN core protein was reported to interact with a nuclear transcription factor, heterogeneous nuclear ribonucleoprotein K, and regulate the C/EBP- β -mediated transcription (7). Furthermore, the HCV core protein was also shown to associate with host nuclear proteins such as heterogeneous nuclear ribonucleoprotein K (18) and PA28 γ (42) and was suggested to regulate the transcription of host cells (18, 45). The nuclear localization of core proteins of *Flaviviridae* might change the suitability of the host-cell environment for viral propagation by producing factors that enhance RNA replication or by suppressing those that reduce it.

Other cytoplasmic RNA viruses, such as members of the families *Picornaviridae* (1, 2, 12), *Coronaviridae* (8, 15, 62), *Arteriviridae* (48, 56), *Togaviridae* (41), and *Rhabdoviridae* (46), may also feed their proteins into the nucleus to facilitate viral propagation. For example, it is suggested that the protein 2A of encephalomyocarditis virus is localized to the nucleoli and inhibits cellular mRNA transcription (1) and that point mutations within the NLS of another nucleolar protein, 3D^{pol}, were lethal due to the inhibition of viral RNA replication (2). The coronavirus nucleoproteins were also found to be localized in the nucleoli of the host cells (8, 15, 62), and the expression of the coronavirus nucleoproteins by transfection inhibits host cell division (8, 62).

Surprisingly, the M4243 virus exhibited a high level of neurovirulence in mice comparable to that of the wild-type JEV despite the fact that the M4243 virus had a 100-fold-lower replication efficiency than the wild-type JEV *in vivo*. Encephalitis induced by flavivirus infection is thought to arise from direct injury of brain neurons by viral replication (49, 64) or indirect injury of brain neurons by immune responses (60). However, the idea that the direct injury of neurons is responsible for encephalitis induced by flavivirus is difficult to reconcile with the results showing that the virulence of the M4243 virus is equivalent to that of the wild-type virus. A previous study indicated that a low dose of WNV-induced encephalitis was associated with inflammatory cell infiltration in mice (60). Alternatively, the defective particles contained in the inoculum or produced by M4243 infections might stimulate a signal path-

way via reactive oxygen species in neuronal cells and induce cell death, as described by Lin et al. (33).

In contrast to the neurovirulence results, a striking difference in levels of neuroinvasiveness was observed between the wild-type and M4243 viruses. Although the magnitude and duration of viremia were suggested to be major determinants for neuroinvasion (19, 32), the precise mechanism by which flaviviruses breach the blood-brain barrier and enter the brain tissue remains uncertain. Encephalitis flaviviruses injected by peripheral routes are thought to replicate in lymphatic tissues, such as peripheral lymph nodes or spleen, and to induce further viremia (32, 40). Although we had no evidence that JEV replicated in peripheral tissues prior to neuroinvasion as described by others (32), the wild-type virus, but not M4243, was present in small amounts (100 to 200 FFU/ml) in blood samples at 1 and 3 days postinoculation (data not shown). In this context, it might be possible that the M4243 virus was unable to replicate in peripheral tissues at a level sufficient to develop viremia and breach the blood-brain barrier, resulting in the low level of neuroinvasiveness. The novel attenuation of neuroinvasiveness observed in M4243 may be applicable to the development of new live vaccines against flavivirus infection.

The life cycles of most flaviviruses are sustained between arthropods and vertebrates. The present finding that nuclear localization of the core protein enhances viral replication in mammalian cells may lead to an improved understanding of the evolutionary adaptation strategy of flaviviruses in expanding their host range from arthropods to vertebrates. It might also be possible to speculate that JEV transgressed its host barrier by translocating the core protein into the nucleus of porcine cells and incorporated the pig as an amplifier in the life cycle of JEV. Furthermore, it is of interest for evolutionary studies on the family *Flaviviridae* that the NLSs of core proteins were well conserved between mosquito-borne flaviviruses and bloodborne and human-adapted HCV.

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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 PA28 γ (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-hSRP1 α (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 μ g of streptomycin per ml, and 10% fetal bovine serum at 37°C in a 5% CO₂ 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-H₂XRos

(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 NH₄Cl 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 \times g for 10 min. The supernatant was then collected and centrifuged at 105,000 \times 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 \times 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 \times g for 10 min to remove large debris and nuclei. The resulting supernatant was then centrifuged at 20,000 \times 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 \times 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 μ g 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 (Immobilion; 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-

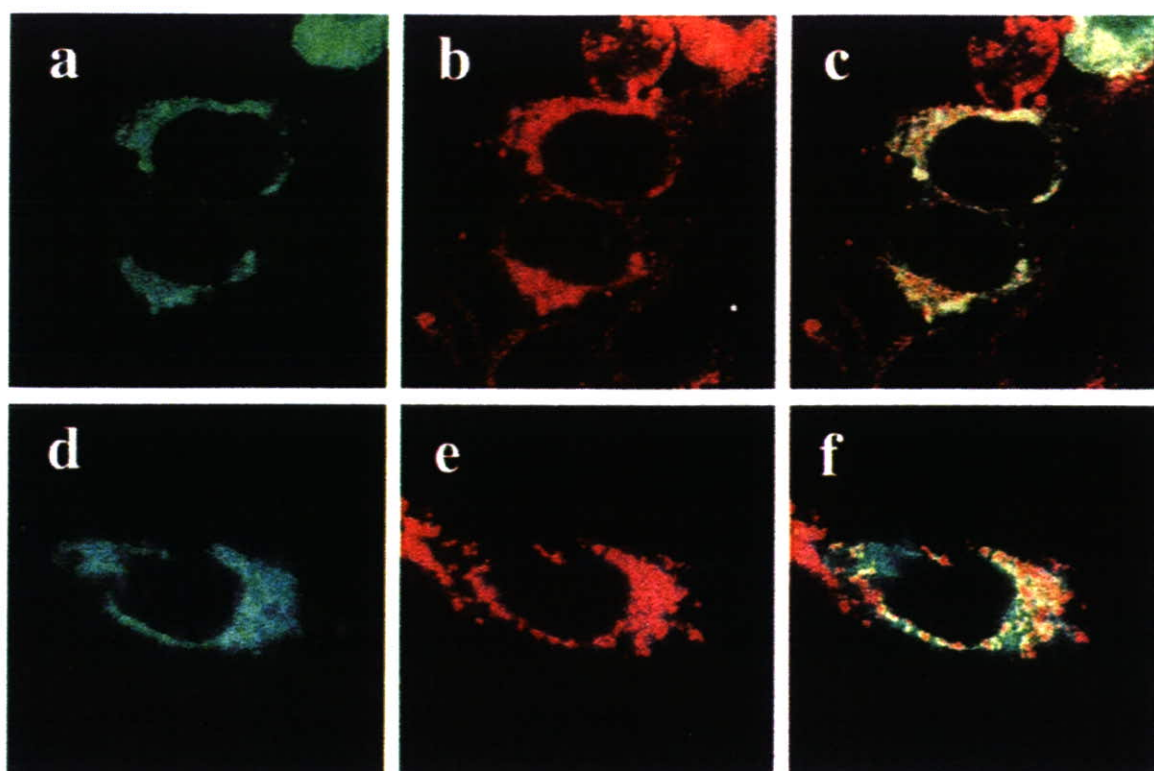


FIG. 1. Confocal analysis of double staining for HCV core protein and ER or mitochondria. 293T cells transfected with full-length HCV core expression plasmid, pCAGC191 were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c) or fixed after loading with Mitotracker (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-hSRP1 α 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 [³⁵S]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,

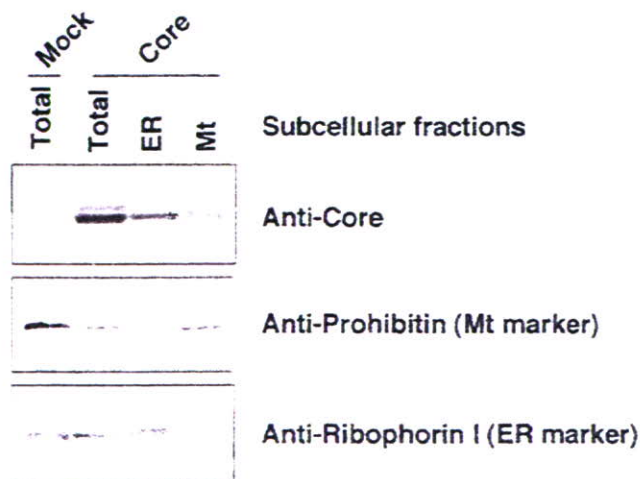


FIG. 2. Subcellular distribution of HCV core protein analyzed by immunoblotting. ER and mitochondrial (Mt) fractions were isolated from 293T cells expressing the full-length core protein (Core) or non-transfected cells (Mock) 2 days after transfection. Equal amounts of protein from each fraction as well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

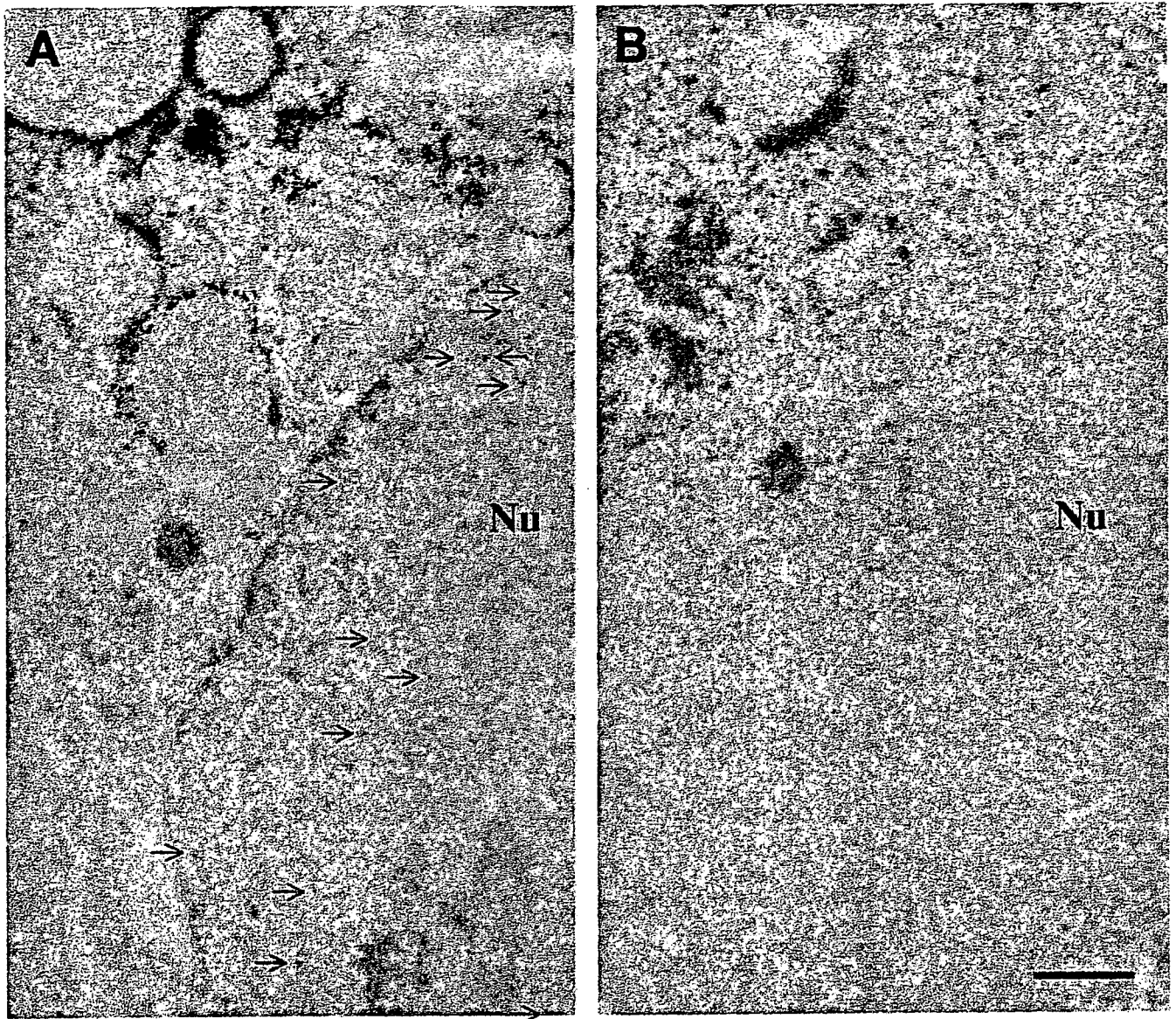


FIG. 3. Immunoelectron microscopy of HCV core protein. 293T cells expressing the full-length core protein (A) and nonexpressing cells (B) fixed 2 days after transfection. Immunoelectron microscopic analysis was performed with a mouse anticore antibody and a secondary anti-mouse IgG conjugated with gold 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-

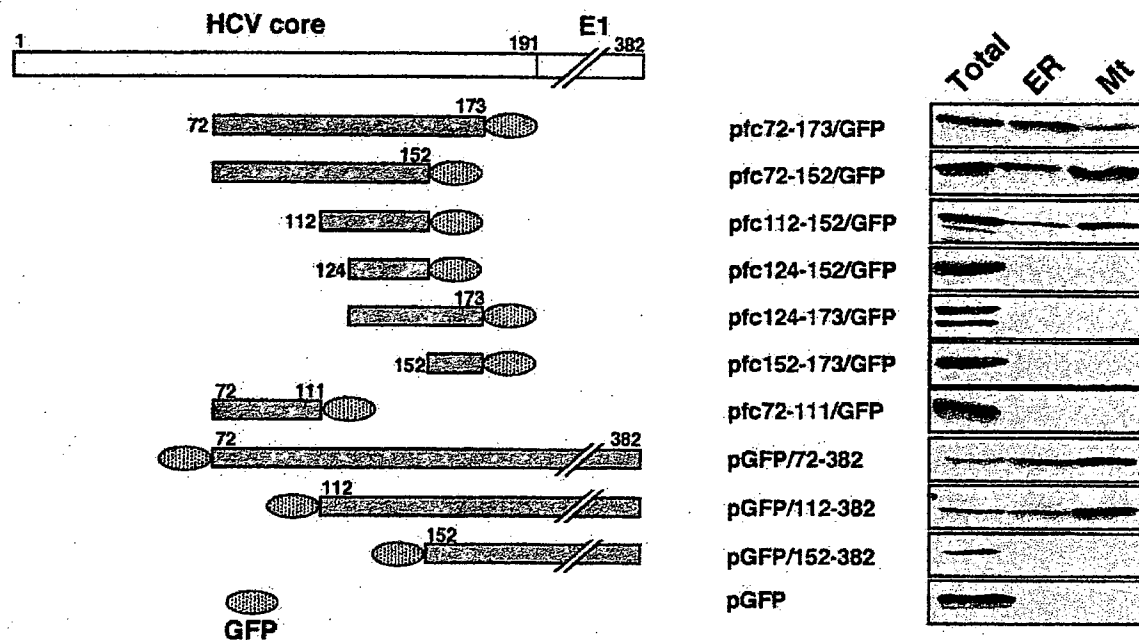


FIG. 4. Identification of segments that mediate association with ER and mitochondria in the core protein. Schematic diagram (left) and nomenclature (middle) of the core-GFP fusions are shown. Gray bars, expressed core and E1 regions. Subcellular distribution of fusion proteins is indicated on the right. 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.

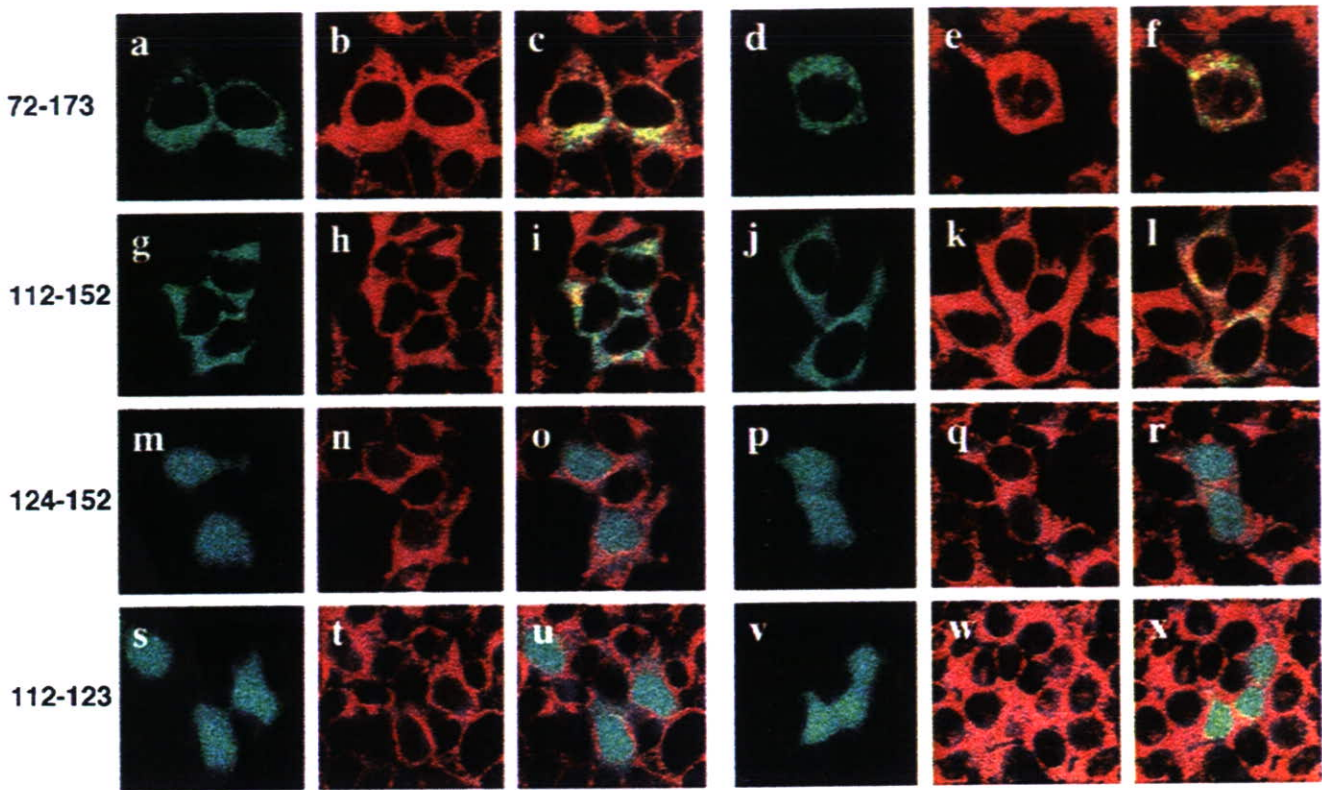


FIG. 5. Confocal analysis of double staining for core-GFP fusion protein and ER or mitochondria. 293T cells transfected with core-GFP expression plasmids (72-173, 112-152, 124-152, and 112-123) were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c, g to i, m to 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 antiregulin 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- β 1, 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 pull-down 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* pull-down 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