るタマリンに GBV-B 分子クローン由来 RNA を接種することによりウイルス感染が成立し急性C型肝炎様症状を呈することが確認された。本研究では分子クローンを出発材料としていることから、今後のワクチンや遺伝子治療研究において既知のウイルス遺伝子情報を利用可能であることから、動物モデルとしての有用性が高いものと考えられる。

　興味深いことに、GBV-B 感染タマリン Tm5 は ALT 低値&plasma RNA 高値を示した。これは、肝外組織、特に血液リンパ系組織において顕著にウイルスが増殖したことが原因であることが明らかとなった。このことは、GBV-B が HCV と同様に肝臓のみならず多様な組織への指向性を有する、いわゆる「pleiotropic virus」であることを初めて実証するものであり、非常に意義深い。特に HCV 感染者においても多様な臓器における肝外病変が報告されていることを考慮すると、本モデルは C 型肝炎を含めた HCV 感染症全体の感染モデルとしての有用性を示すものである。

　上記 pleiotropism に関して、Tm5 由来ウイルスゲノムの遺伝子全シークエンス解析を行ったところ、残念ながら Tm3,4 と異なる特異的な変異は認められなかった。このことから、本ウイルスが Tm5 において特異な変異が起こったことにより pleiotropism を獲得したのではなく、Tm5 個体に何らかの要因があったものと考えられた。今後その原因についてさらに詳細な解析を行っていきたい。

　GBV-B 感染モデルとして、今回新世界サルであるタマリン及びマーモセットを用いてその感受性について比較検討を行った結果、ウイルス増殖効率及び肝炎発症といった観点からタマリンが有用であると考えられた。タマリン使用に際しては、当初入手の困難さが問題となっていた。しかし最近では、唯一のタマリン輸出可能国であるスリナムからの輸入が可能となりつつあり、安定的な供給が期待出来る状況となっている。従って、今後はタ

マリンを中心とするモデル開発を主眼として検討を進めていく予定である。

E．結論

　本研究ではC型肝炎のサロゲート病態動物モデルの開発を試みた。HCV に最も近縁なサル肝炎ウイルスである GBV-B を新世界ザルの一種であるタマリンにチャレンジすることにより、ウイルス増殖に伴う急性C型肝炎様症状を再現性良く発症させることに成功した。また GBV-B が HCV と同様に多様な組織へのトロピズムを有するウイルスである事が明らかとなった。これらの結果より、C型肝炎のサロゲートモデル確立のための基礎がほぼ確立出来たと考えられた。

F．研究発表
1.学会発表
　1. 飯島沙幸、石井孝司、李永仲、八木慎太郎、山口健次郎、槇昇、吉崎佐矢香、町田早苗、木村展之、揚山直英、寺尾恵治、鈴木哲朗、宮村達男、明里宏文：サル類を用いたC型肝炎の新規感染病態モデルの樹立．第 140 回日本獣医学会学術集会、平成 17 年 9 月。
　2. Koji Ishii, Sayuki Iijima, Nobuyuki Kimura, Naoko Iwata, Shintaro Yagi, Kenjiro Yamaguchi, Noboru Maki, Sayaka Yoshizaki, Sanae Machida, Tetsuro Suzuki, Tetsutaro Sata, Tatsuo Miyamura, Hirofumi Akari: GBV-B is a pleiotropic virus in vivo. 12th International symposium on hepatitis C and related viruses, Montreal, October 2-6, 2005.
　3. 石井孝司、飯島沙幸、山口健次郎、槇昇、八木慎太郎、森健一、吉崎佐矢香、李永仲、木村展之、揚山直英、鈴木哲朗、宮村達男、明里宏文：タマリンを用いたC型肝炎のサロゲートモデル：tissue

tropism に関する解析．第 53 回日本ウ
イルス学会学術集会、平成 17 年 11 月。
4. 明里宏文：新世界ザルを用いた C 型肝炎
のサロゲートモデル開発．第 12 回サル
類疾病国際ワークショップ、平成 17 年
12 月。

H. 知的財産権の出願・登録状況
1. 特許取得
なし
2. 実用新案登録
なし
3. その他
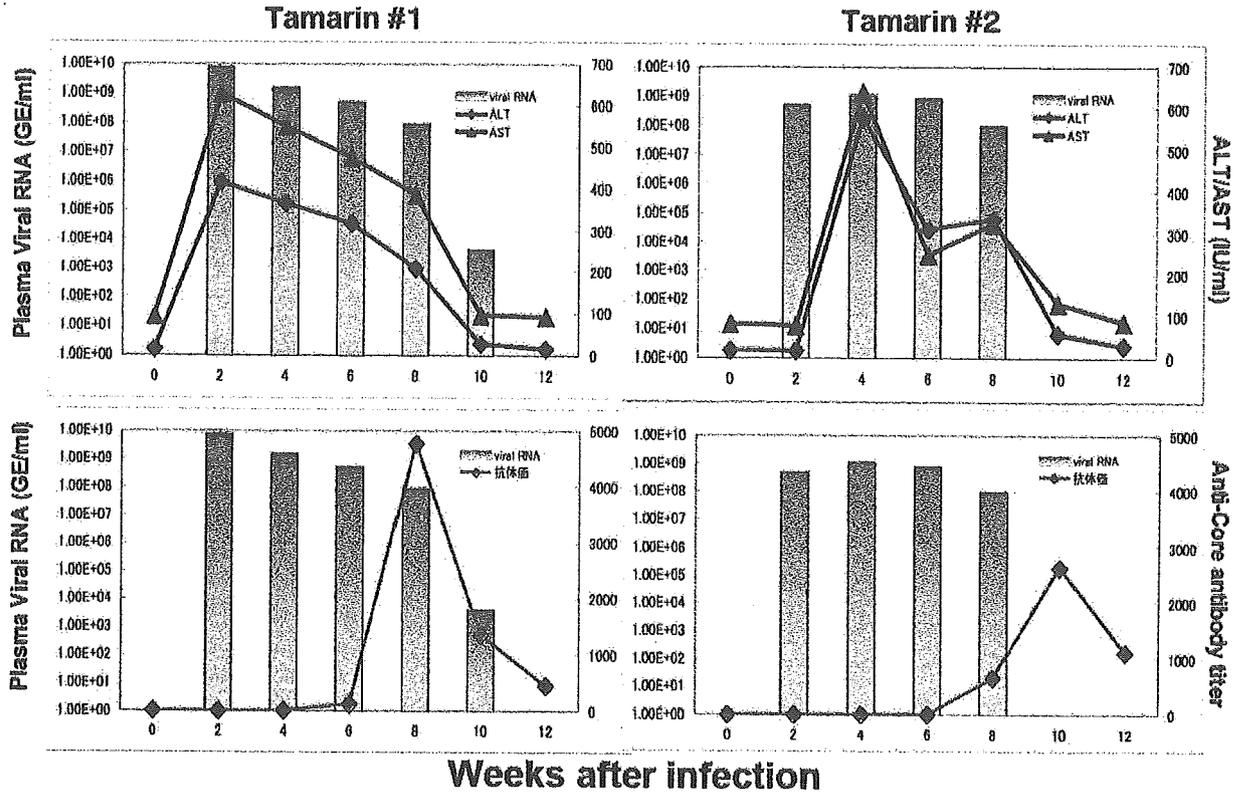なし

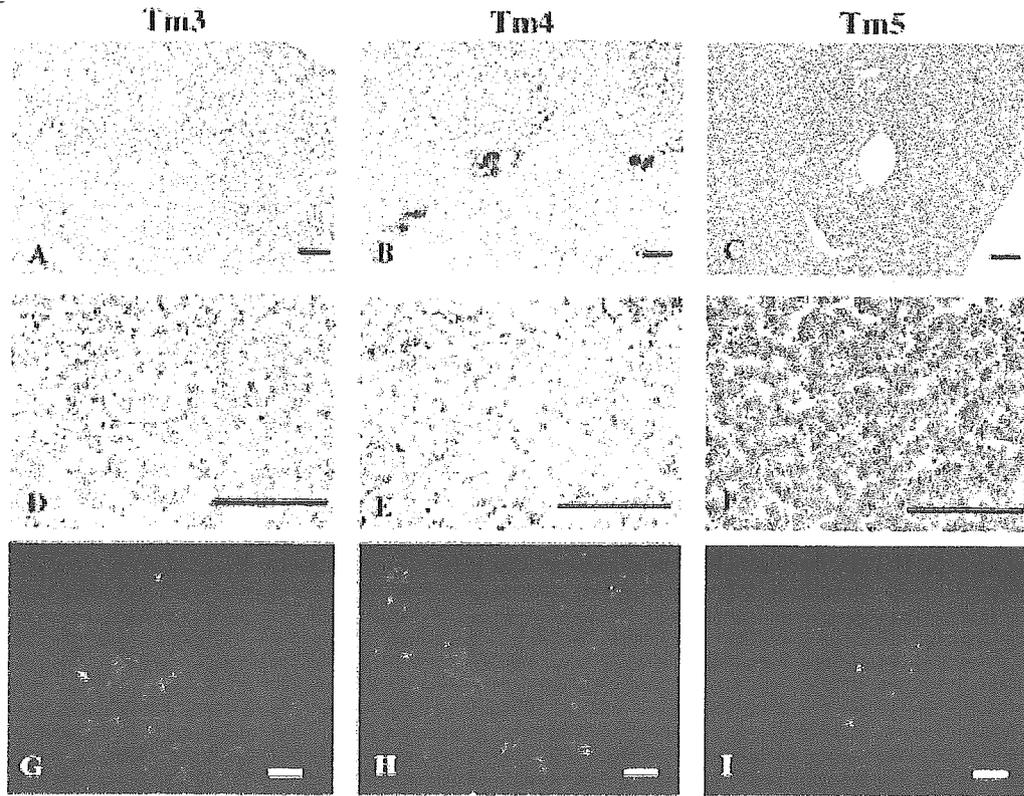**図 1 ： GBV-B RNA/tamarin 接種実験結果**

図2 ： GBV-B感染tamarin由来肝臓における病理組織検索
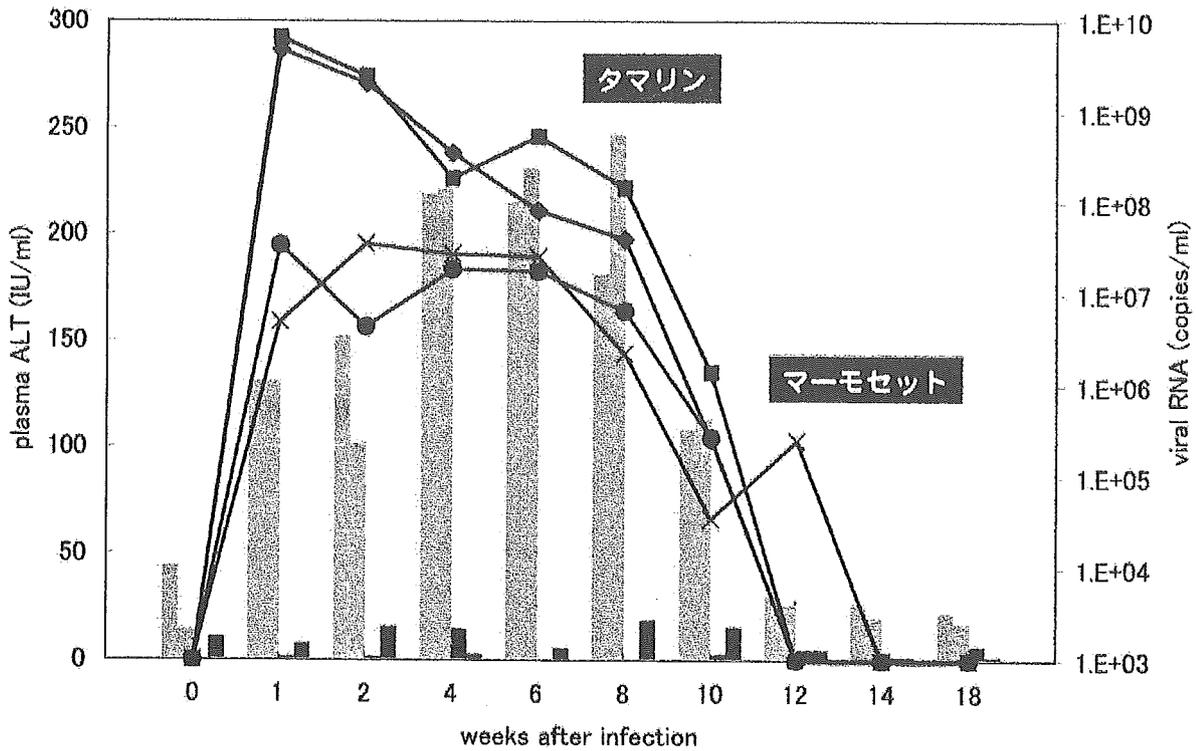

図3 ： タマリンおよびマーモセットにおけるGBV-B感染実験結果

表1：GBV-B感染タマリンにおけるウイルス体内分布

		Tm3	Tm4	Tm5	Tm6
animals		S. labiatus	S. midas	S. labiatus	S. midas
materials for GBV-B inoculation		plasma	plasma	RNA	RNA
weeks at necropsy		4	4	3	alive
ALT		321	522	38	554
viral loads in:					
blood	plasma	3.8×10^8	5.9×10^8	1.3×10^{10}	2.8×10^9
	PBMC	273	1630	35645	ND
spleen		0.1	540	5976	ND
lymph nodes	inguinal	3.7	11	3089	ND
	intestinal	25	0.3	641	ND
liver		70075	33483	16077	ND
kidney		77	39	374	ND
testis (ovary)		1291	154	600	ND

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Zhang J, Yamada O, Sakamoto T, Yoshida H, Araki H, Shimotohno K.	Exploiting cis-Acting Replication Elements To Direct Hepatitis C Virus-Dependent Transgene Expression.	J Virol	79	5923-5932	2005
Takahashi H, Yamaji M, Hosaka M, Kishine H, Hijikata M, Shimotohno K.	Analysis of the 5' end structure of HCV subgenomic RNA replicated in a Huh7 cell line.	Intervirology	48	104-111	2005
Obata Y, Yamamoto K, Miyazaki M, Shimotohno K, Kohno S, Matsuyama T.	Role of cyclophilin B in activation of interferon regulatory factor-3.	J Biol Chem	280	18355-18360	2005
Kato N, Nakamura T, Dansako H, Namba K, Abe K, Nozaki A, Naka K, Ikeda M, Shimotohno K.	Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture.	J Gen Virol	86	645-656	2005
Murata T, Ohshima T, Yamaji M, Hosaka M, Miyanari Y, Hijikata M, Shimotohno K.	Suppression of hepatitis C virus replicon by TGF-beta.	Virology	331	407-417	2005
Naka K, Takemoto K, Abe K, Dansako H, Ikeda M, Shimotohno K, Kato N.	Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors.	J Gen Virol	86	2787-2792	2005
Murata T, Hijikata M, Shimotohno K.	Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis C virus by PD98059.	Virology	340	105-115	2005
Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K.	Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase.	Mol Cell	19	111-122	2005
Nomura-Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K. Hotta H.	The nonstructural protein 4A of hepatitis C virus accumulates on mitochondria and renders the cells prone to undergo mitochondria-mediated apoptosis.	J Gen Virol	87	In press	2006
Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y,	The NS3 protein of hepatitis C virus associates with the tumour suppressor p53 and	J Gen Virol	87	in press	2006

Ikeda M, Kato N, Sada K, Hotta H.	inhibits its function in an NS3 sequence-dependent manner.				
Tanaka M, Nagano-Fujii M, Deng L, Ishido S, Sada K, Hotta H.	Single-point mutations of hepatitis C virus NS3 that impair p53 interaction and anti-apoptotic activity of NS3.	Biochem Biophys Res Commun	340	792-799	2006
Hidajat R, Nagano-Fujii M, Deng L, Tanaka M, Takigawa Y, Kitazawa S, Hotta H.	Hepatitis C virus NS3 protein interacts with ELKS-δ and ELKS-α, members of a novel protein family involved in intracellular transport and secretory pathways.	J Gen Virol	86	2197-2208	2005
Kadoya H, Nagano-Fujii M, Deng L, Nakazono N, Hotta H.	Nonstructural proteins 4A and 4B of hepatitis C virus transactivate the Interleukin 8 promoter.	Microbiol Immunol	49	265-273	2005
Shingai M, Seya T.	A mouse model for wild-type measles virus infection: CD11c-positive dendritic cells are nidus for systemic viral spreading.	J Immunol	175	3252-3261	2005
Okahira S, Seya T.	Interferon-β induction through Toll-like receptor 3 depends on double-stranded RNA structure.	DNA Cell Biol	24	614-623	2005
Tsuji Y, Seya T.	Differential-expression and tyrosine-phosphorylation profiles of caveolin isoforms in human T cell leukemia cell lines.	Int J Mol Med	16	889-893.	2005
Tsujita T, Seya T.	Fish soluble Toll-like receptor (TLR)5 amplifies human TLR5 response via physical binding to flagellin.	Vaccine	24	2193-2199	2006
Muromoto R, Seya T.	Physical and functional interactions between STAT3 and Kaposi's sarcoma-associated herpesvirus-encoded LANA.	FEBS Lett	580	93-98	2006
Ii M, Seya T.	A novel cyclohexene derivative, TAK-242, selectively inhibits Toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling.	Mol Pharmacol		Dec 22 [Epub ahead of print]	2005
Murai M, Seya T.	IRAK-4-dependent phosphorylation and ubiquitination of IRAK-1 is a negative feedback signal for TLR-mediated NF-kB	FEBS J		in press	

	activation.				
Sukenobu N, Seya T.	Protein kinase Cd associates TIRAP/Mal and regulates NF-kB and mitogen-activated protein kinases in Toll-like receptor signaling.	J Immunol		in press	
Seya T, et al.	Antibodies against human Toll-like receptors (TLRs): TLR distribution and localization in human dendritic cells.	J Endotoxin Res	11	369-374	2005
Sasai M, Seya T.	The TICAM-1 pathway in Toll-like receptor signaling.	J Biochem	139	171-175	2006
Seya T, et al.	Application of Toll-like receptor agonists to vaccine adjuvant therapy.	eCAM (review)		in press	
Umehara T, Fukuda K, Nishikawa F, Kohara M, Hasegawa T, Nishikawa	Rational design of dual-functional aptamers that inhibt protease and helicase activities of HCV NS3	J Biochem	137	339-347	2005
Higashi K, Tsukiyama-Kohara K, Tanaka T, Tanaka E, Kiyosawa K, Kohara M.	Characterization of hypervariable region in hepatitis C virus envelope protein during acute and chronic infection.	Arch Virol	150	883-898	2005
Hayashi K, Hasuike S, Kusumoto K, Ido A, Uto H, Kenji N, Kohara M, Stuver SO, Tsubouchi H.	Usefulness of a new immuno-radiometric assay to detect hepatitis C core antigen in a community-based population.	J Viral Hepatol	12	106-110	2005
Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, Ohta A, Tsukuda T, Shimma N, Aoki Y, Arisawa M, Kohara M, Sudoh M.	Host sphingolipid biosynthesis as a target for hepatitis C virus therapy.	Nature Chemical Biology	1	333-337	2005
Hayashida K, Daiba A, Sakai A, Tanaka T, Kaji K, Inaba N, Ando S, Kajiyama N, Terasaki H, Abe A, Ogasawara M, Kohara M, Harada M, Okanoue T, Ito S, Kaneko	Pre-treatment prediction of interferon-aefficacy in chronic hepatitis C patients.	Clin Gastroenterol Hepatol	3	1253-1259	2005
Akashi H, Miyagishi M, Yokota T, Watanabe T, Hino T, Nishina K, Kohara M, Taira K.	Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA.	Molecular BioSystems		in press	2006
Watanabe T, Sudoh	: Targeting the Hepatitis C	Gene		In press	2006

M, Miyagishi M, Arai M, Inoue K, Taira K, Yoshiba M, Kohara M.	Virus genome with RNA interference using highly effective and non-toxic long double-stranded RNA.	Theraphy			
Miyata K, Kakizawa Y, Nishiyama N, Yamasaki Y, Watanabe T, Kohara M, Kataoka K.	Freeze-dried formulations for in vivo gene delivery of PEGylated polyplex micelles with disulfide crosslinked cores to the liver	J Controlled Release		In press	2006
渡邊綱正、小原道法	RNAi による C 型肝炎ウイルス治療戦略	細胞工学	24	373-377	2005
楳原琢哉、渡邊綱正、小原道法	RNAi 医療による C 型肝炎治療	アニムス	10	34-38	2005
Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N.	Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system.	Biochem Biophys Res Commun	329	1350-1359	2005
Naka K, Ikeda M, Abe K, Dansako H, Kato N.	Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-α.	Biochem Biophys Res	330	871-879	2005
Dansako H, Naka K, Ikeda M, Kato N.	Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells.	Biochem Biophys Res Commun	336	458-468	2005
Kato N, Nakamura T, Dansako H, Namba K, Abe K, Nozaki A, Naka K, Ikeda M, Shimotohno K.	Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture.	J Gen Virol	86	645-656	2005
Li K, Chen Z, Kato N, Gale Jr M, Lemon S. M.	Distinct poly-I: C and virus-activated signaling pathways leading to interferon-β production in hepatocytes.	J Biol Chem	280	16739-16747.	2005
Abe K, Ikeda M, Dansako H, Naka K, Shimotohno K, Kato N.	cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells.	Virus Res	107	73-81	2005
Naka K, Takemoto K, Abe K, Dansako H, Ikeda M, Shimotohno K, Kato N.	Interferon resistance of hepatitis C virus replicon-harboring cells is caused by functional disruption of type I interferon receptors.	J Gen Virol	86	2787-2792	2005
Tamura K, Oue A, Tanaka A, Shimizu N, Takagi H, Kato N, Morikawa A, Hoshino H.	Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication.	Microbes Infect	7	29-40	2005
Naka K, Dansako H, Kobayashi N, Ikeda	Hepatitis C virus NS5B delays cell cycle	Virology		in press	

Authors	Title	Journal	Vol	Pages	Year
M, Kato N.	progression by inducing interferon-β via Toll-like receptor 3 signaling pathway without replicating viral genomes.				
Naka K, Abe K, Takemoto K, Dansako H, Ikeda M, Shimotohno K, Kato N.	Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells.	J Hepatol		in press	
Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, Sada K, Hotta H.	The NS3 protein of hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner.	J Gen Virol		in press	
Ishii N, Watashi K, Hishiki T, Goto K, Inoue D, Hijikata M, Wakita T, Kato N, Shimotohno K.	Characterization of the replication sensitivity to cyclosporin A among strains of hepatitis C virus.	J Virol		in press	
Koike K, Moriya K.	Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH.	J Gastroenterol	40	664-665	2005
Koike K	Steatosis in chronic hepatitis C: fuel for overproduction of oxidative stress?	Gastroenterol	40	664-665	2005
Miyoshi H, Fujie H, Shintani Y, Tsutsumi T, Shinzawa S, Makuuchi M, Kokudo N, Matsuura Y, Suzuki T, Miyamura T, Moriya K, Koike K.	Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression.	J Hepatol	43	757-763	2005
Koike K	Hepatitis C as a metabolic disease: implication for the pathogenesis of NASH.	Hepatol Res	33	145-150	2005
Koike K.	Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies.	Clin Gastroenterol Hepatol	3	S132-S135	2005
Koike K.	Hepatitis C virus infection presenting with metabolic disease by inducing insulin resistance.	Intervirology	49	51-57	2006
Koike K, Miyoshi H.	Oxidative stress and hepatitis C viral infection.	Hepatol Res		in press	2006
Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, Abe T, Suzuki T, Lai MM,	Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B.	J Virol	79	13473-13482	2005

Authors	Title	Journal	Vol	Pages	Year
Miyamura T, Moriishi K, Matsuura Y.					
Li TC, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, Hammar L, Xing L, Cheng RH.	Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus.	J Virol	79	12999-13006	2005
Abe T, Hemmi H, Moriishi K, Tamura S, Takaku H, Akira S, Matsuura Y.	Involvement of the toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus.	J Virol	79	2847-2858	2005
Kitagawa Y, Tani H, Limn CK, Matsunaga TM, Moriishi K, Matsuura Y.	Ligand-directed gene targeting to mammalian cells by pseudotype baculoviruses.	J Virol	79	3639-3652	2005
Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, Hasebe F, Tadano M, Konishi E, Moriishi K, Matsuura Y.	Nuclear localization of Japanese encephalitis virus core protein enhances viral replication.	J Virol	79	3448-3458	2005
Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, Moriishi K, Iwasaki T, Mizumoto K, Matsuura Y, Miyamura T, Suzuki T.	Molecular determinants for subcellular localization of hepatitis C virus core protein.	J Virol	79	1271-1281	2005
Masumi A, Fukazawa H, Shimazu T, Yoshida M, Ozato K, Komuro K, Yamaguchi K.	Nucleolin is involved in Interferon Regulatory Factor-2 dependent transcriptional activation.	Oncogene		in press	
Masumi A, Aizaki H, Suzuki T, DuHadaway LB, Prendergast GC, Komuro K, Fukazawa H.	Reduction of Hepatitis C virus NS5A phosphorylation through its interaction with amphiphysin II.	Biochem Biophys Res Commun	336	572-578	2005
Ishikawa T, Fukushima Y, Shiobara Y, Kishimoto T, Tanno S, Shoji I, Suzuki T., Matsui T, Shimada Y, Ohyama T, Nagai R, Miyamura T.	Outbreak of hepatitis C virus infection in an outpatient clinic.	J Gastroenterol Hepatol	20	1087-1093	2005
Suzuki T, Omata K, Satoh T, Miyasaka T, Arai C, Maeda M,	Quantitative detection of hepatitis C virus (HCV) RNA in saliva and gingival	J Clin Microbiol	43	4413-4417	2005

Matsuno T, Miyamura T.	crevicular fluid of HCV-infected patients.				
Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, Abe Y, Suzuki T, Lai MMC, Miyamura T, Moriishi K, Matsuura Y.	Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B.	J Virol	79	13473-13482	2005
Miyoshi H, Fujie H, Shintani Y, Tsutsumi T, Shinzawa S, Makuuchi M, Kokudo N, Matsiira Y, Suzuki T, Miyamura T, Moriya K, Koike K	Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression.	J Hepatol	43	757-763	2005
Masumi A, Aizaki H, Suzuki T, DuHadaway JB, Prendergast GC, Komuro K, Fukazawa H.	Reduction of hepatitis C virus NS5A phosphorylation through its interaction with amphiphysin II.	Biochem Biophys Res Commun.	33	572-578	2005
Shimoike T, Koyama C, Murakami K, Suzuki R, Matsuura Y, Miyamura T, Suzuki T.	Down-regulation of the internal ribosome entry site (IRES)-mediated translation of the hepatitis C virus: critical role of binding of the stem-loop IIId domain of IRES and the viral core protein.	Virology	345	434-445	2006
Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, Aizaki H, Kohara M, Yoshioka H, Mori Y, Manabe N, Shoji I, Sata T, Bartenschlager R, Matsuura Y, Miyamura T, Suzuki T.	Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b.	Virology		in press	

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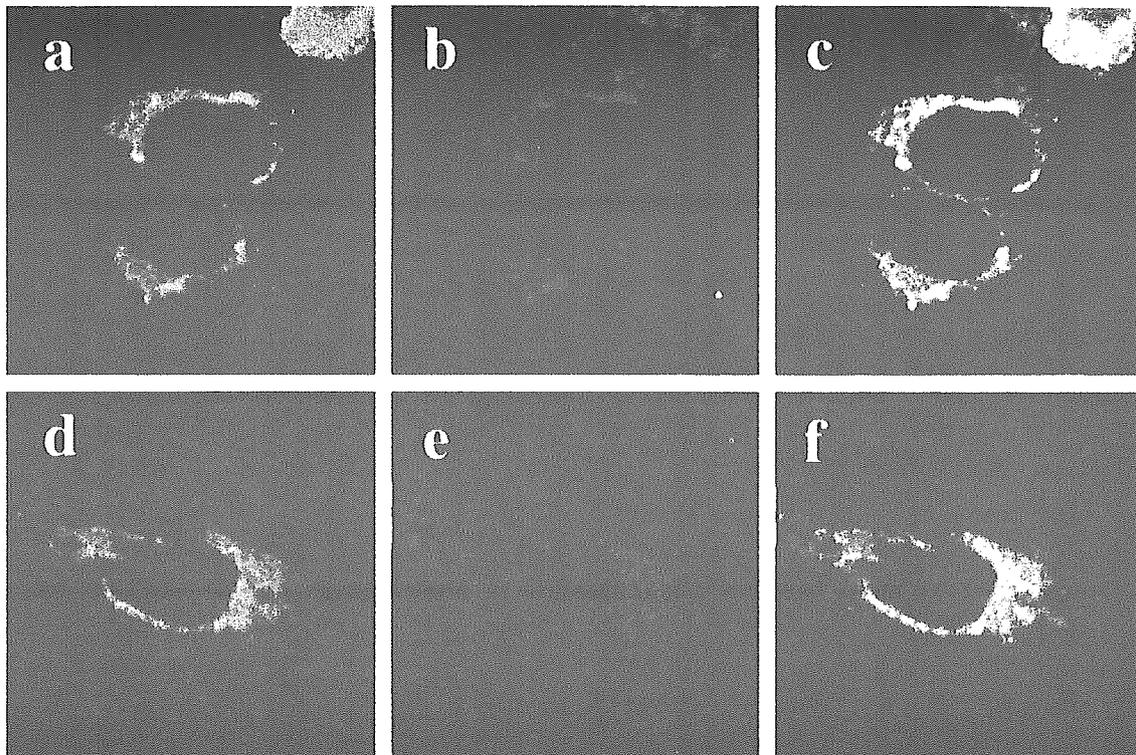
FIG. 1. Confocal analysis of double staining for HCV core protein and ER or mitochondria. 293T cells transfected with full-length HCV core expression plasmid, pCAGC191 were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c) or fixed after loading with Mitotracker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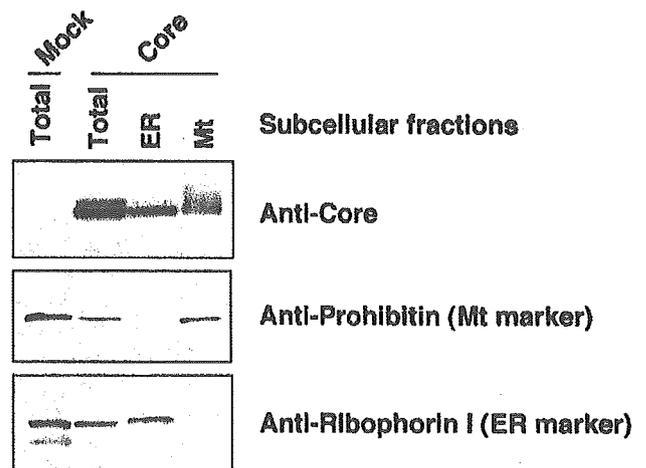

FIG. 2. Subcellular distribution of HCV core protein analyzed by immunoblotting. ER and mitochondrial (Mt) fractions were isolated from 293T cells expressing the full-length core protein (Core) or nontransfected cells (Mock) 2 days after transfection. Equal amounts of protein from each fraction as well as whole cell lysates (Total) were subjected to immunoblotting with a monoclonal antibody against either HCV core, prohibitin, or ribophorin I.
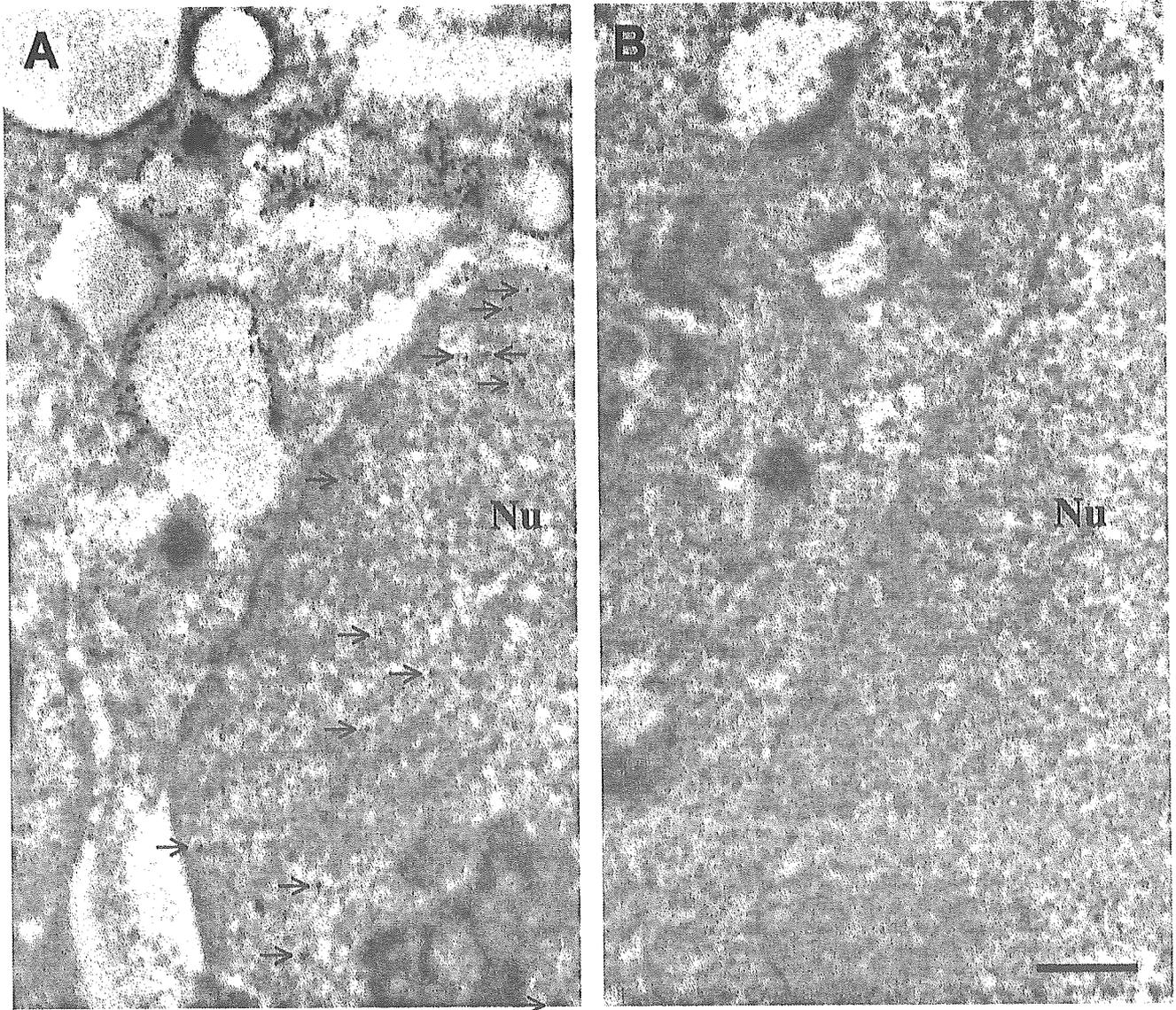
FIG. 3. Immunoelectron microscopy of HCV core protein. 293T cells expressing the full-length core protein (A) and nonexpressing cells (B) fixed 2 days after transfection. Immunoelectron microscopic analysis was performed with a mouse anticore antibody and a secondary anti-mouse IgG conjugated with gold 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-
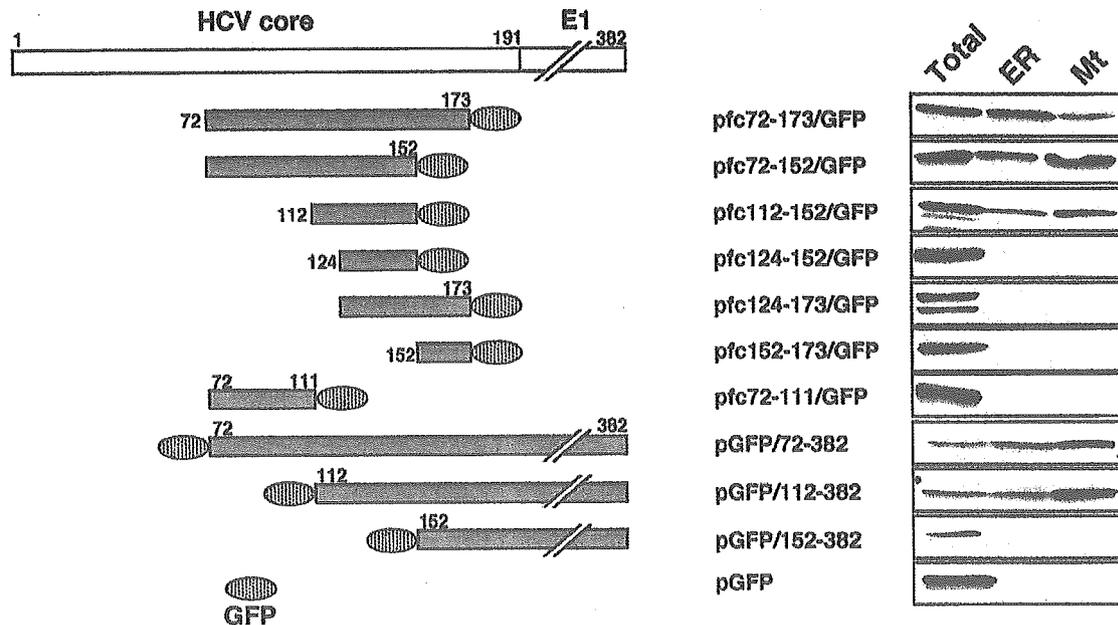
FIG. 4. Identification of segments that mediate association with ER and mitochondria in the core protein. Schematic diagram (left) and nomenclature (middle) of the core-GFP fusions are shown. Gray bars, expressed core and E1 regions. Subcellular distribution of fusion proteins is indicated on the right. 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.
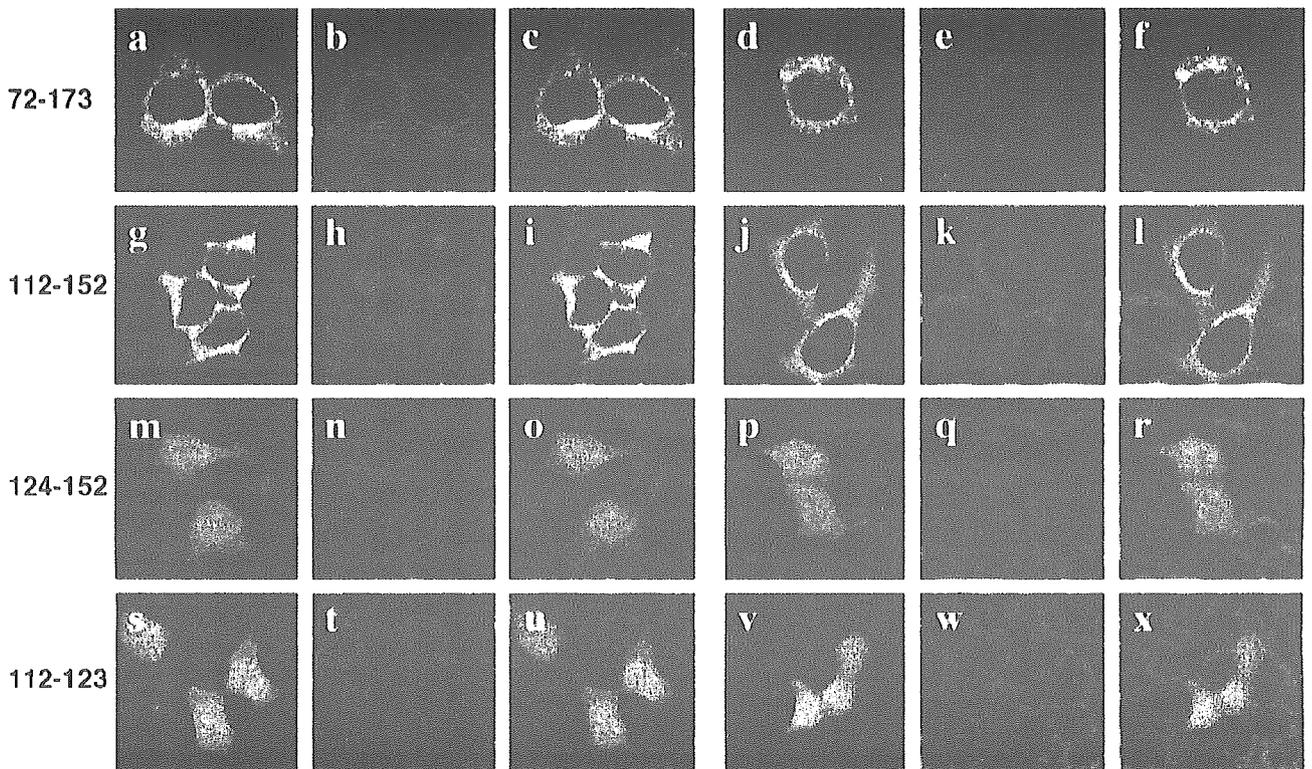
FIG. 5. Confocal analysis of double staining for core-GFP fusion protein and ER or mitochondria. 293T cells transfected with core-GFP expression plasmids (72–173, 112–152, 124–152, and 112–123) were allowed to express the plasmid for 2 days. Transfected cells were fixed directly (a to c, g to i, m to 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.

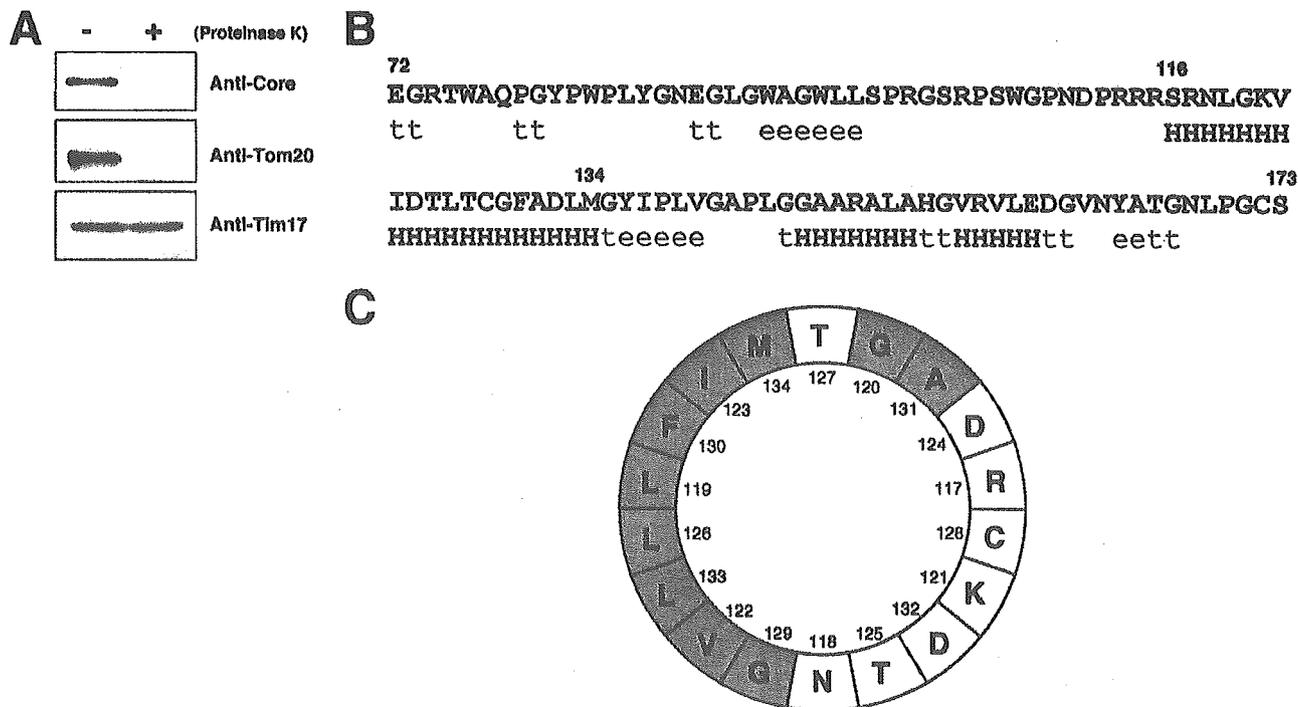Finally, we examined the subcellular localization of core

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-

A

```
      1    5           13       30    43        50                71
M--NPKPQRKTKRN---LPRRGPRL---QPRGRRQPIPKARRP   Wild

M--NP?PQ??T??N---LPRRGPRL---QPRGRRQPIPKARRP   NLS/m1

M--NPKPQRKTKRN---LP??GP?L---QPRGRRQPIPKARRP   NLS/m2

M--NPKPQRKTKRN---LPRRGPRL---QP?G??QPIP?A??P   NLS/m3

M--NP?PQ??T??N---LP??GP?L---QPRGRRQPIPKARRP   NLS/m4

M--NPKPQRKTKRN---LP??GP?L---QP?G??QPIP?A??P   NLS/m5

M--NP?PQ??T??N---LPRRGPRL---QP?G??QPIP?A??P   NLS/m6

M--NP?PQ??T??N---LP??GP?L---QP?G??QPIP?A??P   NLS/m7
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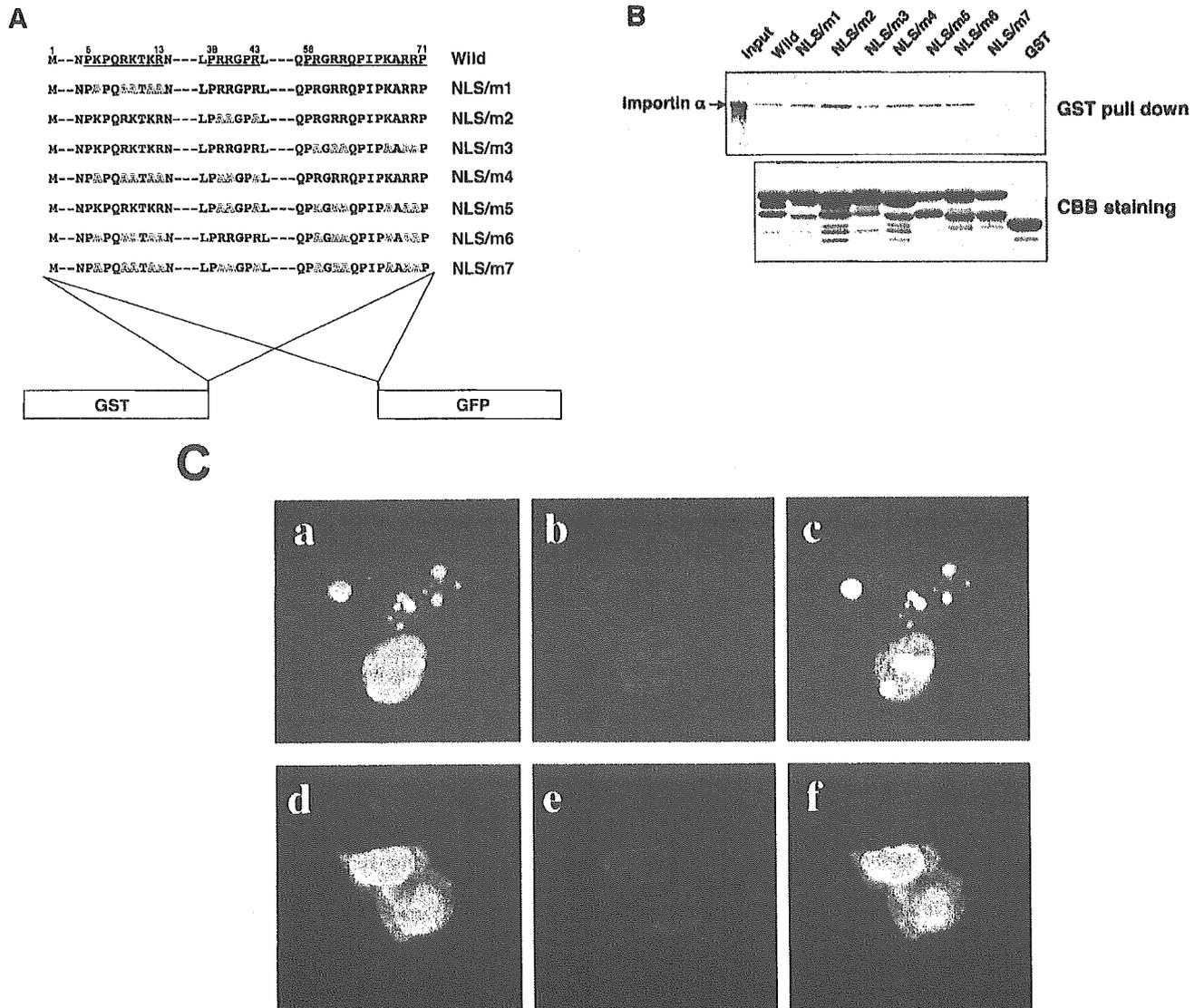

B

C

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
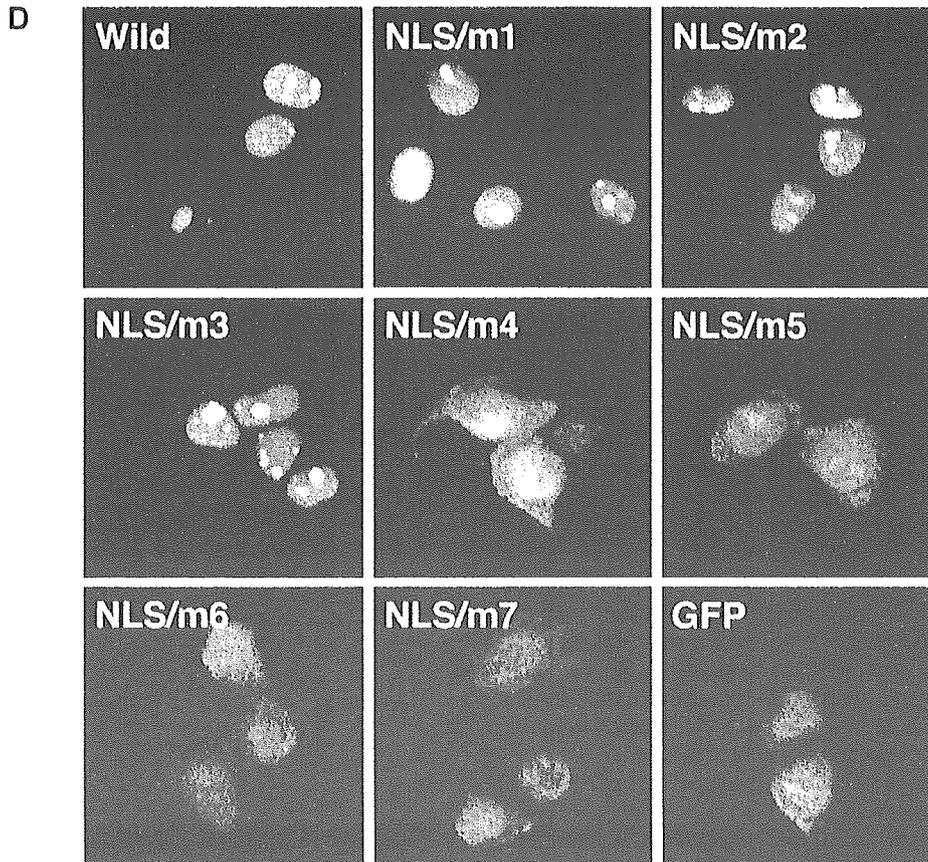
D

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-