厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業

慢性Ｃ型肝炎に対する治療用ヒト型抗体の開発に関する研究

平成１４年度～平成１６年度　総合研究報告書

主任研究者　松浦　善治

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主任研究者　松浦　善治

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厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
総合研究報告書

慢性C型肝炎に対する治療用ヒト型抗体の開発に関する研究

主任研究者　松浦善治　大阪大学微生物病研究所　教授

研究要旨　C型肝炎ウイルス(HCV)の感染機構や宿主免疫からの逃避機構は未だ明らかにされていない。1) 慢性C型肝炎から自然治癒した症例のリンパ球や、ヒト型抗体を産生できるマウスを免疫したヒト型抗 HCV エンベロープモノクローナル抗体を作製した。2) これらヒト型抗体のウイルス排除活性を HCV 持続感染チンパンジーで評価したが、ウイルスの減少は一過的なものであり、HCV を生体から排除するには至らなかった。3) HCV のエンベロープ蛋白質を被ったシュードタイプウイルスや、昆虫細胞で作製した HCV 様粒子を用いて、HCV 感染に重要な宿主因子を探索した結果、ヒト繊維芽細胞成長因子がシュードタイプウイルスの感染を特異的に阻害し、その受容体を恒常的に発現する CHO 細胞がシュードタイプウイルスの感染を許容した。4) HCV コア蛋白質は宿主プロテアーゼによりプロセスされ、小胞体から核に移行して PA28γ依存的に分解される。コア蛋白質の成熟機構と肝疾患発症との関連を明らかにするとともに、細胞性免疫を誘導可能な HCV 抗原としての可能性を検討した。4) 弱毒化ワクチニアウイルス DIs 株は安全なワクチンベクターであることが証明された。

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A. 研究目的

HCV に感染すると肝硬変を経て高率に肝細胞癌を発症する。我が国には2百万人もの HCV 感染者が存在すると推定され、既感染者に対する有効な肝癌進展阻止法の開発が急務である。しかしながら、HCV を効率よく複製できる細胞培養系を欠くため、HCV の感染様式や発癌機構は依然として謎に包まれたままである。本研究では、これまでに得られた HCV エンベロープ蛋白質に対するヒト型モノクローナル抗体の *in vivo* でのウイルス排除活性を HCV に持続感染しているチンパンジーを用いて評価した。また、HCV の宿主細胞への侵入を担うリセプター分子を明らかにし、各ステップをターゲットとした新しいC型肝炎治療法の開発の可能性を探る。HCV コア蛋白質はウイルス粒子を構成するだけでなく、宿主細胞の機能を多様に調節して、脂肪肝や肝細胞癌の発症にも深く関与している。コア蛋白質の成熟機構を詳細に解析し、コア蛋白質と肝疾患発症との関連を明らかにするとともに、コア蛋白質は HCV 蛋白質の中でもアミノ酸配列が最も保存されていることから、CTL ワクチンのターゲットとしての可能性を検証する。HCV の感染機構が解明できれば、悲願であった信頼できる HCV の細胞培養系や小型実験動物の開発、そしてワクチンや治療薬の開発が急展開することが期待でき、社会的貢献度も極めて高いものと思われる。

B. 研究方法

1)ヒト型抗体の HCV 持続感染チンパンジーでの活性評価：慢性C型肝炎の自然治癒例の末梢リンパ球から抗体遺伝子の cDNA ライブラリーを作製し、ファージディスプレイ法を用いて、NOB 活性を持ったヒト型抗 E2 モノクローナル抗体を得た。また、ヒト型抗体を産生できるトランスジェニックマウスを用いて、HCV エンベロープ蛋白質による膜融合を中和できる抗 E1/E2 ヒト型モノクローナル抗体を作製した。これらのヒト型抗体を精製し、安全性が担保された抗体を HCV に持続感染しているチンパンジーに 3mg/kg と 10mg/kg の用量で各2頭ずつ、計4頭に1週間隔で9回、静脈内に Infusion pump を用いて投与した。抗体投与後のウイルス価と肝機能の動き、抗体の半減期、さらに、抗ヒト抗体の産生の有無について検討した。

2)キメラおよび authentic な HCV エンベロープ蛋白質を被ったを被ったシュードタイプ水疱性口内炎ウイルス(VSV-pp)を作製した。さらに、組換えバキュロウイルスを用いて作製した HCV 様粒子(HCV-LP)、および、ウインドウ期の患者血清中の HCV 粒子の各種細胞への感染および結合をリポーター遺伝子の発現、定量 ELISA、ならびに定量 PCR で解析した。

3)HCV コア蛋白質の成熟機構の解析：HCV のコア蛋白質は、前駆体蛋白質からシグナルペプチダーゼ（SP）により切り出され、さらにそのC末端膜貫通領域が切断されて、成熟型のコア蛋白質にプロセスされると考えられている。最近、蛋白質のシグナル配列が SP によって切断された後、そのシグナルペプチドをさらに小胞体の膜内で切断する膜内蛋白質分解酵素、シグナルペプチドペプチダーゼ（SPP）の存在が明らかにされた。HCV コア蛋白質のC末端膜貫通領域の切断に

もSPPが関与しているものと考えられており、SPPとHCVコア蛋白質の相互作用とSPPによるコア蛋白質のC末端膜貫通領域のプロセスに必須な領域を解析した。また、コア蛋白質と結合する宿主因子として、プロテアソーム調節蛋白質PA28γを単離したが、哺乳動物細胞内での局在の一致ならびに正常な発現レベルでの相互作用を検証する。

4)ワクチニアウイルスのプロモーターmH5の下流にHCV遺伝子を挿入したトランスファーベクターを作成してDIsに導入し、目的蛋白の発現の確認を行った。また、これらの組換えDIsの液性及び細胞性免疫誘導能の検討を行った。液性免疫は組換えバキュロウイルスを用いて発現させたHCV蛋白を抗原としてELISA法により、細胞性免疫は、ウイルスが発現するHCVの蛋白に対応するoverlapping peptidesを用いてマウス脾臓細胞を刺激し、ELISPOT assayによるIFN-g分泌細胞数の測定及びMTT assayによる細胞増殖能を測定した。

5) 全長のHCVコアタンパク質発現プラスミドに加えて、ユビキチン-プロテアソーム系により選択的分解を受けるC末端側を欠損させた核局在型のコアタンパク質、C末端側を欠損させ、さらにユビキチン化を受ける可能性のあるすべてのリジン残基をアルギニンに置換した変異体、C末端側を欠損させユビキチン-プロテアソーム系により選択的分解を受けるが、そのC末端に核外移行シグナルを融合させ、細胞質に局在する変異体をそれぞれ発現するプラスミドを構築した。これらのコア蛋白質発現プラスミド10ugを、マウスの右大腿筋にエレクトロポレーション法により接種した。最初の接種から2週、6週後に同量を接種し、7週目に脾臓を摘出してT 細胞傷害活性を測定した。

（倫理面への配慮）

本研究にあたっては、試料提供者、その家族、および同様の肝疾患患者の人権、尊厳、利益が保護されるよう十分に配慮する。具体的には、厚生労働省等で検討されている「ヒトゲノム解析研究に関する共通指針」に則り各研究実施機関の医学研究倫理審査委員会に申請し、インフォームドコンセントに係る手続きを実施し、また提供試料、個人情報を厳格に管理、保存した。実験動物に関しては「動物の保護及び管理に関する法律」（昭和48年法律第105号）及び「実験動物の飼養及び保管に関する基準」（昭和55年総理府公示第6号）の法律及び基準の他、「大学等における実験動物について」（文部省国際学術局長通知、文学情第141号）の通知を踏まえつつ、動物実験が有効かつ適切に行われるよう配慮した。また、本研究に関しては、平成14年7月3日にチンパンジー実験審査委員会において承諾を得ている。

C. 研究結果

1）ヒト型抗体のHCV持続感染チンパンジーでの抗ウイルス活性の評価：抗E2ヒト型抗体を3mg/kgと10mg/kg投与したHCV持続感染チンパンジーの抗ウイルス活性ならびに肝炎改善に有意な差は認められなかった。抗体投与により一過性にウイルス価の減少が認められたが、1週間後には元に戻る傾向が全例に認められ、4頭中2例に於いて、肝機能の改善傾向が認められた。抗E2ヒト型抗体の体内動態はこれまでのヒト抗体と同様のものであり、3mg/kgの方が10mg/kgより半減期が長かった。また、全てのチンパンジーに於いてヒト抗体に対する抗体の産生は認められなかった。また、ヒト抗体を産生できるトランスジェニックマウスを免疫して作製した、膜融合を中和する抗E1および抗E2抗体を大量に培養・精製し、同様にHCV持続感染チンパンジーに投与したところ、一過性にウイルス価の減少が認められた。

2）VSV-ppはHepG2細胞に特異的に感染し、HCV-LPの添加により濃度依存的に感染が阻害された。また、ヘパリンがVSV-ppの感染を阻害することから、ヘパリンとの結合が活性発現に重要な各種成長因子によるVSV-ppの感染阻止活性を調べたところ、ヒト繊維芽細胞成長因子(FGF)、特にFGF2とFGF7がVSV-ppの感染を特異的に阻害した。また、その受容体(FGFR)の中で、FGFR4とFGFR5がVSV-ppの感染を特異的に阻害し、HCV-LPやC型肝炎患者血清中のHCV粒子とも結合した。また、セファロースに固層化したFGFR4とFGFR5はHCV-LPのみならず、ウインドウ期血清中のHCVを特異的に沈降した。さらに、FGFR4あるいはFGFR5を恒常的に発現するCHO細胞はHCV-LPや患者血清中のHCVと特異的に結合した。特にFGFR5を発現するCHO細胞株はVSV-ppの感染を許容できるようになり、siRNAによってFGFR5をHepG2細胞からノックダウンさせると感受性の低下が観察されたことから、FGFR5がVSV-ppの侵入受容体である可能性が示唆された。一方、HepG2細胞から、siRNAによってFGFR4をノックダウンさせても、VSV-ppに対する感受性の低下は観察されなかった。

3) HCVコア蛋白質の成熟機構の解析：ヒト肝臓よりSPP遺伝子をクローニングし、活性中心を失活させた変異体（SPPD219A）を作製した。また、エピトープタグを付加したHCVコア蛋白質とその変異体を準備した。これらを培養細胞に発現させ、イムノブロット法と免疫沈降法により、コア蛋白質のプロセシングならびに両者の相互作用を解析した。また、コア蛋白質とE1蛋白質をシスに発現させ、E1蛋白質の糖鎖付加の有無によって、コア蛋白質C末端膜貫通領域のシグナル活性を評価した。培養細胞にSPPとHCVコア蛋白質を発現させたところ、SPPとコア蛋白質は共沈し、さらに、SPPD219AはコアSPPと結合してプロセスを受けることを確認した。コア蛋白質の変異体を用いた解析から、SPPの切断に必須な領域は既報の成績と異なっており、コア蛋白質のC末端膜貫通領域のみならず、その上流の少なくとも3つのアミノ酸が重要であることが示された。また、その領域はコア蛋白質の小胞体局在にも関与していた。さらに、SPPでプロセスされないコア蛋白質の変異体でも、E1蛋白質のシグナル活性を保持していたことから、SPPによるプロセスとシグナル活性に必須な領域は異なることが

示された。
HCV コア蛋白質は前駆蛋白質として発現された後、上述のごとく SPP によって切断され、成熟蛋白質として小胞体に留まり、一部は核へ移行する。HCV コア蛋白質の宿主内標的蛋白質候補としてプロテアソームの活性化蛋白質である PA28γを同定し、PA28γが HCV コア蛋白質の安定性と細胞内局在を調節していることを明らかにした。
4) DIs 株に HCV の Core-E1-E2 領域、NS3 領域、NS5A 領域及び全長に相当する遺伝子領域を組み込んだ組換え DIs を作成し、それぞれの組換え DIs を哺乳類細胞に感染させると目的蛋白が発現されることを確認した。Core-E1-E2 領域を発現する組換え DIs をマウスに投与した場合、Core、E2 いずれの蛋白に対する抗体も検出され、NS3、NS5A を発現するウイルスでもそれぞれに対する抗体が検出されたことから、組換え DIs の投与により目的の組換え蛋白に対する液性免疫を誘導できることが確認された。また、いずれの組換え DIs を投与した場合も、ELISPOT assay 及び MTT assay により目的蛋白に対する細胞性免疫が誘導されることを確認することができた。
5) 抗原刺激に対する T 細胞傷害活性測定の指標として、IFN-γを分泌する細胞数の測定を行った。その結果、全長のコアタンパク質発現プラスミドで免疫したマウスでのみ、抗原特異的刺激により IFN-γ分泌細胞数の顕著な増加が認められた。ユビキチン-プロテアソーム系により選択的分解を受ける変異体を含めた他の群では、陰性コントロール群との差が認められなかった。また血中のコア蛋白質に対する抗体価を測定したが、すべての群において抗コア抗体は検出できなかった。

D. 考察
1) NOB 活性を持った抗 E2 ヒト型モノクローナル抗体、および、細胞融合阻止活性を持った抗 E1 および抗 E2 ヒト抗体の HCV 持続感染チンパンジーでの抗ウイルス活性は、一過性なものであり、ウイルスを生体から排除することは出来なかった。いずれのヒト型抗体もシュードタイプウイルスの感染を中和出来なかったことから、シュードタイプウイルスの感染を中和できるヒト型抗体の作製が必要と思われる。NOB 活性は精製した E2 蛋白質が細胞表面の CD81 分子に結合するのを阻止するものであり、HCV 感染における関与は依然として否定的な意見が多い。しかし、精製 E2 蛋白質と強いアフィニティーを示すことから、ウイルスの侵入には直接関与しなくても、結合によりシグナルを細胞に入れて、HCV の感染に必須な分子の誘導や肝炎病態に関与している可能性は充分考えられる。今後、本抗体による E2 と CD81 の結合阻害による肝炎病態の改善の可能性も検討課題と思われる。
2) HCV 研究の最重要課題は、信頼できる細胞培養系の開発である。我々が開発した HCV シュードタイプウイルスは、これまでの精製エンベロープ蛋白質を用いた結合アッセイや PCR でようやくウイルスの複製が検出できる細胞培養系に比べ、吸着ならびに侵入のステップを定量的に解析できる点で優れている。FGFR が HCV の新規受容体候補分子であることが示され、FGFR をターゲットとした慢性C型肝炎に対する新たな創薬の可能性を提示できた。HCV は高率に持続感染を成立させることから、C 型肝炎患者のほとんどが中和抗体を保持している CD81 依存的な感染を示すシュードタイプウイルスよりも、中和抗体が検出できない FGFR 依存的なエントリーを示すシュードタイプウイルスが、真の HCV 感染を反映している可能性が十分に考えられる。これらの成果は、信頼できる HCV の細胞培養系や小型実験動物の開発、そしてワクチンや治療薬の開発が急展開することが期待でき、社会的貢献度も極めて高いものと思われる。
3) HCV コア蛋白質の SPP によるプロセス、ならびにコア蛋白質の小胞体局在を規定する領域が同定された。また、小胞体から遊離したコア蛋白質は核内で PA28γ依存的に分解されることが示された。
4) 組換え DIs をマウスに投与することにより、HCV 蛋白に対する液性及び細胞性免疫を誘導することができるという結果が得られた。DIs 株は親株のワクチニアウイルスと異なりヒト生体内で増殖しないため、安全な組換えワクチンとして有望であると考えられている。
5) ユビキチン-プロテアソーム系により選択的分解を受けるコア蛋白質は、Balb/c マウスにおいて強い CTL 誘導能は見られなかった。このコア蛋白は核に局在するが、このタンパク質の局在を細胞質に変えても、同様の結果であった。本来のコアタンパク質は半減期の長い安定な蛋白であるが、主に ER に局在することが知られている。この事から、CTL 活性の誘導には、ユビキチン-プロテアソーム系による分解だけでなく、ER 膜などへの局在が重要である可能性が考えられた。

E.結論
1. C型肝炎の自然治癒例リンパ球から、また、ヒト型抗体を産生できるマウスを免疫して、ヒト型抗 HCV エンベロープモノクローナル抗体を作製した。
2. これらヒト型抗体のウイルス排除活性を HCV 持続感染チンパンジーで評価したが、ウイルスの減少は一過的なものであり、HCV を生体から排除するには至らなかった。
3. ヒト FGFR は HCV の新規受容体候補である。
4. HCV コア蛋白質は SP と SPP により成熟し、小胞体から核に移行して PA28γ依存的に分解される。
5. 弱毒化ワクチニアウイルス DIs 株は安全な組換えウイルスベクターである。

F. 研究発表
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G. 知的所有権の取得状況

1) 2004-242937・石井孝司他 12 名・財団法人ヒューマンサイエンス振興財団・SARS-コロナウイルスタンパク質の全部もしくは一部のタンパク質をコードする DNA をゲノム DNA 上に保有し、該タンパク質を発現し得る組換えワクチニアウイルス DIs 株。・2004 年８月 23 日出願
2) 2004-225043・石井孝司他４名・独立行政法人医薬品医療機器総合機構・HCV ウイルスタンパク質をコードする DNA を保有する組換えワクチニアウイルス DIs 株、およびその利用・2004 年８月２日出願

研究成果の刊行に関する一覧表

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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 Gly^{42} and Pro^{43} in the core protein are essential for the nuclear and nucleolar localization. A mutant M4243 virus in which both Gly^{42} and Pro^{43} 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 secondary 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 impotin-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 Gly^{42} and Pro^{43} 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 DH5α. 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 AFFINITI (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 μg of RNase A (QIAGEN)/ml, the nuclei were stained with 500 μM propidium iodide (Molecular Probes, Eugene, Oreg.). Endogenous nucleolin, a major nucleolar protein (51), was immunostained by an anti-nucleolin monoclonal antibody and Alexa Flour 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 10^7 cells/ml in PBS. RNA (10 μg) was mixed with 500 μl of cell suspension and transferred to an electroporation cuvette (Thermo Hybrid, Middlesex, United Kingdom). Cells were then pulsed at 190 V and 950 μF 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 Flour 488-conjugated goat anti-rabbit IgG (Molecular Probes) antibody. After treatment with 1 μg of RNase A/ml, nuclei were stained with 500 μM propidium iodide. Samples were examined with a laser scanning confocal microscope.

Subcellular fractionation. At 48 h postinoculation, 2×10^6 Vero cells were fractionalized into cytoplasm and nucleus by using a Nuclear/Cytosol Fractionation kit (BioVision, Mountain View, Calif.) according to the manufacturer's instructions. Finally, 210 μl of the cytoplasmic extracts and 100 μl of the nuclear extracts were recovered and 10 μl 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 PA28α (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 (2×10^5) 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 μl) 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 × *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 × *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 × *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 μg) 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 μl 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 Müench (47). Groups of mice (n = 10 or 11) were inoculated intraperitoneally (ip) with 10^5 FFU (100 μl) 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.

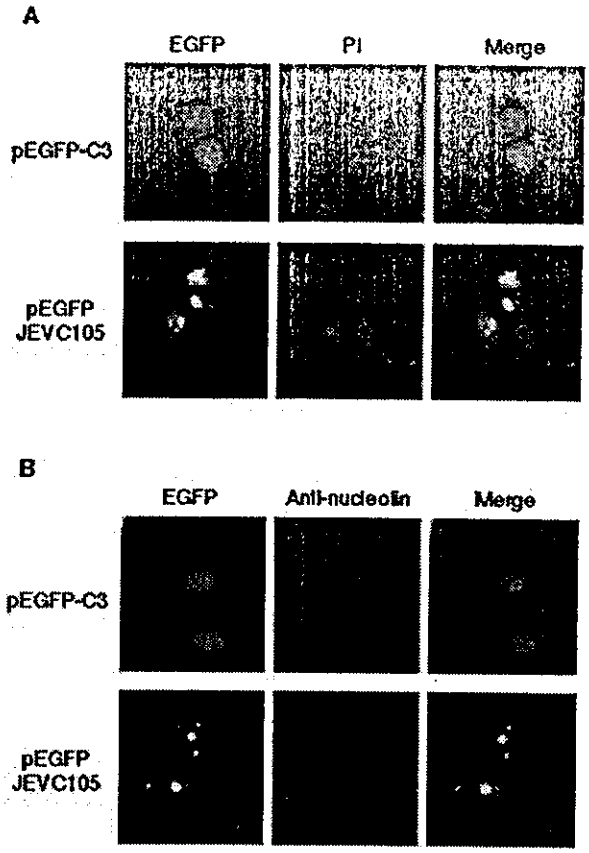


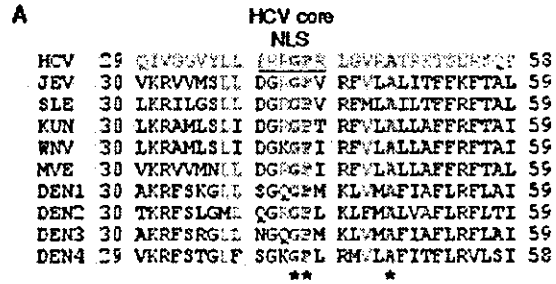
FIG. 1. Intracellular localization of EGFP-fused JEV core protein. Vero cells were transfected with expression plasmids encoding EGFP or EGFP-fused JEV core protein. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. (A) Nuclei were stained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

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 (RKEIGRMLNILNRRKR). A computer program, PSORTII (Institute of Medical Science, Tokyo University [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 [KRELGTLIDAVNKRG RK]). 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 (AAELGTLIDAVNAAGAA) 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

A



B

Constructs		Nuclear localization
C105	39 DGRGPV 44	+
Δ39-44	.....	-
D39A	AGRGPV	+
G40A	DARGPV	+
R41A	DGAGPV	+
G42A	DGRAPV	+
P43A	DGRGAV	+
V44A	DGRGPA	+
GP4243AA	DGRAAV	-

C

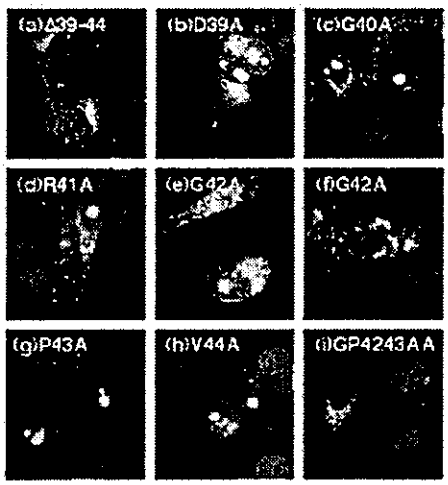


FIG. 2. Role of an HCV core protein NLS in nuclear localization of JEV core protein. (A) A partial alignment of amino acid sequences of core proteins of HCV (HCVJ1 strain, genotype 1b) and flaviviruses, including JEV, SLE (St. Louis encephalitis virus), KUN, WNV, MVE (Murray Valley encephalitis virus), 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 G^{42}-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 Gly^{42} 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 Gly^{42} and Pro^{43} to Ala completely eliminated the nuclear localization of the JEV core protein (Fig. 2C, panel i). These results indicate that Gly^{42} and Pro^{43}, 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 Gly^{42} and Pro^{43} 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

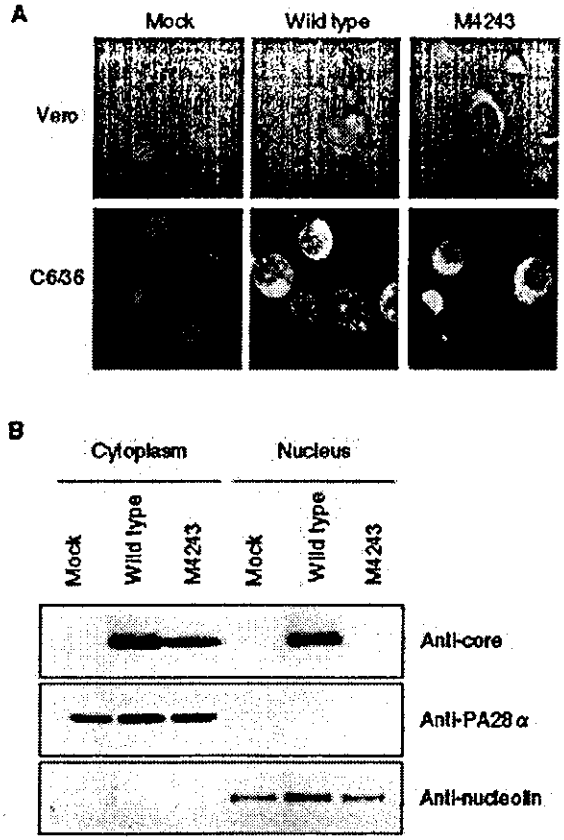


FIG. 3. Intracellular localization of core proteins in cells infected with wild-type or M4243 virus. (A) Intercellular localization of wild-type and mutant JEV. Vero or C6/36 cells were infected with wild-type or M4243 virus, fixed at 2 days postinoculation and immunostained with anti-JEV core rabbit 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 PA28α and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

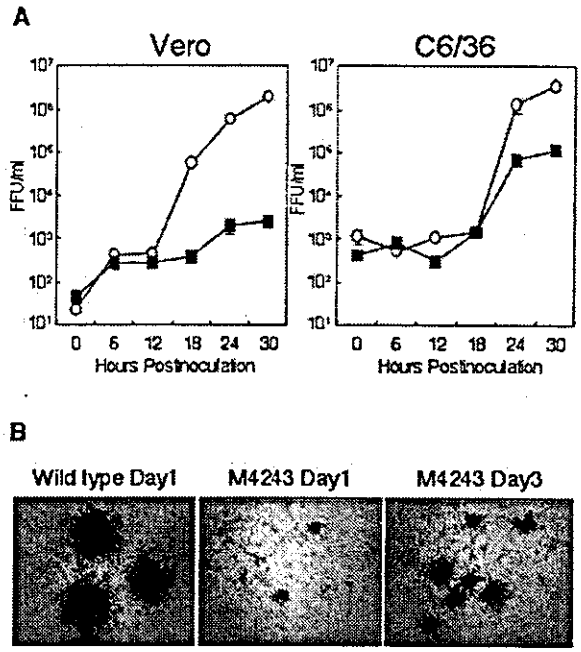


FIG. 4. Growth properties of wild-type and M4243 viruses. (A) Growth kinetics of the viruses in Vero and C6/36 cells. Both cell lines were infected with wild-type or M4243 virus at an MOI of 5. Culture supernatants were harvested at the indicated times postinoculation, and infectious titers were determined 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.

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 Pro^{43} 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 posses 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 particles 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.

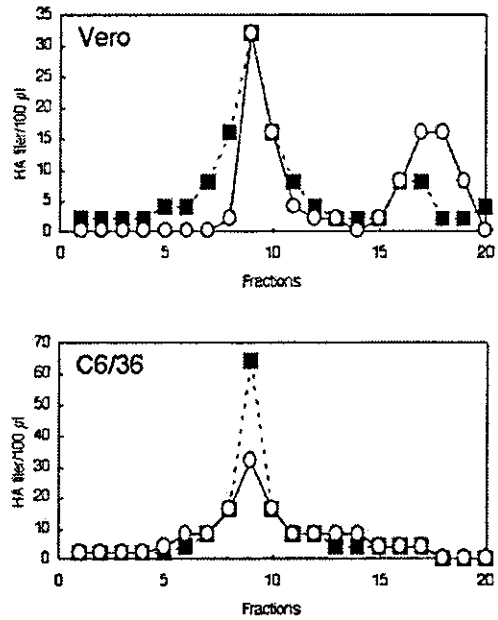


FIG. 5. Gradient fractionation of viral particles of the wild-type and M4243 viruses. The viral particles (400 HA units) derived from Vero or C6/36 cells were applied to 10 to 40% (wt/wt) sucrose gradient and centrifuged at 147,000 × *g* for 90 min. Twenty fractions were collected from bottom to top 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 μl 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-

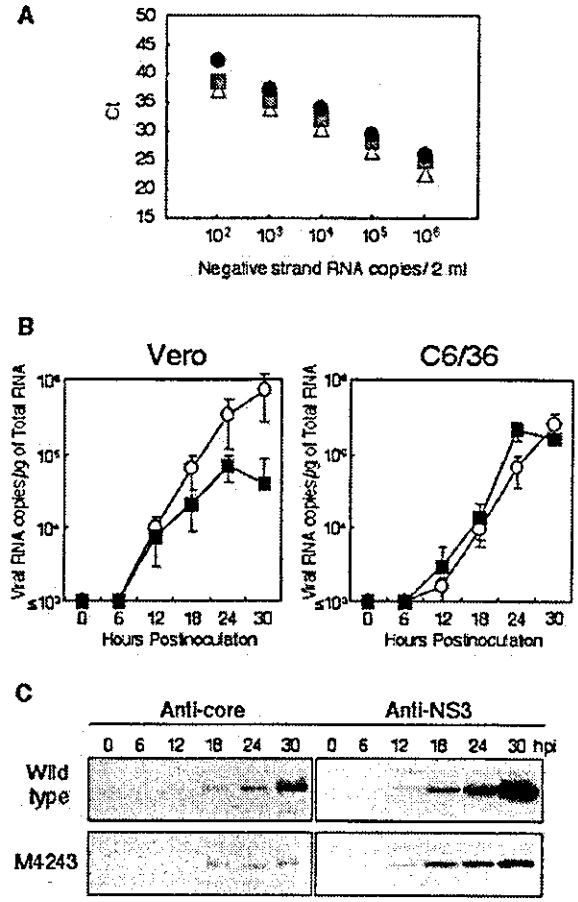


FIG. 6. Viral RNA and protein syntheses in Vero and C6/36 cells infected with wild-type or M4243 virus. (A) Establishment of negative-strand JEV RNA-specific real-time RT-PCR. A series of 10-fold dilutions of synthetic negative-strand RNA in the absence (closed circles) or presence of 100-fold (gray 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/μg 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 μg 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 threefold 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 LD_{50} values by intracerebral inoculation of the viruses into 3-week-old ICR mice. The LD_{50} 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

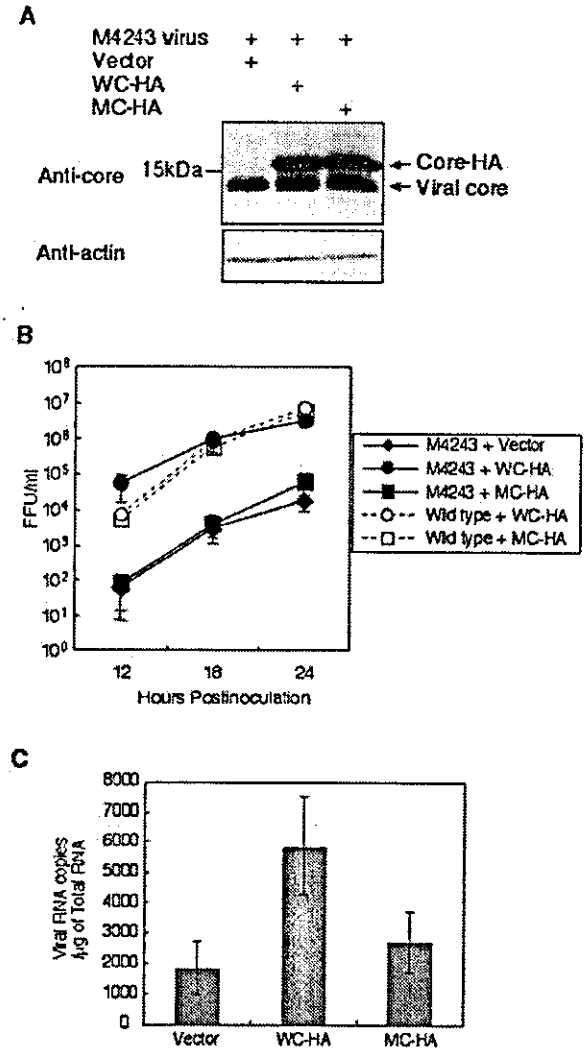


FIG. 7. Complementation of M4243 replication by expression of core proteins in 293T cells. At 4 h after transfection with pCAG-GS (Vector), pCAG-WC-HA (WC-HA), or pCAG-MC-HA (Gly^{42} and Pro^{43} to Ala) (MC-HA), 293T cells were infected with wild-type or M4243 virus at an MOI of 5. At 12, 18, or 24 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 10^5 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-

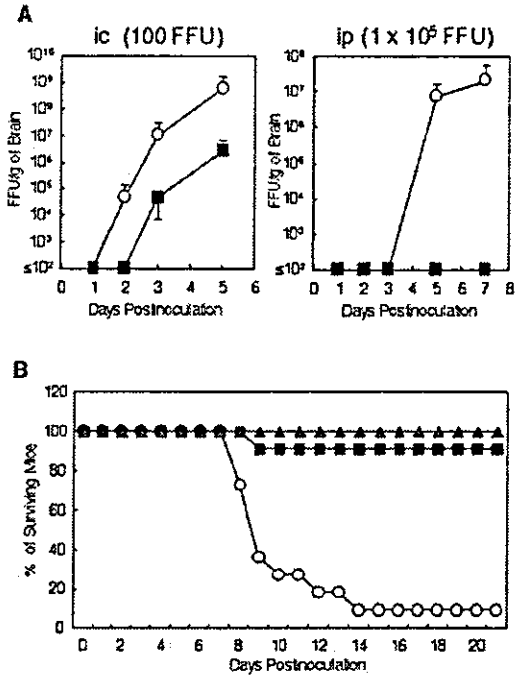


FIG. 8. Virulence of wild-type and M4243 viruses for ICR mice. (A) The infectious titers of wild-type and M4243 viruses in the brains of mice after inoculation with 100 FFU of the viruses intracerebrally (ic) or 10^5 FFU intraperitoneally (ip). Open circles and closed squares indicate wild-type and M4243 viruses, respectively. The detection limit is 10^2 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 10^5 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 Gly^{42} and Pro^{43} were important for the nuclear localization and that Gly^{42} was essential for the nucleolar accumulation of the JEV core protein. The two amino acids Gly and Pro are well conserved not only among mosquitoborne 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 Gly^{42} and Pro^{43} with Ala completely abolished the nuclear localization of the JEV core protein 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 Gly^{42} 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 Gly^{42} and Pro^{43} 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 Gly^{36} corresponding to the Gly^{42} 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 Gly^{42} and Pro^{43} 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 Gly^{42} and Pro^{43} 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 translated 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 PA28γ (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 pathway 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 evolutional studies on the family *Flaviviridae* that the NLSs of core proteins were well conserved between mosquitoborne flaviviruses and bloodborne and human-adapted HCV.

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