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Intracellular localization and determination of a nuclear localization signal of the core protein of dengue virus

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In dengue virus (DEN) particles, the core protein is a structural protein of the nucleocapsid. The core protein is known to be present in the nucleus of DEN-infected cells but there have been conflicting reports as to whether it is also present in the nucleolus. To clarify this, the intracellular location of the core protein was examined using a monoclonal antibody, 15B11, which was produced in this study. Immunofluorescence staining with this antibody demonstrated that the core protein first appeared in the cytoplasm and then in the nuclei and nucleoli of infected cells. Nuclear localization of the core protein was determined to be independent of other DEN proteins, since recombinant core proteins still entered the nuclei and nucleoli of cells transfected with only the core protein gene. Three putative nuclear localization signal motifs have been predicted to be present on the core protein. Deletion of the first one (KKAR), located at aa 6–9, and mutation of the second one (KKSK), located at aa 73–76, did not eliminate the nuclear localization property of the core protein. The third motif with a bipartite structure, RKeigrmlnilnRRRR, located at aa 85–100, was determined to be responsible for the nuclear localization of the core protein, since the core protein without this motif was located exclusively in the cytoplasm of DEN-infected cells and that this motif mediated nuclear localization of a normally cytoplasmic protein.

Introduction

Dengue virus (DEN) is present in over 100 countries (WHO, 2000) and causes a wide range of diseases, including benign febrile illness, dengue fever (DF), plasma leakage syndrome and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) (Halstead, 1980). Approximately 100 million cases of DF and 500 000 cases of DHF/DSS occur every year, with a mortality rate of 5% (Barrett, 1997; Gubler & Clark, 1995; Rigau-Perez *et al.*, 1998).

DEN is composed of a positive-stranded RNA genome and three structural proteins, including the core (C), membrane (M) and envelope (E) proteins. The single-stranded RNA genome, located in the nucleocapsid, is 11 kb in length and encodes a single large polyprotein, which is processed to three structural and seven non-structural (NS) proteins. The order of these proteins on the polyprotein is C–prM–E–NS1–NS2a–NS2b–NS3–NS4a–NS4b–NS5 (Chambers *et al.*, 1990; Westaway *et al.*, 1985).

The building block of the nucleocapsid of DEN is the core protein. Although DEN replicates in the cytoplasm of host cells, its core protein has been detected in both the cytoplasm and the nucleus (Bulich & Aaskov, 1992; Makino *et al.*, 1989) and perhaps in the nucleolus (Tadano *et al.*, 1989) of infected cells. Three putative nuclear localization signal (NLS) motifs have been predicted to be present on the core protein (Bulich & Aaskov, 1992). In this study, we re-examined the intracellular localization of the DEN core protein and determined the NLS motif responsible for nuclear localization.

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Methods

■ **Plasmid construction.** A portion (nt 97–439) of DEN serotype 2 (DEN-2) cDNA was cloned into pBluescript II SK (−) (Stratagene) to generate pBCM, which was used as the template for PCR to amplify the

Table 1. Primers used in this study

Restriction sites (underlined), stop codons (italics) and mutated residues (uppercase letters) that were built into the primers are indicated.

Primer	Sequence	Region amplified
T3	attaaccctcactaaag	Entire C gene from pBCM
P1	atgaatcaccaacgaaaaaag	Entire C gene from pBCM
CMV_{IE}	cgtgtacggtgggaggtct	Entire C gene from pBCM
GFPL	ccgaagatcttgtacagctcgtccatgccg	GFP gene from pEGFP-N2
P15	atgctgaaacgcgagagaaa	aa 15–100 of C gene from pFC1 ~ 100
P37	atgctgcagggacgaggacc	aa 37–100 of C gene from pFC1 ~ 100
P46	atgttcatggccctggtggcg	aa 46–100 of C gene from pFC1 ~ 100
P1–72	*tta*attgttccccatctcttcag	aa 1–72 of C gene from pFC1 ~ 100
P1–84	*tta*gaaccctctcaaaacattaat	aa 1–84 of C gene from pFC1 ~ 100
CoreTerm	*tta*ctgcgtctcctgttcaagat	Entire C gene from pFC1 ~ 100
CoreERI	ggaattctctgcgtctcctgttcaagat	Entire C gene from pFC1 ~ 100
$C^{73}GG^{74}F$	attGGGGGGtcaaaagccattaatgttttg	Mutation of second NLS motif from pFC1 ~ 100
$C^{73}GG^{74}R$	tgaCCCCCCaattgttccccatctcttc	Mutation of second NLS motif from pFC1 ~ 100
P85	gaagatctaggaaagagattggaagg	Amplification of third NLS from pFC1 ~ 100
Core*Sma*I	ccccccgggtctgcgtctcctgttcaagat	Amplification of third NLS from pFC1 ~ 100
$C^{85}GN^{86}$	gaagatctGGCAACgagattggaagg	Mutation of the left-hand side consensus sequence of the third NLS (RK → GN)
$C^{97}G^{100}$	ccccccgggGCCGCCGCCGCCgttcaagatgttcagcatcc	Mutation of the right-hand side consensus sequence of the third NLS (RRRR → GGGG)

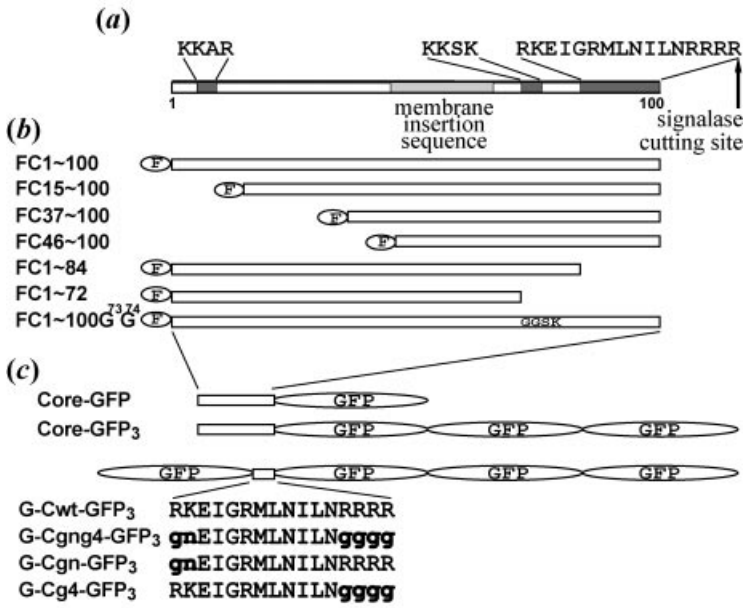

Fig. 1. Schematic representation of the recombinant core proteins used in this study. (a) Predicted NLS motifs of the DEN core protein. (b) Flag-tagged core proteins. The Flag tag on each recombinant core protein is denoted as 'F' in ellipses. (c) GFP-tagged proteins and the sequences of wild-type or mutant NLS motifs. GFP is denoted as 'GFP' in ellipses.

coding region of the core protein gene without the prM signal sequence using primers T3 and CoreTerm (Table 1). Since the core protein gene does not have a stop codon, the stop codon was built in to the primer CoreTerm. The PCR product was digested with *Eco*RI, resulting in an *Eco*RI end (derived from the polylinker region of the vector) at the 5′ terminus and a blunt end at the 3′ terminus of some molecules of the PCR product. The digested PCR product was inserted between the *Eco*RI and *Eco*RV sites of pFlagCMV2 (Sigma) to generate pFC1 ~ 100 (Fig. 1b), which expresses the entire core protein with a Flag tag at the N terminus.

Plasmids were also constructed to express the core protein tagged with the green fluorescence protein (GFP). The core protein gene was amplified from pBCM using primers T3 and CoreERI (Table 1). This PCR product had a *Bam*HI site derived from the polylinker region of the vector at the 5′ end and an *Eco*RI site derived from primer CoreERI at the 3′ end. After digestion with *Bam*HI/*Eco*RI, the PCR product was inserted between the *Bgl*II and *Eco*RI sites located in the polylinker region of pEGFP-N2 (Clontech), which contains the GFP gene driven by the cytomegalovirus (CMV) immediate early promoter. The resulting

plasmid, named pCore-GFP (Fig. 1c), expressed the core protein tagged to GFP at the C terminus.

To investigate whether the core protein enters the nucleus of infected cells by active transport, the core protein was fused to three copies of GFP in tandem, resulting in a fusion protein with a molecular mass of 96 kDa, which is too big to enter the nucleus by simple diffusion. To join three copies of the GFP gene together, the GFP gene was first amplified from pEGFP-N2 with primer CMV_{IE} (Table 1), which anneals to the CMV promoter and primer GFPL (Table 1), which, in turn, anneals to the 3′ end of the GFP-encoding region. Primer GFPL was designed so that the stop codon of the GFP gene was not amplified. This PCR product contained a *Bgl*II site located 75 bp downstream from the 5′ end and a second *Bgl*II site derived from the primer at the 3′ end. After digestion with *Bgl*II, the fragment was inserted between the *Bgl*II and *Bam*HI sites of pEGFP-N2, resulting in $pGFP_2$, which contained two tandem copies of the GFP gene fused in-frame. Since the 3′ end *Bgl*II site of the PCR fragment was fused with the *Bam*HI site on the vector, these two restriction sites were lost. The same PCR fragment containing the GFP gene with a *Bgl*II site on both ends was inserted between the *Bgl*II and *Bam*HI sites of $pGFP_2$, generating $pGFP_3$, which contained three copies of the GFP gene fused head-to-tail in tandem. These three tandem copies of the GFP gene, situated between the *Bam*HI site located 23 bp upstream from the initiation codon of the first copy of the GFP gene and the *Xba*I site located right at the stop codon of the last copy of the GFP gene, was then isolated by digesting $pGFP_3$ with *Bam*HI/*Xba*I. The resulting 2231 bp *Bam*HI–*Xba*I fragment was used to replace the single copy GFP gene on pCore-GFP by inserting the fragment into the corresponding restriction sites. The new recombinant plasmid obtained was named pCore-GFP_3 (Fig. 1c), which encodes the core protein fused with three copies of GFP, with a molecular mass of 96 kDa. The production of this 96 kDa GFP in cells transfected with pCore-GFP_3 was confirmed by Western blot analysis using a rabbit anti-GFP antibody (Clontech), indicating that pCore-GFP_3 was stable.

To determine the NLS of the core protein, plasmids containing different portions of the core gene were constructed. Primer CMV_{IE} was paired with primer P1–72 or P1–84 (Table 1) for PCR using pFC1 ~ 100 as the template to generate two products encoding aa 1–72 and 1–84, respectively, of the core protein. Since these PCR products have a single *Sst*I site (derived from the polylinker region of the vector) at their 5′ ends, they were cloned between the *Sst*I and *Eco*RV sites of pFlagCMV2, generating plasmids pFC1 ~ 84 and pFC1 ~ 72 (Fig. 1b), which express Flag-tagged, truncated core proteins missing aa 85–100 and 73–100, respectively. With a similar approach, pFC15 ~ 100, pFC37 ~ 100 and pFC46 ~ 100 (Fig. 1b) were constructed to express Flag-tagged core proteins missing the first 14, 36 and 45 N-terminal amino acids, respectively.

To mutate the sequence KKSK located at aa 73–76 of the core protein to GGSK, PCR-directed mutagenesis was performed. Primers $C^{73}GG^{74}F$ and $C^{73}GG^{74}R$ (Table 1) with the desired mutations incorporated were used. Primer CMV_{IE} was paired with primer $C^{73}GG^{74}R$ to amplify a fragment from pFC1 ~ 100 encoding aa 1–75 of the core protein. In a separate PCR, primer $C^{73}GG^{74}F$ was coupled with primer CoreTerm to amplify a fragment from pFC1 ~ 100 encoding aa 72–100. These two PCR products were mixed, denatured and then re-natured. After incubation in the reaction buffer containing *Taq* DNA polymerase and deoxynucleotides for 5 min, primers CMV_{IE} and CoreTerm were added to amplify the entire core gene. The PCR product thus generated was digested with *Sst*I and then inserted into pFlagCMV2 between the *Sst*I and *Eco*RV sites producing pFC1 ~ $100G^{73}G^{74}$, which expressed a core protein with the two lysine residues located at aa 73 and 74 changed to glycine residues.

To determine whether the putative NLS motif, RKeigrmlnilnRRRR, located at aa 85–100 could mediate nuclear localization, a DNA fragment encoding this 16 aa motif was amplified from pFC1 ~ 100 with primers P85 and Core*Sma*I (Table 1). The amplified product was inserted between the *Bgl*II and *Sma*I sites of pEGFP-C1 (Clontech), fusing this 16 aa motif in-frame to the C-terminal end of GFP, which does not naturally have an NLS sequence. This newly constructed plasmid was named pGFP-NLS. The DNA fragment encoding the GFP–NLS fusion protein was isolated by digesting pGFP-NLS with *Nhe*I (located 750 bp upstream from codon 85 of this NLS) and *Sma*I (derived from primer Core*Sma*I) and then cloned into the corresponding sites on $pGFP_3$, generating pG-Cwt-GFP_3, which encodes a fusion protein in the order of GFP–NLS–GFP–GFP–GFP (Fig. 1c). This putative NLS has a bipartite structure of RK–n_{10}–RRRR. To determine the role of residues RK in nuclear transport, these two residues were changed to GN using primer $G^{85}GN^{86}$ (Table 1), which had the desired mutations built in. Similarly, residues RRRR were changed to GGGG using primer $C^{97}G^{100}$ (Table 1). Primer pairs $C^{85}GN^{86}$ and Core*Sma*I, $C^{85}GN^{86}$ and $C^{97}G^{100}$, and P85 and $C^{97}G^{100}$ were used to generate DNA fragments encoding GNeigrmlnilnRRRR, GNeigrmlnilnGGGG and RKeigrmlnilnGGGG, respectively. These DNA fragments were cloned into pEGFP-C1, then $pGFP_3$ using the same cloning strategy described above to generate pG-Cgng4-GFP_3, pG-Cgn-GFP_3 and pG-Cg4-GFP_3, respectively, containing the mutated NLS fused in-frame with four copies of the GFP gene (GFP–NLS–GFP–GFP–GFP; Fig. 1c). The stability of these NLS–GFP_4 fusion proteins was again confirmed by Western blot analysis using the anti-GFP antibody as described above. A GFP fusion protein of approximately 120 kDa was detected in samples from cells transfected with any of these recombinant plasmids (data not shown). The inserts of all plasmids constructed in this study were sequenced to confirm the accuracy of the inserts.

■ **Production of anti-core antibodies.** Mice were injected intravenously with 1×10^8 p.f.u. of live DEN (serotype 2 strain PL046, referred to as DEN-2 PL046) and booster injected with the same dosage of DEN-2 PL046 every 2 weeks. Spleen cells were obtained from these mice 8 weeks after the initial injection and fused with mouse myeloma F_o cells (Lane *et al*., 1986). BHK-21 cells were infected with DEN-2 PL046 at an m.o.i. of 5 for 24 h, fixed with cold acetone and then used as the antigen for ELISA to screen hybridoma clones. Since the core protein is known to be present in the nucleus, ELISA-positive clones were assayed by immunocytochemistry to detect those that react with antigens in the nucleus of DEN-infected cells. One clone, designated monoclonal antibody (mAb) 15B11, showed positive nuclear staining. This clone was characterized further and confirmed to specifically react with the core protein. Another clone (mAb 13F4), which reacted with DEN-infected cells but not with the core protein, was also selected and used as a control. A polyclonal antibody against the core protein (anti-Core) was also produced by immunizing rabbits with a synthetic peptide corresponding to aa 3–22 of the core protein, according to the methods described by Reed *et al*. (1997).

■ **Immunofluorescence staining.** BHK-21, HeLa or HuH-7 cells were infected with DEN-2 PL046 or transfected with plasmids encoding Flag- or GFP-tagged core proteins. Transfection of recombinant plasmids was performed using liposomes (Lipofectamine 2000 reagent, Life Technologies). DEN-2 PL046-infected or core gene-transfected cells were fixed with 4% paraformaldehyde for 30 min and then permeated with 0·2% Triton X-100 for 5 min at room temperature. The fixed cells were reacted with the appropriate antibodies described below and examined under a fluorescence or confocal microscope (Leica TCS SP2). The core protein expressed in transfected cells was detected by immunofluorescence staining using mAb 15B11 as the primary anti-core antibody and

FITC-conjugated goat anti-mouse IgG (Sigma) as the secondary antibody. Alternatively, mAb 15B11 was labelled with biotin using the EZ-Link Biotinylation kit (Pierce) and then reacted with UltraAvidin–Rhodamine (Leinco Technologies). FITC-conjugated anti-Flag M2 mAb (Sigma) was used to detect Flag-tagged core proteins. Expression of the GFP-tagged core protein in transfected cells was visualized directly under a fluorescence microscope (Olympus BX50). The nuclei of cells were stained with a DNA-specific dye, Hoechst 33258. The nucleoli that reacted with mAb CP2, which reacts against the human nucleolar protein p130 (Pai *et al.*, 1995), were visualized by reaction with FITC-conjugated goat anti-mouse IgG2a (Sigma).

■ **Immunoprecipitation and Western blot assays.** Antibodies were bound to protein A–agarose beads (Sigma) and then incubated overnight with lysates of DEN-2 PL046-infected or core gene-transfected BHK-21 cells in NET buffer (1 mM PMSF, 0·1% NP-40, 150 mM NaCl, 0·5 mM EDTA and 50 mM Tris–HCl, pH 7·5). After washing three times with NET buffer, the beads were boiled for 2 min in 50 µl of SDS sample buffer. Proteins dissolved in this sample buffer were electrophoresed on a 12·5% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk, the membrane was incubated with an appropriate primary antibody. Bound antibody was detected with an HRP-conjugated secondary antibody and the Renaissance Western Blot Chemiluminescence Reagent Plus (Perkin Elmer).

Results

Characterization of antibodies

A mAb directed against the core protein (mAb 15B11) was produced in order to determine the intracellular location of the core protein. To confirm the specificity of mAb 15B11, it was used to probe a Western blot containing electrophoretically separated proteins from DEN-infected BHK-21 cells and cells expressing the recombinant core protein FC1 ~ 100 (Fig. 1). No protein bands were seen to react with mAb 15B11 (data not shown), suggesting that mAb 15B11 does not react with denatured DEN antigens.

To be able to examine the core protein by Western blot analysis, a polyclonal antibody (anti-core) against a synthetic peptide corresponding to aa 3–22 of the core protein was produced. The specificity of this antibody was demonstrated by Western blotting, in which it reacted with a 16 kDa protein band in the sample from BHK-21 cells transfected with pFC1 ~ 100 (Fig. 2a, lane 5) but not from cells transfected with pFC37 ~ 100 (Fig. 2a, lane 4) or the vector (Fig. 2a, lane 6). This result indicates that the anti-core antibody reacted with the protein encoded by pFC1 ~ 100. Since the only difference between pFC1 ~ 100 and pFC37 ~ 100 is that the protein encoded by pFC37 ~ 100 is missing the first 36 aa of the core protein where the antibody recognizes, this 16 kDa protein must be the Flag-tagged core protein encoded by pFC1 ~ 100.

A protein of approximately 24 kDa was also seen in all three samples (Fig. 2a, lanes 4–6), indicating that the anti-core antibody also reacts with a protein from BHK-21 cells. To ensure that the recombinant core protein FC37 ~ 100 (Fig. 1) was expressed in cells transfected with pFC37 ~ 100, a Western blot containing the same proteins was reacted with the anti-Flag M2 antibody. No protein bands were detected from the sample of cells transfected with the vector (Fig. 2a, lane 3), whereas the 16 kDa protein (Fig. 2a, lane 2) and the 11 kDa protein (Fig. 2a, lane 1) were detected in samples obtained from cells transfected with pFC1 ~ 100 and pFC37 ~ 100, respectively. These results indicate that both FC1 ~ 100 and FC37 ~ 100 (Fig. 1) recombinant core proteins were expressed in transfected cells.

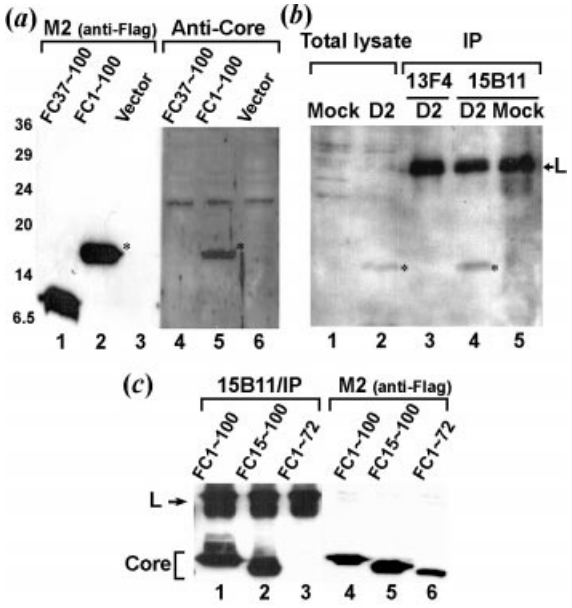

Fig. 2. Characterization of the polyclonal anti-core antibody and mAb 15B11. (a) Western blots of electrophoretically separated proteins from BHK-21 cells transfected with pFC1 ~ 100, pFC37 ~ 100 and the vector pFlagCMV2 were probed with the anti-Flag M2 (lanes 1–3) or the polyclonal anti-core (lanes 4–6) antibody. Bands marked with asterisks are full-length core proteins. (b) Total cell lysate of mock- (Mock, lane 1) or DEN-infected (D2, lane 2) BHK-21 cells. Immunocomplexes precipitated from these cell lysates with the control mAb 13F4 (lane 3) and mAb 15B11 (lanes 4 and 5) were electrophoresed on a 12·5% SDS–polyacrylamide gel. The Western blot of the gel was reacted with rabbit anti-core polyclonal antibody followed with a HRP-conjugated goat anti-rabbit IgG and a luminol substrate for HRP. Bands marked with asterisks are full-length core proteins. The immunoglobulin light chains (L) of the primary antibodies (mAbs 13F4 and 15B11) were seen in immunoprecipitates of both mock- and DEN-infected cell lysates because it also reacted with the HRP-goat anti-rabbit IgG. (c) Total cell lysate (lanes 4–6) or immunocomplexes precipitated with mAb 15B11 (lanes 1–3) of BHK-21 cells transfected with pFC1 ~ 100 (lanes 1 and 4), pFC15 ~ 100 (lanes 2 and 5) or pFC1 ~ 72 (lanes 3 and 6) were electrophoresed and then examined by Western blotting. The Western blot was reacted with anti-Flag M2 antibody followed with HRP-goat anti-mouse IgG and a luminol substrate for HRP. The immunoglobulin light chains (L) derived from mAb 15B11 were seen because it also reacted with the HRP-goat anti-mouse IgG. IP, immunoprecipitation.

Since mAb 15B11 does not recognize the denatured core protein on Western blots, its specificity was examined by immunoprecipitation. mAb 15B11 was bound to protein A-conjugated agarose beads and then reacted with cell lysates of DEN- or mock-infected BHK-21 cells. A Western blot of the gel containing electrophoresed cell lysates (Fig. 2b, lanes 1 and 2) and immunoprecipitated proteins (Fig. 2b, lanes 3–5) was reacted with the polyclonal anti-core antibody. With this

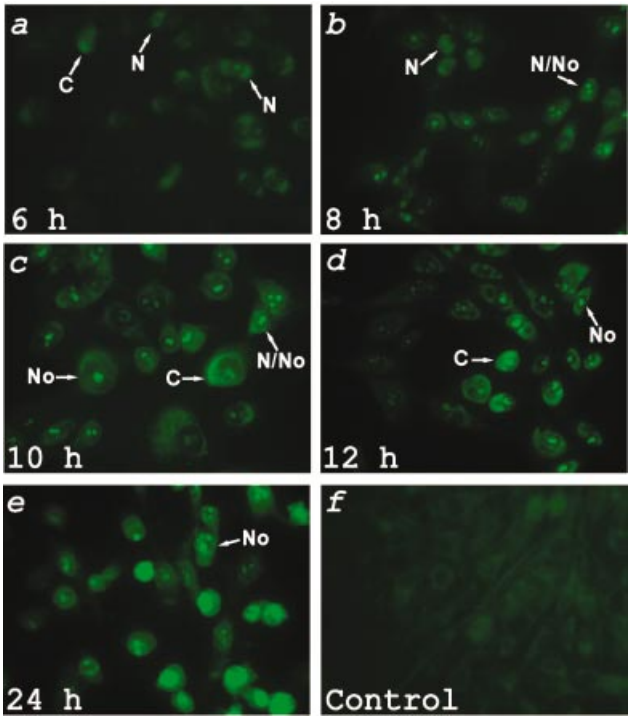

Fig. 3. Immunofluorescence staining with mAb 15B11 of DEN-infected cells. BHK-21 cells were infected with DEN (m.o.i. = 5). At 6 (a), 8 (b), 10 (c), 12 (d) and 24 (e) h p.i., the infected cells were reacted with mAb 15B11 followed with an FITC-conjugated goat anti-mouse antibody and then examined under a fluorescence microscope. Cytoplasmic, nuclear and nucleolus staining of infected cells are indicated as C, N and No, respectively. BHK-21 cells infected with UV-inactivated DEN was used as a negative control (f).

approach, mAb 15B11 was found to precipitate a protein of approximately 15 kDa in size from DEN-infected cells (Fig. 2b, lane 4) but not from mock-infected cells (Fig. 2b, lane 5). Since the anti-core antibody reacts specifically to the core protein, this 15 kDa band should be the core protein. It should be noted that this 15 kDa protein band is slightly larger than the size (14 kDa) deduced from the amino acid sequence, probably because the core protein is rich in positively charged amino acids and thus migrates more slowly on the gel. A control mAb (13F4), which does not react with the core protein but which is capable of binding to protein A-conjugated agarose beads, did not precipitate the 15 kDa protein (Fig. 2b, lane 3). Since the HRP-conjugated goat anti-rabbit IgG that was used as the secondary antibody cross-reacted with the light chain of mouse antibodies, the light chain of mAbs 15B11 or 13F4 also appeared on the Western blot (Fig. 2b, lanes 3–5, indicated as L).

Immunoprecipitation using mAb 15B11 was also performed on cell lysates derived from BHK-21 cells transfected with pFC1 ~ 100, pFC15 ~ 100 and pFC1 ~ 72. To ensure that the recombinant core proteins were expressed in transfected cells, the cell lysates were analysed by Western blotting using anti-Flag M2 antibody. Only one band, which is the size of the respective recombinant core protein, was seen from each sample (Fig. 2c, lanes 4–6). Immunoprecipitation with mAb 15B11 was then performed on these cell lysates. The immunoprecipitates were analysed on a Western blot probed with the anti-Flag M2 antibody. mAb 15B11 was found to precipitate recombinant core proteins from cell lysates of pFC1 ~ 100- and pFC15 ~ 100-transfected cells but not from pFC1 ~ 72-transfected cells (Fig. 2c, lanes 1–3). This result suggests that the epitope that is recognized by mAb 15B11 is located in the last C-terminal 28 aa of the core protein or that deletion of the last C-terminal 28 aa changed the conformation of the core protein so that it was no longer recognized by mAb 15B11. The light chain of mAb 15B11 was also seen in Fig. 2(c, indicated as L) because the HRP-conjugated secondary antibody, goat anti-mouse IgG, reacted with it, as described above.

Intracellular localization of the core protein during DEN infection

To determine the intracellular location of the core protein during DEN infection, BHK-21 cells infected with DEN-2 PL046 were examined with mAb 15B11 by immunofluorescence staining at 6, 8, 10, 12 and 24 h after infection (Fig. 3). At 6 h post-infection (p.i.), a slight positive staining was observed in both the cytoplasm and the nuclei of infected cells (Fig. 3a). At 8 h after infection, the staining became more intense and appeared as spots located mainly in the nuclei of infected cells (Fig. 3b). At 10 h p.i. (Fig. 3c), the cytoplasmic staining and the spots in nuclei became even more visible. The intensity of the immunofluorescence in both the nucleus and the cytoplasm increased over time during infection (Fig. 3a–e). The highest intensity was observed at 24 h p.i. No immunofluorescence was observed in BHK-21 cells mock-infected with UV-inactivated DEN at 24 h p.i. (Fig. 3f).

The observation that immunofluorescence staining with mAb 15B11 appeared as spots in the nuclei of infected cells suggests that the core protein is located in nucleoli. To prove this, DEN-infected HuH-7 cells (a human hepatoma cell line) were examined simultaneously with mAb 15B11 and a nucleolus-specific antibody CP2, which reacts with the human nucleolar protein p130 (Pai *et al.*, 1995). Since CP2 does not react with the p130 counterpart in BHK-21 cells, BHK-21 cells were replaced with HuH-7 cells that are also susceptible to DEN (Lin *et al.*, 2000).

To distinguish reactions from the two different mAbs, mAb 15B11 was labelled with biotin and detected with Ultra-Avidin–Rhodamine (red fluorescence) and mAb CP2 was detected with FITC-conjugated goat anti-mouse IgG2a (green fluorescence). HuH-7 cells were infected with DEN-2 PL046 for 24 h, reacted with both antibodies and then examined by confocal microscopy. As seen in BHK-21 cells, DEN-2 PL046-infected HuH-7 cells also showed spots in the nuclei when they were stained with mAb 15B11 (Fig. 4a). Staining with the anti-nucleolus mAb CP2 resulted in tiny green granules in the nucleoli (Fig. 4b), as reported previously (Pai *et al.*, 1995). When these two images were superimposed, yellow dots were

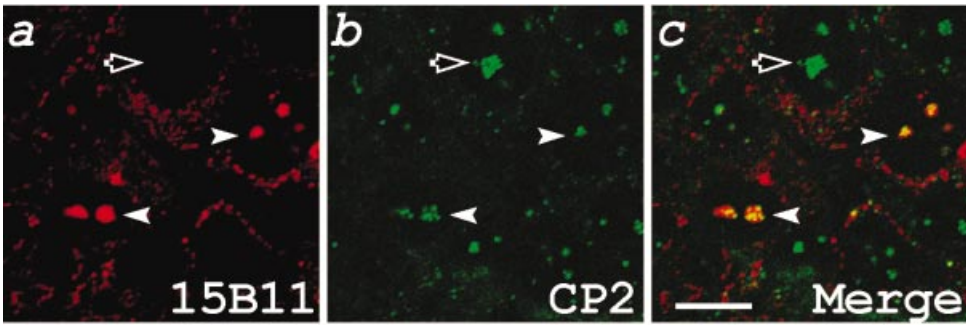

Fig. 4. Nucleolus localization of the core protein in DEN-infected cells. HuH-7 cells were infected with DEN (m.o.i. = 5) for 18 h. The infected cells were fixed and reacted simultaneously with mAb 15B11 and the nucleolus-specific mAb CP2. (a) Reaction with biotinylated mAb 15B11 detected with UltraAvidin–Rhodamine (red fluorescence). (b) Reaction of mAb CP2 to the nucleolar protein p130 detected with FITC-conjugated goat anti-mouse IgG2a (green fluorescence). (c) Superimposed images of (a) and (b). Yellow spots represent the subcellular structure that reacted with both mAb 15B11 and CP2. Closed arrowheads indicate nucleoli of DEN-infected HuH-7 cells. Open arrows indicate HuH-7 cells that are not infected with DEN. Images were taken with a confocal microscope. Bar, 10 μm.

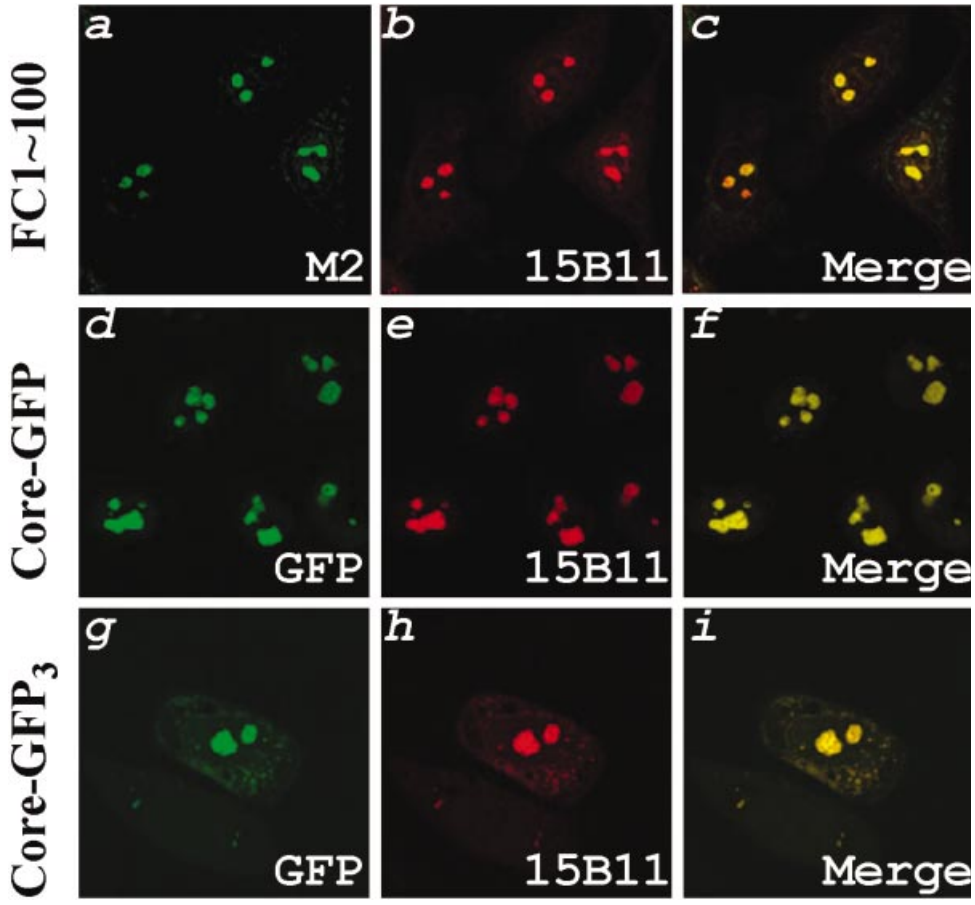

Fig. 5. Nuclear localization of recombinant core protein of DEN. HeLa cells were transfected with pFC1 ~ 100, pCore-GFP or pCore-GFP_3. At 18 h after transfection, pFC1 ~ 100-transfected cells were simultaneously stained with FITC-conjugated anti-Flag M2 antibody (a) and biotinylated mAb 15B11 followed with UltraAvidin–Rhodamine (b) and then examined under a confocal microscope. Green spots are antigens that reacted with the FITC-conjugated anti-Flag antibody. Red spots are those that reacted with biotinylated mAb 15B11. Yellow spots in the merged image (c) represent the same antigen that reacted with both antibodies. HeLa cells transfected with pCore-GFP (d–f) or pCore-GFP_3 (g–i) were examined directly or reacted with biotinylated mAb 15B11 and then examined by confocal microscopy. The GFP-tagged core protein exhibited green fluorescence (d, g) when examined directly and red fluorescence when reacted with mAb 15B11 (e, h). Yellow spots are seen (f, i) when the two images are superimposed. Bar, 10 μm.

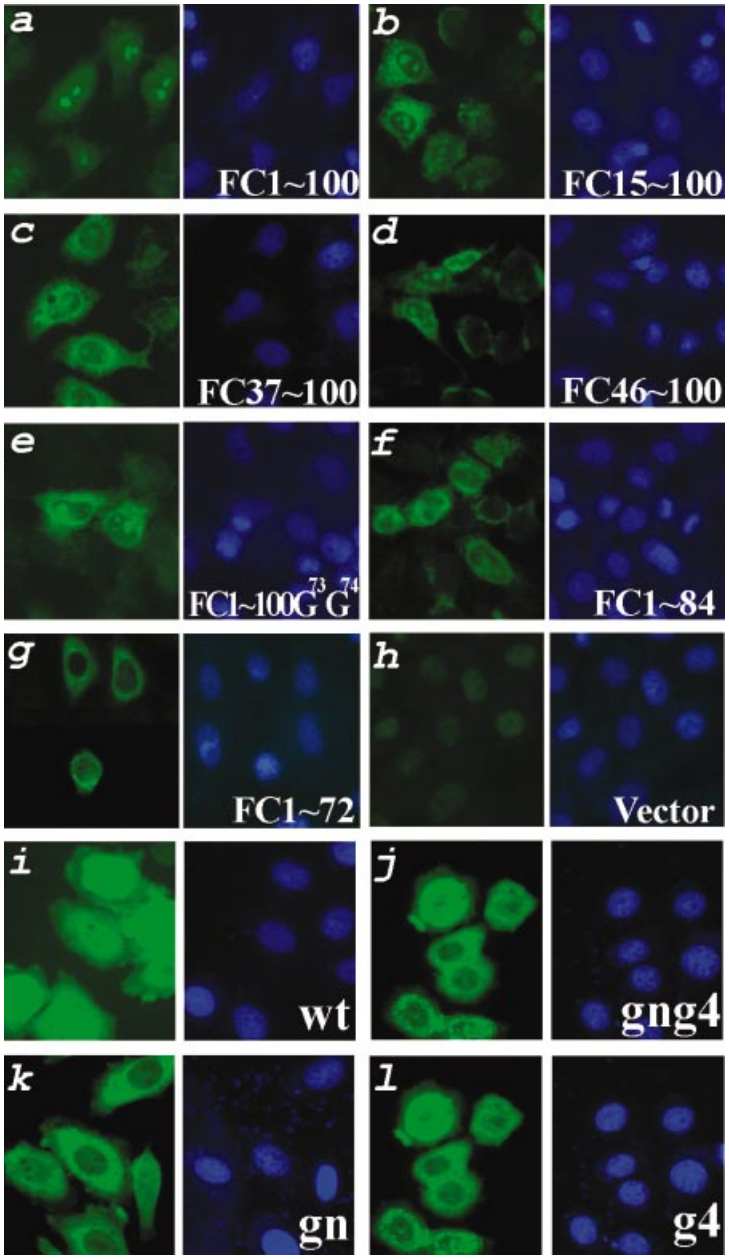

Fig. 6. Determination of the NLS of the core protein. HeLa cells were transfected with pFC1 ~ 100 (a), pFC15 ~ 100 (b), pFC37 ~ 100 (c), pFC46 ~ 100 (d), pFC1 ~ 100G^{73}G^{74} (e), pFC1 ~ 84 (f), pFC1 ~ 72 (g), pFlagCMV2 vector (h), pG-Cwt-GFP$_3$ (i), pG-Cgng4-GFP$_3$ (j), pG-Cgn-GFP$_3$ (k) or pG-Cg4-GFP$_3$ (l). At 18 h after transfection, the cells were reacted with FITC-conjugated anti-Flag M2 antibody (a–l, left panels) or the blue fluorescence dye Hoechst 33258 (a–l, right panels) and then examined under a fluorescence microscope.

seen (Fig. 4c), indicating that these two antibodies reacted with the same subcellular structure, the nucleolus.

Lack of involvement of other DEN proteins in nuclear localization of the core protein

To determine whether other DEN proteins are involved in nuclear transport of the core protein, pFC1 ~ 100 encoding only the core protein was transfected into HeLa cells, which have larger nuclei and nucleoli and thus make it easier to observe nuclear localization of the core protein. At 18 h after transfection, cells were examined with FITC-conjugated anti-Flag M2 antibody and green fluorescent spots similar to those seen in DEN-infected cells were observed (Fig. 5a). The same transfected cells were also reacted with biotinylated mAb 15B11 and detected with UltraAvidin–Rhodamine resulting in red spots (Fig. 5b). When the two images were superimposed, the same spots in the nuclei exhibited yellow fluorescence (Fig. 5c), suggesting that the core protein is transported into the nucleus and then to the nucleolus without the involvement of other DEN proteins.

To ensure that the nuclear localization of the recombinant core protein was not due to the Flag tag, cells transfected with the GFP-tagged core protein gene were examined with mAb 15B11 in a similar manner. Both pCore-GFP and pCore-GFP$_3$ were transfected separately into HeLa cells and the transfected

cells were examined 18 h after transfection. The same pattern of immunofluorescence as that seen in cells transfected with the Flag-tagged core protein gene was observed (Fig. 5d–i). These results suggest that other DEN proteins are not required for nuclear localization of the core protein and that the core protein alone is responsible for its nuclear localization. The observation that pCore-GFP (Fig. 5d–f) and pCore-GFP_3 (Fig. 5g–i) transfected cells showed identical staining patterns suggests that the length of the tag does not affect the nuclear localization of the core protein. Since the core protein tagged with three copies of GFP has a molecular mass of 96 kDa, the GFP-tagged core protein must have entered the nucleus and nucleolus by active transport instead of simple diffusion.

Determination of the NLS motif of the core protein

To determine the role of the three predicted NLS motifs in nuclear localization of the core protein, recombinant clones containing the Flag-tagged wild-type or the truncated core protein gene were transfected into HeLa cells. At 18 h after transfection, subcellular location of the expressed core proteins was determined by immunofluorescence microscopy using the FITC-conjugated anti-FLAG M2 antibody. The same cells were also stained with the blue fluorescence dye Hoechst 33258, which stains cell nuclei (Fig. 6). The results showed that recombinant core proteins expressed in cells transfected with pFC1 ~ 100, pFC15 ~ 100, pFC37 ~ 100 or pFC46 ~ 100 were all located in nucleoli (Fig. 6a–d). Since FC15 ~ 100, FC37 ~ 100 and FC46 ~ 100 are Flag-tagged truncated core proteins missing the N-terminal 14, 36 and 45 aa, the results suggest that the putative NLS motif (KKAR) located at aa 6–9 does not play a role in nuclear localization of the core protein. These N-terminal truncations of the core protein did result in accumulation of more core proteins in the cytoplasm, with a slight reduction in the amount of core protein in the nucleoli (Fig. 6a–d).

To determine the function of the predicted NLS motif (KKSK) located at aa 73–76 in nuclear localization of the core protein, the first two amino acids of this motif were changed from lysine to glycine. Plasmid pFC1 ~ $100G^{73}G^{74}$ containing these two mutations was transfected into HeLa cells and then examined for subcellular location of the expressed core protein (Fig. 6e). The results showed that the core protein expressed in HeLa cells transfected with this plasmid was still located in the nucleoli (Fig. 6e). More cytoplasmic core protein was also observed in cells transfected with pFC1 ~ $100G^{73}G^{74}$. These results suggest that neither of these two putative NLS motifs play a major role but both can enhance nuclear localization of the core protein.

The possibility that the putative NLS motif located at aa 85–100 is essential to the nuclear localization of the core protein was investigated by examining the subcellular location of the core protein that is missing this motif. HeLa cells were transfected with pFC1 ~ 84 and pFC1 ~ 72, which encode core proteins missing the last C-terminal 16 and 28 aa, respectively. The results showed that both of these two recombinant core proteins were located exclusively in the cytoplasm (Fig. 6f–g), suggesting that this NLS is responsible for the nuclear localization of the core protein. There was no difference in the staining pattern between cells transfected with pFC1 ~ 84 (Fig. 6f) and those transfected with pFC1 ~ 72 (Fig. 6g), implying that deletion of the last 16 or 28 aa from the C-terminal end resulted in the same effect on subcellular localization of the core protein.

To confirm the nuclear localization ability of this putative NLS motif, experiments were performed to determine if it can drive the GFP protein, which lacks an NLS signal, into the nucleus. pG-Cwt-GFP_3, which encodes the GFP–NLS–GFP–GFP–GFP fusion protein, was transfected into HeLa cells. With a fluorescence microscope, 300 transfected cells were examined; 178 (59·3%) cells were found to have a pattern of nuclear fluorescence (Fig. 6i). No cells exhibited nuclear fluorescence when they were transfected with the negative control $pGFP_4$ (data not shown); a similar observation was reported by Chatterjee *et al*. (1997). These results indicate that this motif has nuclear localization function. Since this motif has a bipartite structure of RK–n_{10}–RRRR, the role of the consensus sequences at both sides of the bipartite motif in nuclear localization was examined by mutating either or both consensus sequences. Three such NLS mutants, GNeigrmlnilnGGGG, GNeigrmlnilnRRRR and RKeigrmlnilnGGGG, were generated (Fig. 1c). Plasmids pG-Cgng4-GFP_3, pG-Cgn-GFP_3 and pG-Cg4-GFP_3 containing these mutants were transfected separately into HeLa cells (Fig. 6j–l). The GNeigrmlnilnGGGG mutant was found to mediate transport of the core protein into the nucleus in only 21·7% of transfected cells (Fig. 6j). This is a 37·6% decrease in nuclear localization as compared to the wild-type NLS RKeigrmlnilnRRRR (Fig. 6i). A similar decrease in nuclear localization was observed in constructs with mutants GNeigrmlnilnRRRR (Fig. 6k) and RKeigrmlnilnGGGG (Fig. 6l), which mediated nuclear localization in only 20 and 14·7%, respectively, of transfected cells. These results further confirm that this bipartite motif is a functional NLS.

Discussion

The core protein of DEN was first detected in the nucleolus in addition to the nucleus and cytoplasm of infected cells by Tadano *et al*. (1989). However, Bulich & Aaskov (1992) used different mAbs against the core protein in a similar experiment and found that the core protein was not located in the nucleolus during infection. To clarify this discrepancy, we independently produced another mAb (15B11) against the core protein to re-examine the intracellular location of the core protein. Using mAb 15B11, we clearly observed the core protein in the nucleoli of cells infected with DEN (Fig. 4) or transfected with the wild-type core protein gene (Fig. 5), indicating that the core protein has a very strong nucleolus

Table 2. Sequence comparison of the bipartite NLS located at aa 86–100 of the core protein of various serotypes of DEN

The sequences of aa 85–100 are referenced as follows: DEN-1, Chu *et al.* (1989); DEN-2 (NGC), Irie *et al.* (1989); DEN-3, Osatomi & Sumiyoshi (1990); DEN-4, Zhao *et al.* (1986).

	NLS motif		
Serotype	Left	Middle	Right
DEN-1	RK	eisnmlniln	RRKR
DEN-2 (NGC)	RK	eigrmlniln	RRKR
DEN-3	RK	eisnmlniln	RRKR
DEN-4	RK	eigrmlnilng	RKR
DEN-2 (PL046)	RK	eigrmlniln	RRRR
Consensus*	$^{R}/_{K}$ $^{R}/_{K}$	10–12 residues	$(^{R}/_{K})_3$ in five residues

* The consensus sequence is published by Dingwall & Laskey (1991).

localization preference and that the core protein itself has the ability to enter the nucleus.

Three NLS signal motifs had been predicted to be present on the core protein (Bulich & Aaskov, 1992). The first putative NLS motif (KKAR) located at aa 6–9 of the core protein is identical to the NLS motif of the polyomavirus large T antigen (Richardson *et al.*, 1986). The second putative NLS motif (KKSK) located at aa 73–76 resembles that of nucleoplasmin (KKKK) (Burglin & De Robertis, 1987). Although these two motifs have sequences characteristic of NLS motifs, deletion or mutation of these two motifs did not abolish nuclear localization of the core protein (Fig. 6). However, deletion of the third putative NLS motif, RKeigrmlnilnRRRR, located at aa 85–100 completely eliminated the ability of the core protein to enter the nucleus. In addition, this motif was shown to have the ability to translocate a cytoplasmic protein, GFP, into the nucleus (Fig. 6i). Mutation of the consensus sequences, which are well conserved in various serotypes of DEN (Table 2), of this bipartite motif diminished its nuclear localization ability (Fig. 6j–l). Interestingly, the motif RRRR located at this bipartite motif is also seen in the bipartite NLS (MGFVKVVK-NKAYFKRYQVKFRRRRE) of the ribosomal protein L5, although the function of this motif is not clear (Claussen *et al.*, 1999). Recently, the bipartite NLS from nucleoplasmin shows binding activity to importin-α, a nuclear import receptor (Fontes *et al.*, 2000). The bipartite NLS of DEN core protein may have a similar function.

The DEN core protein is responsible for encapsidation of the viral RNA which takes place in the cytoplasm (Chambers *et al.*, 1990; Westaway *et al.*, 1985). The function of the core protein in the nucleus and nucleolus remains to be investigated. It has been hypothesized that the DEN core protein serves as a transcription modulator (Chang *et al.*, 2001), similar to the core protein of hepatitis C virus (HCV) (Hsieh *et al.*, 1998). The HCV core protein has been shown to interact with the transcription factor hnRNP K, which is located in the nucleus (Michelotti *et al.*, 1996) and is known to enhance c-Myc to promote apoptosis (Prendergast, 1999). Apoptosis due to DEN infection has been observed in the liver of infected patients (Couvelard *et al.*, 1999), primary cultured endothelial and hepatic cells (Avirutnan *et al.*, 1998; Marianneau *et al.*, 1999) and neuroblastoma and hepatoma cell lines (Despres *et al.*, 1996; Jan *et al.*, 2000; Marianneau *et al.*, 1997; Su *et al.*, 2001). It is possible that the DEN core protein also modulates apoptosis of host cells, leading to the development of illness.

The function of the core protein in the nucleolus is more elusive. The core proteins of Kunjin virus and HCV, which belong to the same virus family (*Flaviviridae*) as DEN, have been shown to bind RNA (Hwang *et al.*, 1995; Khromykh & Westaway, 1996; Santolini *et al.*, 1994; Shimoike *et al.*, 1999). Since the nucleolus contains enormous amounts of RNA, it is likely that the core protein of DEN binds to those RNA molecules. The C terminus of the core protein is rich in positively charged amino acids such as lysine and arginine. This may be the region where the core protein interacts with RNA. Although nucleolus localization signals (NoLS) have been discovered in several other proteins (Liu *et al.*, 1997), no consensus sequence of the NoLS motif has been proposed. Therefore, it is not possible to predict the NoLS of the DEN core protein at present. Nevertheless, the nucleus and nucleolus localization implies that the core protein is involved in regulating the life cycle of DEN in addition to being a building block of the virus. Identification of the NLS will enable investigations on the role of the core protein in the pathogenesis of DEN.

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