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Identification of Transmembrane Domain of a Membrane Associated Protein NS5 of *Dendrolimus punctatus* Cytoplasmic Polyhedrosis Virus

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We examined the intracellular localization of NS5 protein of *Dendrolimus punctatus* cytoplasmic polyhedrosis virus (DpCPV) by expressing NS5-GFP fusion protein and proteins from deletion mutants of NS5 in baculovirus recombinant infected insect *Spodoptera frugiperda* (*Sf*-9) cells. It was found that the NS5 protein was present at the plasma membrane of the cells, and that the N-terminal portion of the protein played a key role in the localization. A transmembrane region was identified to be present in the N-terminal portion of the protein, and the detailed transmembrane domain (SQIHMVWVKSGLVFF, 57-71aa) of N-terminal portion of NS5 was further determined, which was accorded with the predicted results, these findings suggested that NS5 might have an important function in viral life cycle.

Keywords: Cytoplasmic polyhedrosis virus, Immuno-gold labeling, Immunofluorescent, Membrane association protein, NS5

Introduction

Cytoplasmic polyhedrosis viruses (CPVs) are members of the genus Cypovirus in the family Reoviridae, and characterized by the synthesis of large proteinaceous occlusion bodies (polyhedra) in the cytoplasms of midgut epithelial cells of the wide range insects during a late stage of infection (Belloncik, 1989; Belloncik and Mori, 1998), The infectious icosahedral CPV is the structurally simplest member of the Reoviridae with only a single shell, which is made up of five structural proteins with 12 turret-like projections, and encompasses a dsRNA genome of 10 segments (S1-S10) (Zhang *et al.*,1999;

Xia *et al.*, 2003). Each genomic dsRNA segment which encodes one protein is composed of an mRNA (plus-strand) and its complementary (minus strand) in an end-to-end base-paired configuration except for a protruding 5' cap in the plus strand (Furuichi *et al.*, 1973a; 1975b). The segments are transcribed by virus-associated RNA polymerase to form capped mRNA which also function as templates for a replicase in virus-infected cells (Kuchino *et al.*, 1982; Arella *et al.*, 1988). CPV has been characterized and classified as many as 17 types on the basis of the various patterns of electrophoretic mobility of the 10 equimolar segments of the dsRNA to the date (Alexandra *et al.*, 2005). However, they usually have no significant nucleotide sequence similarity to the corresponding segment among different types of cypoviruses, which might mean various characteristics and functions of the genomes.

The complete nucleotide sequences of *Dendrolimus punctatus* cytoplasmic polyhedrosis virus (DpCPV) have been recently deposited in GenBank. They also have the similar conserved terminal sequences that each dsRNA segment (1-10) has the motif "AGUAA" at the 5' terminal and "GUUAGCC" at 3' terminal of the plus RNA of DpCPV (Du *et al.*, 2001; Zhao *et al.*, 2003a; Zhao *et al.*, 2003b; Hong *et al.*, 2004). But little is known about the protein functions of DpCPV due to lack of host cell culture suitable for DpCPV to infect, although it have been identified that the RNA-binding functions of the VP4 and p44 *in vitro* which were encoded by the segment 6 and 8 respectively (Zhao *et al.*, 2005c; Zhao *et al.*, 2005d). In the current study, we report that NS5 of DpCPV is found to locate at the cytoplasm membrane of infected insect *Spodoptera frugiperda* (*Sf*-9) cells using baculovirus expression system. All these findings may help to illuminate the protein functions of DpCPV for the further studies.

Materials and Methods

Expression of NS5 in E.coli and production of antisera. Infected

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Dendrolimus punctatus walkers (forest pests) were harvested from Jiangxi province of China. After purification of polyhedra by sucrose density gradient centrifugation, the dsRNA was extracted from the purified polyhedra according to SDS-hot phenol treatment (Belloncik *et al.*, 1996), the full length cDNA of S9 genomic dsRNA was then obtained by using single primer RT-PCR amplification method (Paul *et al.*, 1992). The segment S9 ORF was amplified with forward primer of S9F: 5'-CGAACATACTA GGATCCATCATGG-3'; containing *Bam*HI site (underlined) and reversed primer of S9R: 5'-AGACTACACCTCGAGACGACAT TAC-3'; containing *Xho*I site (underline), and cloned into the same sites of bacterial expression vector pET28a (Invitrogen) to generate pET28a-NS5. The plasmid pET28a-NS5 was transformed into competent *E.coli* BL21 (Invitrogen), followed by the induction with the IPTG of 1 mM for 5 h at 37°C, and the NS5 protein was expressed insolubly in form of inclusion bodies. Before purified by Ni-NTA agarose column (Novagen), the inclusion bodies were denatured with 8 M urea in a soluble state, purified NS5 was finally dialyzed and immunized against rabbit for the production of the polyclonal antibodies.

Construction of baculovirus recombinant NSP5-GFP and confocal microscopy. To express fusion protein of NS5-GFP by baculovirus expression system, the ORF of S9 segment was amplified by PCR with primer S9F (above) and reverse primer S92R: 5'-CACCAACGGAATTCCTCTGATACG; containing *Eco*RI site (underlined) without the stop codon (TAA) at its C-terminal. The amplicon was then cloned into the baculovirus transfer vector pFASTBacHTb (gift from Prof.Qi) to generate pBac-NS5. The GFP gene was amplified from pcDNA 3.0 (gift from Dr.Jiang) with forward primer GFP3F: 5'-GACCGGAATTCCGGATGAGTAAA GGAGAAG-3'; containing *Eco*RI site (underlined) and reverse primer GFP3R: 5'-CTAGGTCTCGAGAGTTCATCCCATGC-3'; containing *Xho*I site (underlined), then the GFP gene was inserted into downstream of the NS5 gene in frame with the later. The resulting transfer vector pBac-NS5-GFP was transformed into DH10BAC competent cells, after transposition, the recombinant Bacmid DNA were identified by *LacZ* selection on X-Gal and IPTG plates and isolated from selected colonies according to the manufacturer's instructions. After mixed with lipofectin reagent (Invitrogen), recombinant bacmid DNA was transfected to the monolayer of *Sf*-9 cells (gift from CCTCC) , which cultured with Grace's media (Gibco) containing 10% FBS (Hyclone) at 28°C, and the recombinant baculoviruses vBac-NS5-GFP were harvested 96 h post transfection and amplified with two rounds. Finally, after seeded onto 22-mm coverslips, *Sf*-9 cells were infected with the recombinant baculovirus (m.o.i = 5). After 1,2,3,4 days of infection, infected cells were washed by PBS (PH = 7.4) twice, then observed directly under confocal laser scanning microscopy (Leica instruments, Germany).

Detection of protein on the surface by flow cytometry. The ORF of S9 segment was cloned into the baculovirus transfer vector pFASTBacHTb by digestion with *Bam*HI and *Xho*I from bacteria expression vector pET28a-NS5 (above), recombinant baculoviruses vET28a-NS5 were obtained as described above. After infected with recombinant baculovirus at an m.o.i of 5., the *Sf*-9 cells were harvested by centrifugation at 500 g for 10 min 48 h p.i., fixed with 2% formaldehyde and washed three times with PBS (PH = 7.4), then blocked with PBS containing 5% FBS for 45 min, cells were incubated with the primary polyclonal antibodies specific for NS5 for 2 h at room temperature. After washing, FITC goat anti-rabbit IgG was used as a secondary antibody and incubated for 30 min, the unbound antibodies were then washed off. Finally, 10^5 cells were resuspended in PBS and fluorescence on the cells surface was measured using flow cytometry (Beckman Coulter).

Immuno-gold labeling and electron microscope. For post-embedding labeling, *Sf*-9 cells were infected with recombinant baculovirus at an m.o.i of 5 as described above, after 48 h of infection, infected cells were fixed in 4% polyformaldehyde and 0.5% glutaraldehyde for 16 h at 4°C after centrifugation 1,000 g for 10 min, then washed with PBS (PH = 7.4) twice, the samples were dehydrated through a graded series of ethanol 30%, 50%, 70% (1 h in each), then cells were infiltrated at 4°C by two 1 h changes of 70% ethanol and L. R. White resin (sigma) and three 8 h changes of L. R. White. The cells were finally polymerized in L. R. White at 50°C for 48 h. Ultrathin sections were collected on formvar-coated nickel gold. The samples were blocked with 1% BSA in TBS (PH = 7.4) for 60 min, incubated with the primary polyclonal antibodies in TBS (1 : 100, 1%BSA, PH = 7.4) for 60 min, and washed twice with TBS (PH = 7.4) containing 0.2% Tween 20 for 20 min and TBS (PH = 8.4) for 10 min, followed by the incubation of secondary goat anti-rabbit IgG-conjugated 10 nm colloid gold (sigma 1 : 50) in TBS (PH = 8.4) containing 1% BSA for 3 h. Sections were finally stained with 2% aqueous uranyl acetate for 15 min and lead citrate for 5 min before observation with transmission electron microscope, uninfected *Sf*-9 cells were treated the same way and used as a control(Akhtar *et al.*, 1999).

Construction and expression of the deleted proteins of NS5 in *Sf*-9 cells. To construct a series of deletions of NS5 protein, we amplified the different fragments such as NS5A (1-212aa), NS5B (31-320aa), NS5C (57-320aa), NS5D (72-320aa) and NS5E (72-212aa), and cloned into the *Bam*HI and *Xho*I sites of baculovirus transfer vector pFASTBacHTb as described above. Then *Sf*-9 cells were seeded onto the coverslips and infected with these different recombinant baculovirus at m.i.o of 5 for 48 h, then fixed with 4% paraformaldehyde for 30 min at room temperature, followed by permeability buffer with 0.2% Triton X-100 in PBS (PH = 7.4) for 10 min, and washed again with PBS. Cells were incubated with primary antibodies (1 : 100) and secondary antibodies FITC-conjugated goat anti-rabbit IgG (1 : 100) for 60 min respectively. Meanwhile, stained with propidium iodide (PI) (10 μg/ml) for 10 min and washed with PBS (PH = 7.4) for three times (each time 10 min), these stained *Sf*-9 cells were finally observed under confocal laser scan microscope.

Results

Transmembrane lacolization of NS5-GFP in infected *Sf*-9 cell. To evaluate the localization of NS5 protein, we constructed a recombinant baculovirus vBac-NS5-GFP expressing a fusion protein NS5-GFP, with green fluorescent protein at the C-terminal of NS5 (the jellyfish Aequorea
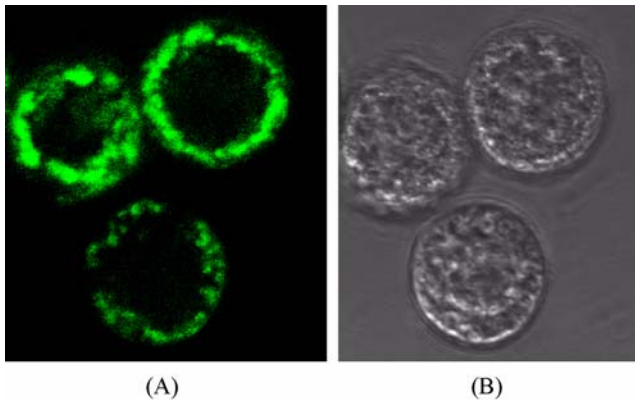
Fig. 1. Transmembrane localization of NS5-GFP expressed in baculovirus recombinant infected *Sf*-9 cells was observed under fluorescence-activated scope at 48 h postinfection (A). The picture B was taken under normal light corresponding to A.

Fig. 2. Analysis of flow cytometry. *Sf*-9 cells were infected with recombinant baculovirus vET28a-NS5 and stained with polyclonal antisera and FITC-conjugated secondary antibodies respectively after 48 h of infection (b), while mock-infected cells (a) were treated with the same antibodies as a control for nonspecific binding.

Victoria green fluorescent protein GFP, a non-membrane associated protein) (Tsien, 1998). *Sf-9* cells were infected with the recombinant baculovirus, and observed continuously at 48, 72, 96 h p.i. respectively, under the confocal laser scanning microscope. The green fluorescence signal was too weak to be detected in the *Sf*-9 cells during the first 24 h (data not shown). As show in Fig. 1, at 48 h p.i., the green fluorescence was observed clearly at the plasma membrane of *Sf*-9 cells (Fig. 1A). At 72h and 96 h p.i., the bright green fluorescence of the infected cells was further strengthened to aggregate obviously at the plasma membrane, and the green fluorescence would last visibly at the plasma membrane till the infected cells were lysed (data not shown). The same observation was performed as control under the dark scope (Fig. 1B). Apparently, the NS5 was the protein which determined the localization of the fusion protein NS5-GFP at the plasma membrane since the GFP is a non membrane associated protein.

Detection of transmembrane protein NS5 by Flow cytometry. The *Sf*-9 cells were infected with recombinant baculovirus vET28a-NS5, after 48 hours of infection, they were incubated with polyclonal antisera specific for NS5 and FITC-conjugated secondary antibodies respectively (see *Materials and Methods*). The samples were finally analyzed by flow cytometer. The profile of the fluorescence of the infected cells could be clearly distinguished from that of the mock-infected cells (Fig. 2). The result showed that approximately 42% (gated) of *Sf*-9 cells were labeled by the NS5 specific polyclonal antisera and FITC-conjugated secondary antibodies (Fig. 2b), compared to the control cells labeled with the same antibodies (Fig. 2a). Because the cells were not treated with the permeability buffer, the antibodies could not penetrate the cell membrane, the difference of fluorescence profile might be due to the presence of the transmembrane protein of NS5 on the baculovirus recombinant vET28a-NS5
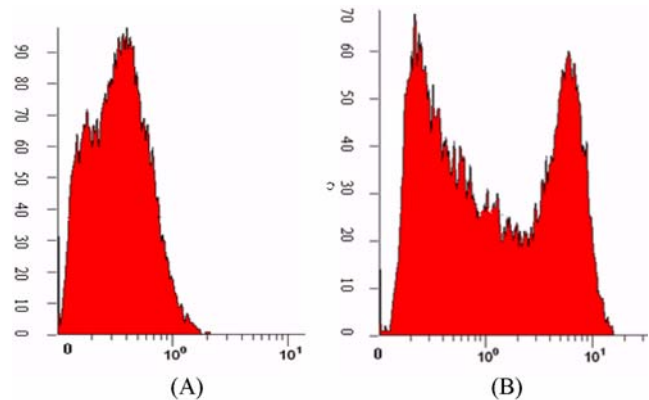
infected cells. This result suggested that the NS5 was distributed on the cell surface across the membrane due to some epitopes of the protein NS5 were exposed onto the surface of *Sf*-9 cells, which could be recognized by polyclonal antibodies special for NS5.

The observation by immuno-gold electron microscope. To provide the more direct evidence, we investigated the localization of the nonstructural protein NS5 of DpCPV by immuno-gold electron microscope. After infection with recombinant baculovirus vET28a-NS5 at m.i.o = 5, the infected *Sf*-9 cells were incubated with rabbit antibodies against NS5 and goat anti-rabbit immunoglobulins conjugated to 10 nm gold particles respectively (Fig. 3). The result revealed that the plasma membrane was labeled with 10 nm gold particles rather than the other cellular areas of *Sf*-9 cells, which suggesting that the expressed NS5 protein was mainly aggregated within the plasma membrane, but not other areas of *Sf*-9 cell. This result had also further demonstrated that NS5 was a plasma membrane associated protein.

Identification of transmembrane region of NS5. A series of baculovirus recombinants of NS5 with deletions in N or C-terminal or both were made and expressed respectively in *Sf*-9 cells. After 48 h p.i., cells were treated with immunofluorescent staining as described as above, whereas the cell nucleus were stained with PI and then examined under confocal microscope. The result showed that when cells were infected with recombinant baculovirus of the full length NS5, the fluorescent was observed only on the plasma membrane (Fig. 4a). When C-terminal portion of NS5 was deleted, the green fluorescence was still distributed at the plasma membrane (Fig. 4b), which suggested that the C- terminal portion of NS5 does not contain a transmembrane signal since its deletion did not have any effect on the transmembrane distribution of the
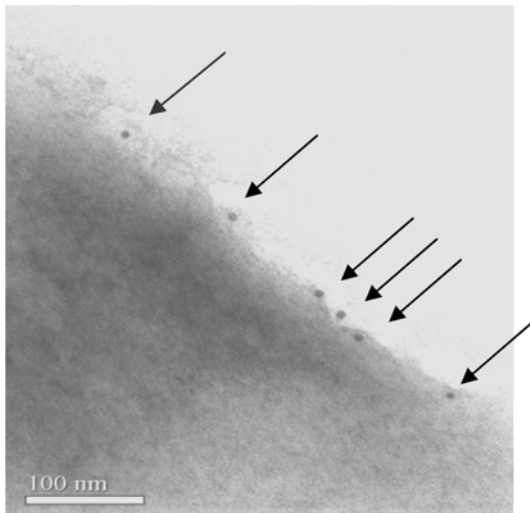
Fig. 3. Ultrastructural localization of NS5. Ultrathin section of LR White-embedded infected *Sf*-9 cells was stained with rabbit special polyclonal anti-NS5 and the goat anti-rabbit IgG-conjugated 10 nm colloid gold. Gold particles (*arrow*) were located at in the plasma membrane of *Sf-9* cell. *Bar*, 100 nm.

NS5. To further determine the precise spanning regions of the N-terminal portion of NS5, we constructed a series of baculovirus recombinants with different deletions of NS5. The results indicated that the proteins expressed from NS5B (31-320aa) and NS5C (57-320aa) did not change their transmembrane localization (Fig. 4c and 4d), suggesting that the critical amino acids to determine the transmembrane feature are not located in the region from 1-57 of the NS5. However, when the 71 amino acids of the N-terminal of the NS5 were deleted (NS5D: 72-320aa), the green fluorescence was almost distributed throughout the whole cells (Fig. 4e). The same results were observed, i.e. when both N- and C-terminal of the NS5 were deleted (NS5E: 72-212aa), the green fluorescence was still spread all over the whole cells (Fig. 4f). These data suggested that the 57-71aa (SQIHMVWVKSG LVFF) of the NS5 was probably the dominative spanning region at N-terminal of NS5.

Discussion

The function of each of 10 genomic dsRNA segments of DpCPV was poorly understood due to lack of the permissive cell culture. In present studies, interestingly, we had obtained an important result that the localization of NS5 was on the plasma membrane of *Sf*-9 cells when it was expressed using Bac-to-Bac baculovirus expression systems, since *Sf*-9 cells may provide the similar cellular environment for the expression and distribution of these viral proteins, because they belonged to the same order of insect: Lepidoptera. Based on the
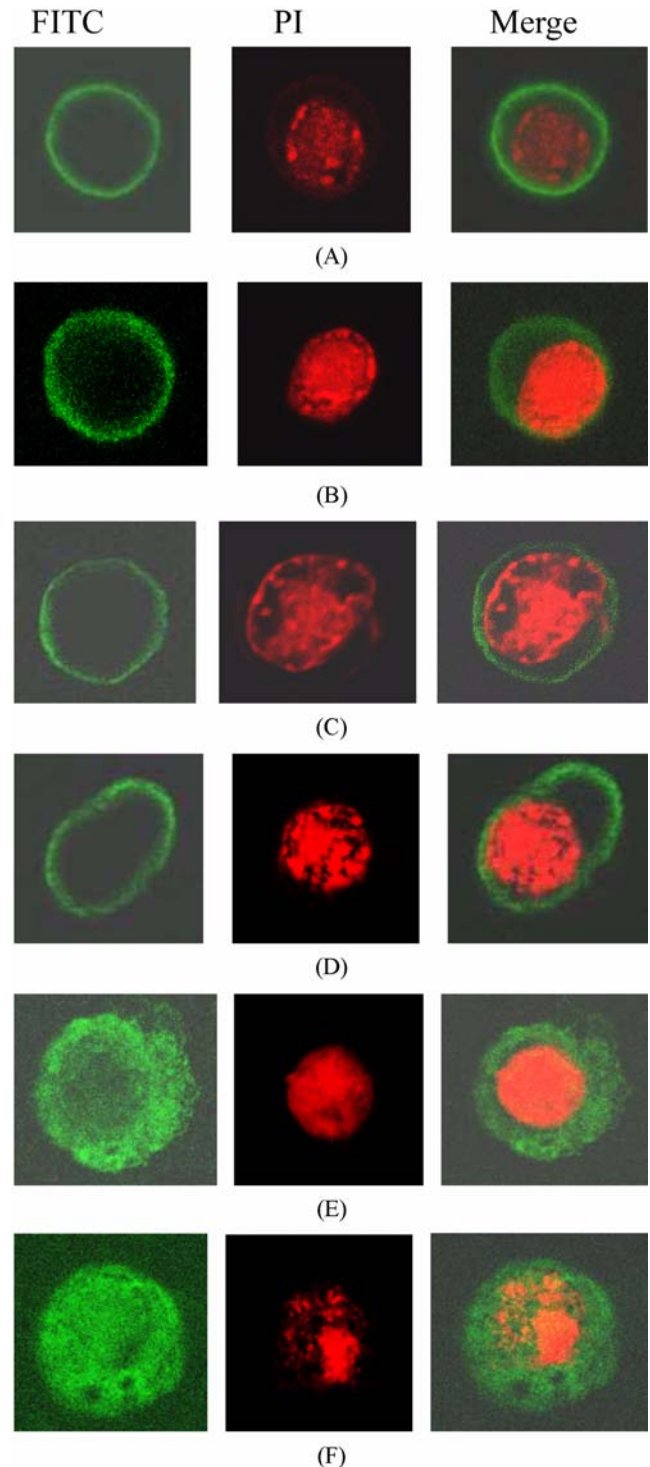

Fig. 4. The distribution of the full length NS5 and its deleted mutants. Infected cells were incubated with anti-NS5 polyclonal antibodies (primary antibody) and FITC-conjugated goat anti-rabbit IgG (secondary antibody), while the nucleus were stained with PI (10 μg/ml), then observed under confocal microscope. (a). NS5 (1-320aa), (b). NS5A (1-212aa), (c). NS5B (31-320aa), (d). NS5C (57-320aa), (e). NS5D (72-320aa), (f). NS5E (72-212aa).

observation that the fusion protein NS5-GFP was localized dominantly on plasma membrane till the infected cells were lysed, moreover, the different deletions of NS5 led to various cellular distributions of fluorescence by indirect immuno-fluorescence assays, when the amino acids from 1-57 were deleted, the NS5 proteins were located on the plasma membrane, however, when the amino acids from the 1-71 were deleted, the NS5 was no longer distributed on the cell membrane in stead of cytoplasm (Fig, 4e and 4f). All these results suggested that NS5 was a transmembrane protein. Intriguingly, the critical amino acid sequences of 57-71 (SQIH MVWVKSGLVFF), which possessed an intense hydrophobic region, were consistented with the prediction of transmembrane characteristic by TMpred, TMHMM, DAS, SVM-Prot and LOCSVMPSI method (Möller *et al.*, 2001; Cai *et al.*, 2003; Xie *et al.*, 2005).

Membrane-protein interactions could be typically separated into two categories, peripheral and integral (Smith *et al.*, 2001). Peripheral membrane proteins generally did not interact significantly with the hydrophobic interior of the lipid bilayer, while integral membrane proteins did interact, either through a transmembrane region or a hairpin loop that did not pass through the bilayer (Sankaram *et al.*, 1993). The analysis of results also suggested that the transmembrane hydrophobic domains of the protein NS5 were possibly bedded in the phospholipids bilayer as a integral protein, and some positively charged residues (Arg-42, Lys-44, Lys-48) which flank the hydrophobic domain contributed to membrane binding via strong electrostatic interactions with the negatively charged phospholipids head groups, and another positions 72-79 was almost occupied by a small residue (Thr-72, Pro-73, Ser-74, Ala-75, Ser-76, Pro-78, Ala-79), which seemed to be flexible and possibly facilitated the spanning of hydrophobic domain, and the more details of transmembrane way were further elucidated (Towner *et al.*, 1996). From the secondary structure predictions of some transmembrane proteins, many transmembrane regions possessed the motifs of putative amphiphathic α-helical wheels, such as the 1-13aa of protein NS3, 1-20aa and 22-41aa of protein VP5 of Bluetongue virus, the 55-72 residues of protein NSP4 of Rotavirus (Newton *et al.*, 1997; Hassan *et al.*, 2001; Beaton *et al.*, 2002). However, with the similar transmembrane structure prediction, the hydrophobic region 57-71aa of NS5 might adopt another structure of β-sheets which probably contained two high degree of β-branched amino acids of hydrophobic residues MVWV (61-64) and LVFF (68-71) (Gromiha *et al.*, 2005).

The possible functions of protein NS5 served within cells might be consistent with its particular localization. Like some eukaryotic plus-strand RNA viruses, their transmembrane nonstructural proteins were related to RNA replication and localized in the various intracellular membranes, such as 3AB of Poliovirus, NS5A of Hepatitis C virus, protein A of Greasy Grouper Nervous Necrosis virus (GGNNV) and proteins 2C and 3D of Poliovirus. It was common property that nonstructural proteins of these viruses appeared to intimate association of

their RNA-synthesizing machinery with intracellular membranes (Towner *et al.*, 1996; Schlegel *et al.*, 1996; Elazar *et al.*, 2003; Guo *et al.*, 2004; Gromiha *et al.*, 2005). On the other hand, as members of non-enveloped cypoviruses of the family *Reoviridae*, some nonstructural proteins which expressed on the plasma membrane were always related with some important functions such as the nonstructural protein NS3 of Bluetongue, which interact with cellular protein p11 (calpactin light chain, part of the annexin II complex), could mediate virus release (Towner *et al.*, 1996). The NSP4 of Rotavirus and NS3 of Afican Horsesickness virus (AHSV), which played a central role in viral pathogenesis through modifying membrane permeability and favored the release of the matured viruses (Newton *et al.*, 1997; Niekerk *et al.*, 2001). The precise transmembrane structure of NS5 and the functions related with the special transmembrane way remained to be further studied.

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