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Strand-like structures and the nonstructural proteins 5, 3 and 1 are present in the nucleus of mosquito cells infected with dengue virus

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ABSTRACT

Dengue virus (DENV) is an arbovirus, which replicates in the endoplasmic reticulum. Although replicative cycle takes place in the cytoplasm, some viral proteins such as NS5 and C are translocated to the nucleus during infection in mosquitoes and mammalian cells. To localized viral proteins in DENV-infected C6/36 cells, an immunofluorescence (IF) and immunoelectron microscopy (IEM) analysis were performed. Our results indicated that C, NS1, NS3 and NS5 proteins were found in the nucleus of DENV-infected C6/36 cells. Additionally, complex structures named strand-like structures (Ss) were observed in the nucleus of infected cells. Interestingly, the NS5 protein was located in these structures. Ss were absent in mock-infected cells, suggesting that DENV induces their formation in the nucleus of infected mosquito cells.

1. Introduction

The four distinct dengue virus (DENV 1–4) serotypes belong to the family *Flaviviridae*, genus *Flavivirus* and are responsible for 390 million of infections each year according to World Health Organization (WHO). DENV is transmitted by *Aedes* mosquitoes mainly *Aedes aegypti* and *Aedes albopictus* in the tropical and subtropical regions of the world (Back and Lundkvist, 2013; Bhatt et al., 2013; Guzman and Harris, 2015). DENV genome consists of a positive-sense RNA of ~11 Kb that during infection is translated into a polyprotein, which is cleaved by cellular and viral proteases into the structural proteins C, prM, and E, and the seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Chambers et al., 1990; Lindenbach and Rice, 2003). The nonstructural proteins are involved in viral replication and assembly of the new viral progeny in the endoplasmic reticulum (ER) of infected cells. Although it has been reported that DENV has a cytoplasmic replicative cycle, some viral proteins such as the structural capside protein (C) (Bulich and Aaskov, 1992; Makino et al., 1989; Netsawang et al., 2010; Sangiambut et al., 2008; Tadano et al., 1989; Tiwary and Cecilia, 2017; Wang et al., 2002), and the nonstructural protein 5 (NS5) (Hannemann et al., 2013; Kapoor et al., 1995; Kumar et al., 2013; Miller et al., 2006; Pryor et al., 2007; Tay et al., 2016) are relocated to the nucleus during infection in mammalian cells. Although the functions of C and NS5 in the nucleus are unclear, it could be related with the control of gene expression of the infected cells (IL-8 expression) (Medin et al., 2005). In this regard, in a recent report, the interaction of NS5 with components of the U5 snRNP was confirmed in mammalian cells. This interaction reduces the efficiency of pre-mRNA splicing and renders a favorable cellular environment for DENV replication (De Maio et al., 2016).

In contrast to mammalian cells, little is known about the localization and function of viral proteins in the nucleus of mosquito cells, however, the presence of C and NS5 proteins in the nucleus has been previously described in infected C6/36 cells (Bulich and Aaskov, 1992; Hannemann et al., 2013; Junjhon et al., 2014; Sangiambut et al., 2008; Tadano et al., 1989). In this study, we perform an immunofluorescence (IF) and immunoelectron microscopy (IEM) analysis to determine which viral proteins are present in the nucleus of DENV-2 infected C6/36 cells.

2. Materials and methods

2.1. Cell culture and viral strain

C6/36 cells, adapted to grow at 35 °C (Igarashi, 1978; Kuno and Oliver, 1989), were grown in a CO_2-free incubator (Lab Line) and were cultured (Corning) in Minimal Essential Medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), vitamins

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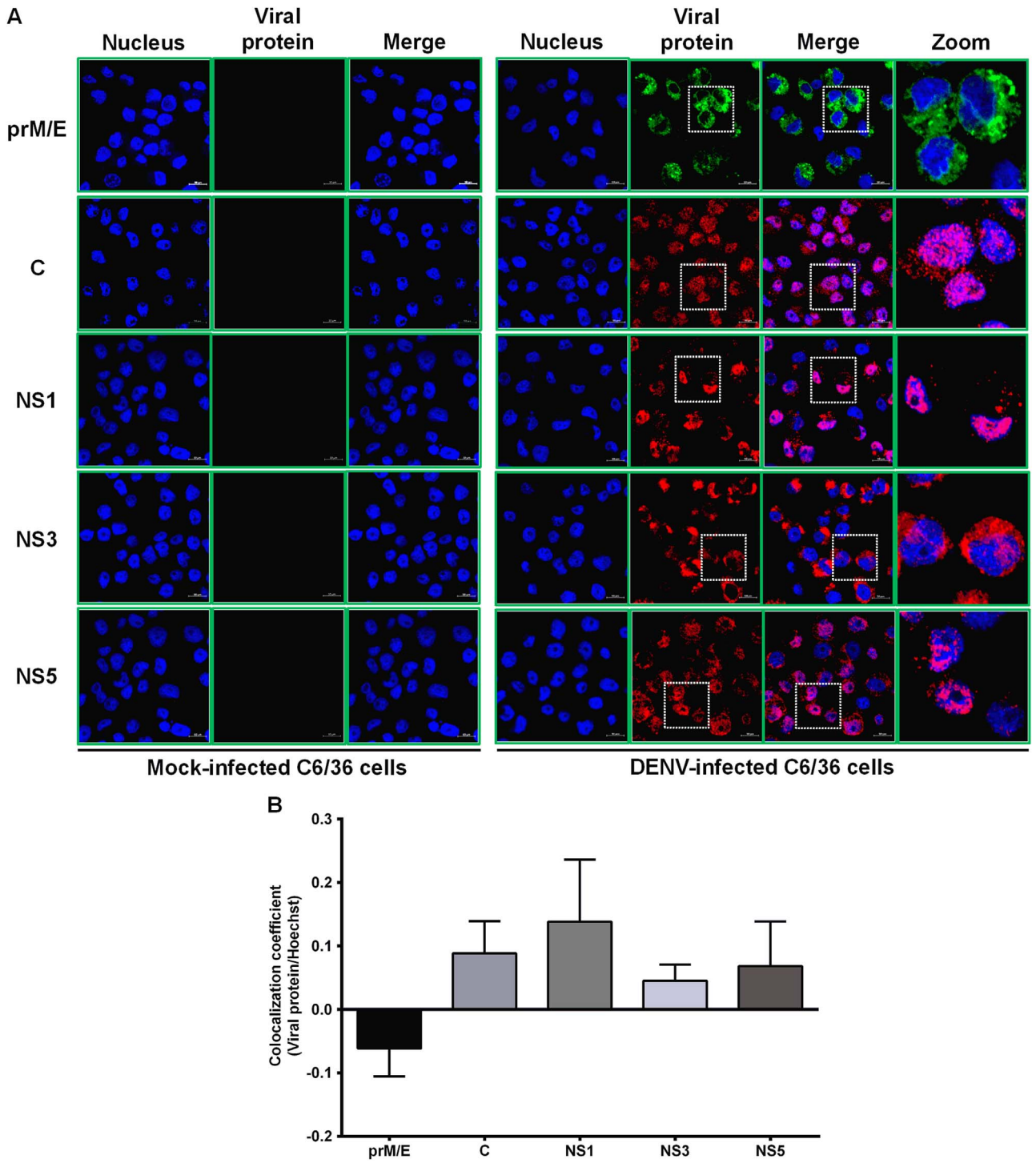

Fig. 1. Immunolocalization of viral proteins in DENV-infected C6/36 cells by immunofluorescence confocal microscopy. (A) C6/36 cells were mock-infected or infected with DENV-2, fixed, and immunolabeled with specific antibodies anti-prM/E, -C, -NS1, -NS3, or -NS5 viral proteins. Nuclei were stained with Hoechst (blue). (B) The graph compares the colocalization coefficient mean ± SD, through Pearson's correlation coefficient, among Hoechst (blue) and viral proteins (green and red).

(Invitrogen), 0.034% sodium bicarbonate (J.T. Baker), 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma).

DENV-2 (New Guinea C strain) (donated by InDRE, Instituto Nacional de Diagnóstico y Referencia Epidemiológica, Mexico) was propagated in BALB/c suckling mice brains (Gould and Clegg, 1991) and viral titer was determined by plaque assay in BHK-21 cells as was previously reported (Juárez-Martínez et al., 2012). BALB/c suckling mice brains from uninfected or mock-infected mice were used as a control.

2.2. DENV infection

C6/36 cells, seeded at 80% confluence, and incubated at 35 °C during 24 h were washed three times with Hanks medium, and mock-infected or infected with DENV serotype 2 at a MOI of 5 for 2 h at 35 °C; then, cells were washed with acid glycine (pH 3) to inactivate non-internalized virus, washed 3 times with PBS and the infection was allowed to proceed for 48 h at 35 °C.

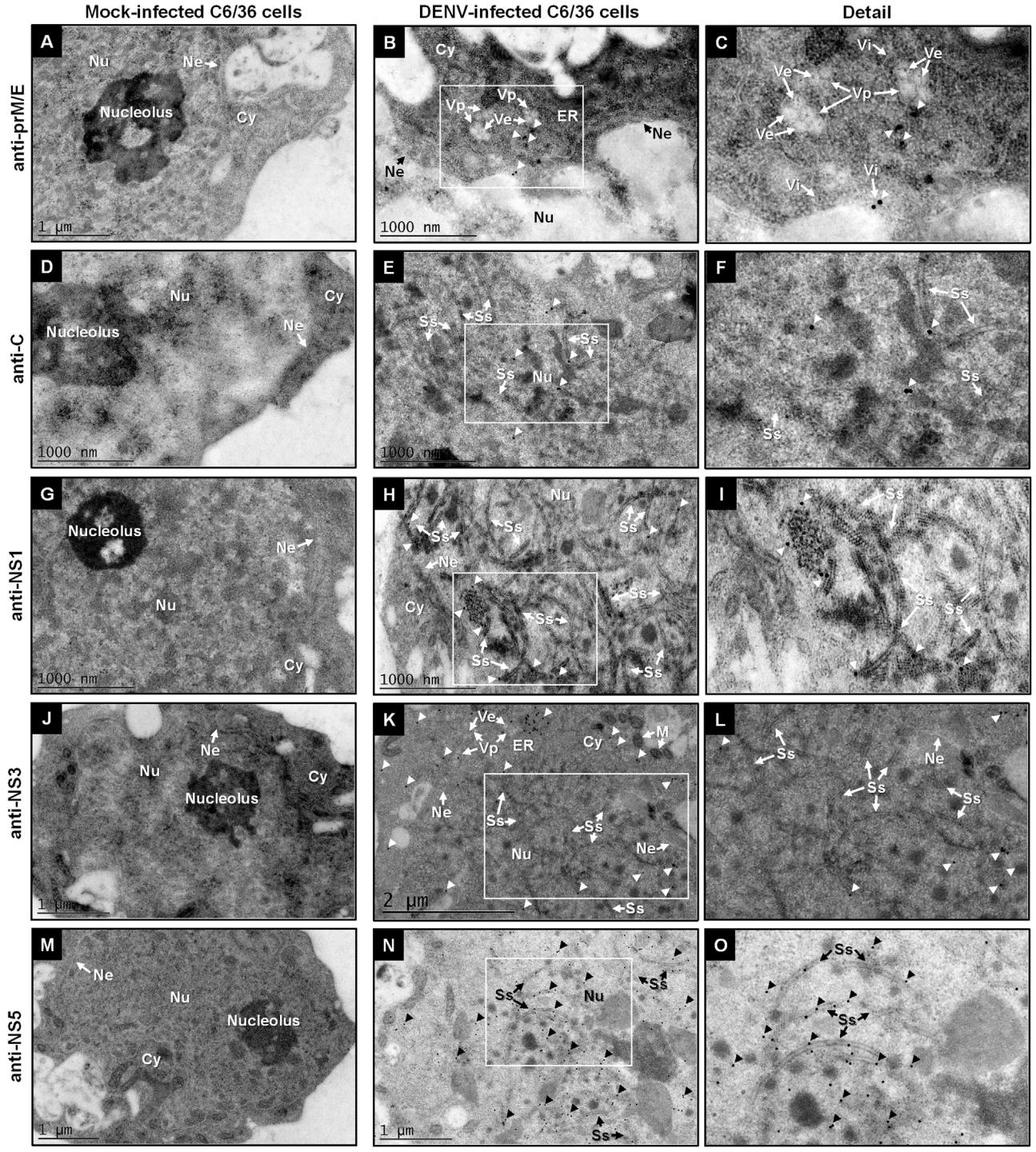

Fig. 2. Immunolocalization of viral proteins in DENV-infected C6/36 cells by immunoelectron microscopy. C6/36 cells were mock-infected or infected with DENV-2, fixed, and labeled with specific antibodies anti-prM/E (A-C), -C (D-F), -NS1 (G-I), -NS3 (J-L), or -NS5 (M-O) viral proteins. Cy, cytoplasm; M, mitochondria; ER, endoplasmic reticulum; Ve, double-membrane vesicles; Vp, membrane packets; Vi, virus-like particles; Ne, nuclear envelope; Nu, nucleus; and Ss, strand-like structures. Arrowheads are used to point the gold particles in the preparation.

2.3. Antibodies

The immunolabeling of DENV proteins was performed using the following antibodies: anti-prM/E DENV complex (ATCC® HB-114), anti-C (Genetex, catalog number 103343), anti-NS1 (kindly donated by Dr. Juan Ludert, Cinvestav, Mexico), anti-NS3 (Genetex, catalog number 124252), and anti-NS5 (Genetex, catalog number 103350).

2.4. Immunofluorescence (IF)

C6/36 cells grown at 80% confluence on slides were mock-infected or DENV-infected. At 48 h post infection (hpi), the cells were fixed with 1% formaldehyde, incubated for 20 min with permeabilized solution (0.1% saponin and 1% FBS in 1X PBS), and incubated overnight at 4 °C with anti-prM/E, anti-C, anti-NS1, anti-NS3 and anti-NS5 antibodies diluted 1:200. As secondary antibodies goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 555 (Life Technologies) were used. Finally,

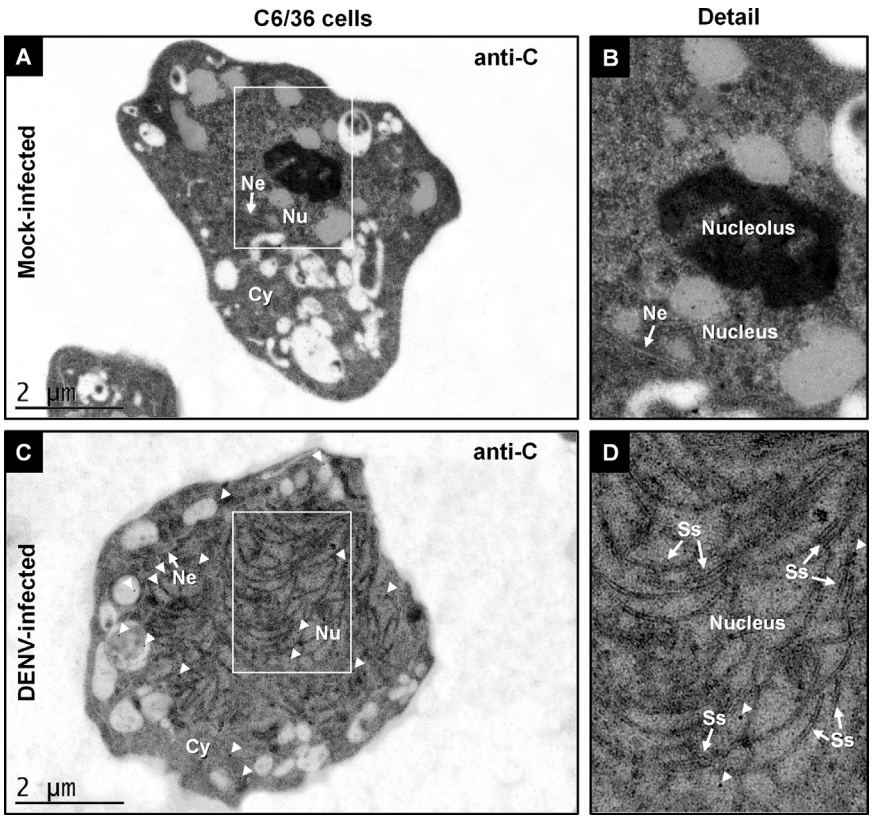

Fig. 3. Ultrastructural differences between mock-infected and DENV-infected cells. Ultrastructural analysis by immunoelectron microscopy of mock-infected (A and B) and DENV2-infected C6/36 (C and D) cells using anti-C protein antibody. The nuclear structure with a regular appearance in the mock-infected cells can be observed (B). In contrast, DENV-2 infected cells showed strand-like structures in the nucleus (D). Cy, cytoplasm; Ne, nuclear envelope; Nu, nucleus; and Ss, strand-like structures. Arrowheads are used to point the gold particles in the preparation.

nuclei were stained with Hoechst (Santa Cruz), and slides were observed in a Zeiss LSM700 laser confocal microscopy. The images were analyzed using the ZEN software, v. 2010. The same software was used to determine Pearson's correlation coefficient between viral proteins and Hoechst.

2.5. Immunoelectron microscopy (IEM)

C6/36 cells seeded in 75 cm^2 flask at 80% confluence were mock-infected or DENV-infected. After 48 h, cells were washed three times with PBS, fixed with 4% paraformaldehyde/0.5% glutaraldehyde for 1 h at room temperature (RT). Then, cells were dehydrated through increasing concentrations of ethanol, embedded in the acrylic resin (LR White) and polymerized under UV irradiation at 4 °C overnight. The Resin-embedded DENV-infected or mock-infected C6/36 cells sections of 70 nm were obtained and mounted on a Formvar-covered nickel grids; then were incubated in PBS with 10% fetal bovine serum for 1 h to block nonspecific binding and reacted with anti-prM/E (undiluted), anti-C (diluted 1:20), anti-NS1 (diluted 1:20), anti-NS3 (diluted 1:20), and anti-NS5 (diluted 1:20) antibodies diluted in PBS with 5% fetal bovine serum. The samples were washed three times and incubated with anti-mouse or anti-rabbit IgGs secondary antibodies conjugated to 20-nm colloidal gold particles (Ted Pella Inc., Redding, CA, USA) at RT for 1 h. Finally, sections were contrasted with uranyl acetate and lead citrate before being examined under the Jeol JEM-1011 transmission electron microscope. The mock-infected cells were treated under the same conditions as infected cells.

3. Results and discussion

To analyze the location of DENV proteins in infected C6/36 cells, immunofluorescence (IF) and immunoelectron microscopy (IEM) analysis using antibodies against structural (prM/E and C) and non-structural proteins (NS1, NS3, and NS5) were performed. As expected, C and NS5 proteins were mainly observed in the nucleus while prM/E was detected in the perinuclear region of the infected C6/36 cells where the replicative complexes are located (Fig. 1A). Interestingly, a proportion of the NS3 and a significant amount of NS1 were detected in the nucleus of DENV-infected cells (Fig. 1A). Since no signal was observed in mock-infected cells, the specificity of the antibodies was confirmed (Fig. 1A). The localization of C, NS1, NS3, and NS5 in the nucleus was corroborated using Pearson's correlation coefficient. While a negative correlation coefficient was observed between prM/E and Hoechst (−0.06), a positive correlation was observed for C, NS1, NS3, and NS5 proteins (0.08, 0.13, 0.04, and 0.06, respectively) (Fig. 1B). This result confirms the presence of C, NS1, NS3, and NS5 in the nucleus of infected cells.

To corroborate these findings an IEM analysis, using the same set of antibodies, was performed in mock-infected and DENV-infected C6/36 cells. Our first observation in infected C6/36 cells was the previously described ultrastructural alterations in the ER such as the formation of membrane packets (Vp) that contained double-membrane vesicles (Ve) (Junjhon et al., 2014) (Fig. 2B and C). As expected, immunolabeling with the anti-prM/E antibody was associated with replication complexes and with some virus-like particles (Vi) (Fig. 2B and C). Although, not all the Vi was labeled with the anti-prM/E antibody at the concentration used, its electron-dense nature, its size and the presence of a membranous layer, suggest that they are viral particles. Additionally, the presence of C (Fig. 2E and F), NS1 (Fig. 2H and I), and NS5 proteins (Fig. 2N and O) in the nucleus of infected cells was confirmed by IEM. Although, we used the same antibody to immunolabel C protein, the labeling of C was more prominent in the IF then in IEM. This may be possible because the sample preparation used in each assay could be modifying antibody reactivity. Moreover, the NS3 protein was found associated with vesicle structures in the ER, but also it was located in the nucleus of infected cells (Fig. 2K and L), confirming our previous results. As it was observed previously, the antibodies were unable to detect any signal in mock-infected cells (Fig. 2A, D, G, J, and M).

In addition to the presence of C, NS1, NS3, and NS5 in the nucleus of mosquito cells, some differences in the nuclear and cytoplasmic structures between mock-infected and DENV-infected cells were observed

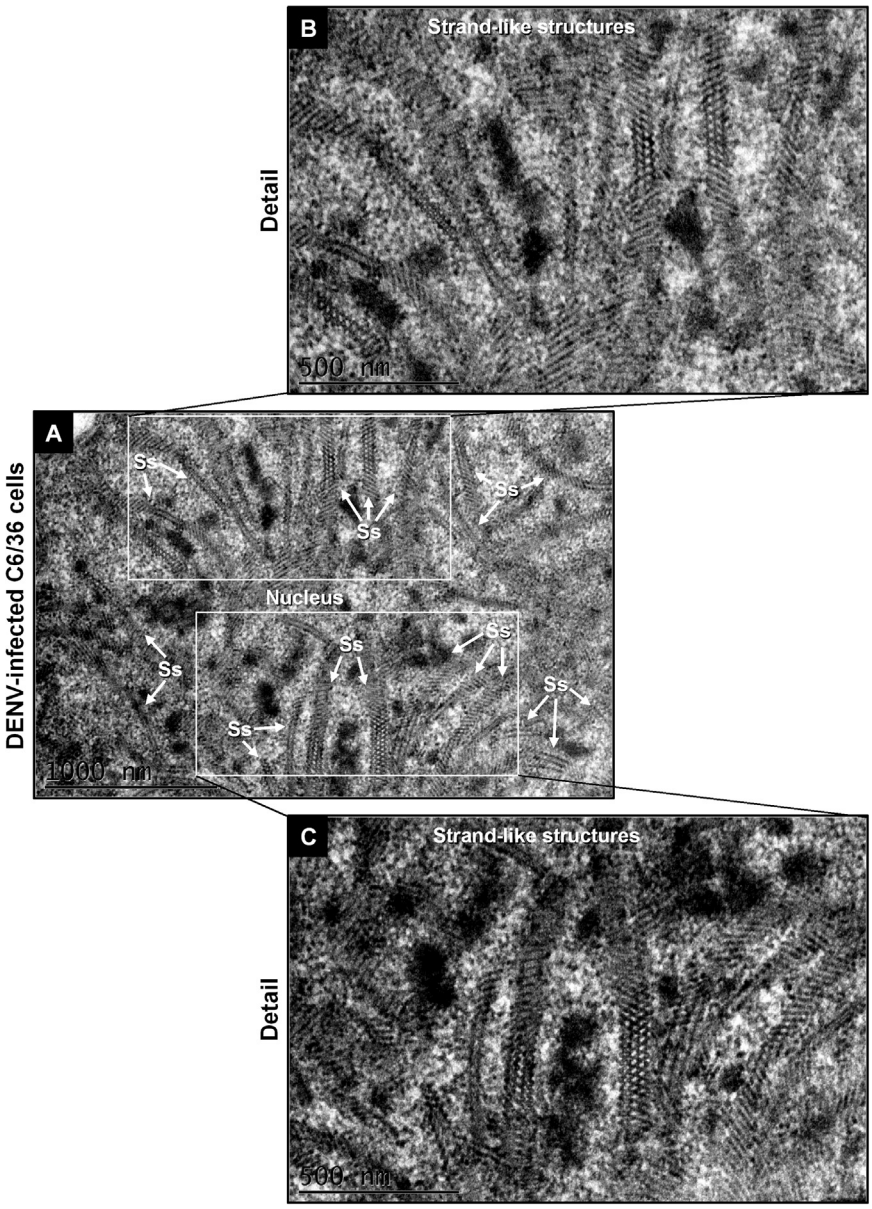

Fig. 4. Identification of the strand-like structures by immunoelectron microscopy analysis. (A-C) Strandlike structures (Ss) were detected in the nucleus of DENV-2 infected C6/36 cells.

(Fig. 3A–C). The nucleus of mock-infected cells (Fig. 3A) was smaller than the nucleus of infected cells (Fig. 3C) and, a higher amount of large-sized vacuoles were observed in the cytoplasm of infected cells compared with mock-infected cells. The nucleolus was not detected in the section examined from infected cells. It is possible that the presence of nucleolus was dependent on the cross-sectional cut. Unexpectedly, complex structures were detected in the nucleus of some infected cells (Fig. 3C). Given the morphology of these structures, they were named strand-like structures (Ss) (Fig. 3D). These complex structures have the following characteristics: they look like strands that intertwine with each other to acquire a helical shape, forming chain-like structures of 200–900 nm (nm) in length and ~40 nm in diameter (Fig. 4A). Although Ss were numerous, they did not show a specific orientation in the nucleus, differing from each other in size and conformation without losing their extraordinary morphological complexity (Fig. 4B). The abundance of Ss in a specific preparation is dependent on the cross-sectional cut. Finally, we observed the interaction of 2 or 3 of these strand-like structures forming structures of higher complexity (Fig. 4C). To determine if these structures were present only in the nucleus of the infected cells, 200 cells were analyzed in the infected and in the mock-infected cell culture. While in none of the mock-infected cells the Ss were detected, they were observed in 26 out of 137 DENV-infected cells positive for C protein. Interestingly, the amount of C protein was higher (at least twice) in the cells containing the Ss in the nucleus than in the infected cells without Ss (data not shown). Remarkably, while C and NS1 were close to the Ss, the NS5 protein was observed along the Ss, suggesting that NS5 protein could be involved in the Ss formation.

In summary, our results obtained using two different techniques confirm that C, NS1, NS3, and NS5 proteins are located in the nucleus of DENV-2 infected mosquito cells. Although it has been described that C protein migrates to the nucleus of DENV-2 infected mammalian (Bulich and Aaskov, 1992; Makino et al., 1989; Netsawang et al., 2010; Sangiambut et al., 2008; Tadano et al., 1989; Tiwary and Cecilia, 2017; Wang et al., 2002) and C6/36 cells (Bulich and Aaskov, 1992; Sangiambut et al., 2008; Tadano et al., 1989) and that it interacts with different nuclear and nucleolar proteins in mammalian cells (Balinsky et al., 2013; Colpitts et al., 2011), its function in this organelle is unclear. Although it has been reported the nucleolar localization of C protein (Bulich and Aaskov, 1992; Sangiambut et al., 2008; Tadano et al., 1989; Tiwary and Cecilia, 2017; Wang et al., 2002), however, in our preparations, the presence of C was more abundant in the nucleus. It is possible that the sections examined do not allow us to observe the

location of C in the nucleolus. The presence of NS5 in the nucleus of DENV-infected mammalian (Hannemann et al., 2013; Kapoor et al., 1995; Kumar et al., 2013; Miller et al., 2006; Pryor et al., 2007; Tay et al., 2016) and C6/36 cells (Hannemann et al., 2013; Junjhon et al., 2014) has also been described previously.

The NS5 protein has two enzymatic activities, the methyltransferase activity (MTase) (Egloff et al., 2007; Kroschewski et al., 2008) required for type 1 cap addition (Dong et al., 2014), and the RNA-dependent RNA polymerase (RdRp) (Lim et al., 2013; Yap et al., 2007) essential for viral RNA replication (Kapoor et al., 1995; Tay et al., 2015). Additionally, this protein has been involved in reducing IFN signaling by STAT2 degradation (Ashour et al., 2009) and in the expression and secretion of immunomediators such as IL-6 and IL-8 associated with dengue hemorrhagic fever (Kelley et al., 2011; Medin et al., 2005). Moreover, NS5 interacts with components of the spliceosome interfering with splicing efficiency and with mature mRNA export (De Maio et al., 2016). All these functions support the nuclear localization of NS5 and show the ability of this protein to interact with cellular proteins and nucleic acids that could be important components of the strand-like structures (Fig. 2N and O). It is possible that the Ss could be involved in the transcriptional machinery and/or splicing processes in the infected cells. Interestingly, although the location of NS1 in the nucleus was suggested previously (Alcalá et al., 2016), this is the first report that confirms its presence in this compartment of mosquito cells.

On the other hand, although an essential proportion of the NS3 protein is located in the perinuclear region where the replication complexes are located (Junjhon et al., 2014; Welsch et al., 2009), this protein is also present in the nucleus; suggesting this as the first report that indicates the nuclear localization of NS3 in DENV-infected cells. In a recent report, the presence of NS1 and NS5 of Zika virus was observed in the nucleus of cells transfected with the recombinant proteins (Hou et al., 2017). Given that NS3 has a protease activity this protein could be playing a role in viral replication or pathogenesis in the nucleus. Some viral proteases such as the ones encoded by enteroviruses (3C) migrate to the nucleus and modulate the immune response and viral replicative cycle by altering the nucleus-cytoplasmic transport (Sharma et al., 2004). Further studies directed to analyze the cellular mechanisms involved in the import of NS1 and NS3 to the nucleus of DENV-infected mosquito and mammalian cells and their roles in this organelle are being performed in our laboratory.

Moreover, previous studies have shown that viruses can induce changes in the organization and structure of chromosomes, by encoding proteins that cause dynamic changes in DNA structure, for example, affecting chromatin structure through changes in histones or other nuclear proteins essential for chromatin function (Lieberman, 2008; Wei and Zhou, 2010). Since we identified C, NS1, NS3, and NS5 proteins inside the nucleus, it is possible that these proteins could be involved in the induction of changes in the cellular DNA structure.

In our analysis, we observed the presence of complex structures exclusively in the nucleus of DENV-infected cells. Then, it is probable that DENV infection induces the formation of these structures. Although it has been reported that DENV infection causes remodeling of the ER to generate the invaginations (Vp and Ve) and convoluted membranes (CM) required for viral RNA replication (Junjhon et al., 2014; Welsch et al., 2009), it has not been described that DENV infection induces changes in the structure of cellular DNA. It has been described that C protein can interact with histones, then the presence of C in the nucleus could sequester some histones disrupting the formation of the nucleosome (Colpitts et al., 2011). On the other hand, since NS5 protein was mainly located in the Ss, (Fig. 2N and O) and it interacts with components of the spliceosome interfering with splicing efficiency (De Maio et al., 2016), it can be suggested that Ss could be related with cellular transcription and splicing processes. Further studies are required to confirm this possibility.

The electron microscopy (EM) is a tool that has been used to characterize the chromatin remodeling complexes (Leschziner, 2011). Interestingly, the strand-like structures were not observed in the transmission electron microscopy (TEM) analysis of DENV-infected cells (data not shown). A critical difference between TEM and IEM is the chemical fixation. It has been described that for the optimal preservation of biological samples it is necessary to consider the selection and fixation efficiencies of a fixative. In IEM, the paraformaldehyde penetrates faster and deeper than glutaraldehyde, used in TEM (Park et al., 2016). Also, the fixation with glutaraldehyde causes changes in molecular bonds in the sample (Frankl et al., 2015), while the paraformaldehyde permits the preservation of molecules for immunolabeling. All these advantages of the paraformaldehyde allow us to observe Ss. Besides, since the Ss were not detected in mock-infected cells, we rule out the possibility that these structures were artifacts (Ayache et al., 2010; Culora et al., 1995).

In conclusion, our observations indicate that the C, NS1, NS3, and NS5 proteins are present in the nucleus of infected mosquito cells and that DENV infection induces changes in the nuclear structure of the cells. One of these changes is the presence of strand-like structures. These results improve our knowledge about the changes in the nuclear composition and structure during DENV infection. Although the origin, function and biological significance of these structures remains to be elucidated, we speculate that these structures contain cellular DNA. These changes in the structure of the nucleus of infected mosquito cells could influence transcriptional machinery, and gene expression in response to viral infection.

Acknowledgments

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