

VERO CELL LINES EXPRESSING NUCLEAR LOCATION SIGNALS OF *Penaeus merguensis* HEPANDENSOVIRUS: AN EARLY STUDY

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author DS principal investigator, analyzed the data and wrote the report. Authors DS and CC designed and performed the experiments. Author JE provided supervision during preparation of materials and methods and checked the writing. Author LO gave assistance in the experimental design, data analyzing and writing. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Penaeus merguensis* hepadensovirus (PmeHDV) (GenBank No. DQ458781) is a shrimp hepatopancreatic parvovirus (HPV), belonging to subfamily *Densovirinae*. Transportation of *Densovirinae* into and out of nucleus is allowed by the binding of nuclear location signals (NLSs) to importins (Imp). PmeHDV has putative NLSs that need to be experimentally tested. The aims of this study is to determine if the three putative NLSs of PmeHDV are functioning by transfecting NLS-inserted-plasmid DNAs into Vero cell lines using a transfection reagent.

Place and Duration of the Study: Data for this study was collected from the Veterinary and Biomedical Sciences Laboratories at James Cook University (JCU) during the duration from May 2015 to December 2016.

Methodology: Each plasmid has been synthetically inserted with each sequence of the putative NLSs and a fluorescent protein. The presence of the NLS in the cell nucleus and cytoplasm was screened. The overlay of visualization of transfected plasmids is presented.

Results: It appears the NLSs are not functioning well as that the proteins are blocked at the nuclear membrane, probably linked to importin beta-1 and not frequently entering the nucleus. Our study demonstrated small noticeable differences in the outer nuclei within transfected-Vero cells with the experimental NLSs genes.

Conclusion: In conclusion, our fluorescent study was not sufficiently sensitive to be confident of the detection in NLS-transfected cells under different filters. The study of crustacean virus-host interactions using proxy cell cultures as models remains a major challenge.

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1. INTRODUCTION

Cellular processes such as cell differentiation and development require protein transport between the nucleus and cytoplasm. The transportation of proteins is also critical to some viral disease and oncogenesis [1-3]. In order to gain entry into the cell nucleus via the nuclear pore complexes (NPCs), proteins with molecular mass greater than 45kDa generally need a nuclear location signal (NLS).

Viruses within the subfamily *Densovirinae* are intranuclear and require cells in their S-phase when DNA is synthesised for all or most of their replication and assembly. Transportation into the nucleus is absolutely required for viral replication. Specific chaperone proteins called importins (Imp) types α/β or β alone mediate the transportation of the viral proteins into the nuclei. Upon the binding of NLSs to the Imp, viral proteins are transported in or out of the nuclei [4].

Some stretches of basic aa such as lysine (K) and arginine (R) are commonly the main components of an NLS and sometimes an NLS sequence is headed by helix-breaking neutral amino acids, proline (P), glutamine (Q) or glycine (G) and less commonly by the negatively charged aspartic acid (D) and glutamic acid (E) [5]. There are three main group of NLSs. When an NLS contains 6 aa (hexapeptides) with at least 4 of them are basic aa without acidic or bulky aa, it can be termed classic monopartite NLS (e.g. SV-40T-antigen) [6,7]. The classic monopartite NLS can be preceded by aa P, Q or G as a helix-breaking residue (e.g. PKRKKVR) [5,7]. Whereas when 2 basics aa separated by at least 9 aa from a cluster of at least 3 basic aa (e.g. DNA helicase Q1) or non-classical (e.g. proline-tyrosine (P-Y)), an NLS can be bipartite. The third group is a Chelsky NLS, which is when three of 4 aa are basic and the dibasic K-K/R-x-K/R are found at the beginning of the NLS [7,8].

PmeHDV (DQ458781) had been demonstrated to have putative NLSs in all of the three ORFs (Table 1) and it was suggested that the NLSs affected the nature

of inclusion bodies of PmeHDV to be basophilic and the site of encapsidation to be nuclear [7].

PmeHDV NS2 has a potential classic human $\alpha 1$, DNA helicase Q1 (KK-15aa-KKRK) NLS, starting at amino acids (aa) 181. This putative NLS in PmeHDV contains only 14 amino acids between the bipartite repeats of the lysine (KK-14aa-KKTK) without an A (alanine) at position aa 274 (Table 1). DNA helicase Q1s are heavily involved in unwinding DNA duplexes in a 3' to 5' direction and may have DNA repair capabilities. This enzyme may be involved with separation of DNA templates during the synthesis of rolling replication [7].

NS1 has a potential classic Chelsky sequence preceded by a disruptive negatively charged aspartic acid (D), starting at aa 559 RKFK. A possible pattern of the Dorsal (RRPS-22-aa-RRKRQK) [4] is observed in NS1 with a bipartite NLS compensating for the negative D might exist as 20 aa upstream there is a KK signal (Table 1). The NLS was suggested functional particularly with an upstream bipartite partner. However, an experimental investigation is needed to prove the functioning of the bipartite NLS as serine (S) of dorsal is absent in the upstream segment of the bipartite NLS [7].

In addition, the capsid protein or VP1, ORF3 shows a very strong NLS (PKKKKKYK) starting at aa position 809. This aa sequence is noticeably very similar to SV40-T-antigen (PKKRKV) [7]. The very powerful NLS would ensure nuclear transport [7].

Various techniques have been developed for decades for efficient introduction of DNA into cultured eukaryotic cells and have allowed expression of various genes cloned in an expression vector for the studies of gene regulation. Although many experiments can be performed with cells transiently expressing foreign genes, it is often necessary to isolate cell lines that permanently express foreign genes. Especially when large quantities of a product are required for the experiments, the success of the experiments depends on the isolation of a cell line highly expressing the foreign gene.

Table 1. Putative NLSs in *P. merguensis* hepadensovirus (PmeHDV)

Coding domains, ORF	Putative NLS sequences	Functional homology
NS2, ORF1	¹⁸¹ KRDQKKT(-)EKKDDE <u>PPKKK</u> TK	DNA Helicase Q1
NS1, ORF2	⁵³⁴ KKNP EL TQFVLASMQYVHSNYMDK <u>PDRK</u> FK	Dorsal
VP1, ORF3	⁸⁰⁹ <u>PKKKKK</u> YR	SV40T antigen

Bold letters indicate conserved basic amino acids (aa); blue underlined are the helix-breaking prolines; and those in red could be disruptive [7]

This present study aims to determine experimentally if of the three putative NLSs of PmeHDV [7] are functioning by transfecting NLS-inserted-plasmid DNAs into mammalian cell culture using a transfection reagent. Originally, it was planned to use C6/36 mosquito cell lines which should have supported the growth of PmeHDV but it was proved that C6/36 was unsuitable for PmeHDV [9]. Each plasmid has been synthetically constructed and inserted with each sequence of the putative NLSs and a fluorescent protein marker. The presence of the NLS in the cell nucleus and cytoplasm was screened under a fluorescent microscope with different filter sets (Ex358/Em461 (blue); Ex488/Em520 (green); Ex 591/Em618 (red)).

2. MATERIALS AND METHODS

2.1 Preparation of Synthetic Plasmid DNA (pDNA) from Glass Microfiber Filters

Each protein NLSs sequence was inserted into a plasmid by DNA2.0, USA which has now changed their name to ATUM (www.atum.bio). In our study, IP-Free© Mammalian transient expression vectors (pD603 and pD763) were designed to express the NLSs protein sequences. Each plasmid contains a fluorescence protein (FP) such as Dasher Green, Cayenne, Yukon, and Match Yellow with different Excitation (Ex) and Emission (Em) wavelength (nm), to express the location signals (Table 2). The neomycin resistance gene (neo-r) was also inserted into each plasmid. A neo gene is included as a selectable marker for successful insertion of the plasmid and confers resistance to neomycin and kanamycin in prokaryotes and eukaryotes (Fig. 6).

The synthesized DNA plasmids that were stored on glass microfiber grade GF/C filter papers, the presence of the NLS-fluorescent hybrids protein and the selection marker, neomycin, were assessed following the manufacture's guidance. Briefly, the filter papers were placed on sterile and clean surfaces separately. One hundred μ L of 10mM Tris-HCl pH

7.5 were added directly into the center of each filter. The filters than were incubated at room temperature for 2 mins. After being incubated, the GF/C filters were placed in 0.6 mL tubes that were punctured using a needle-tipped syringe. Each 0.6 mL tube was placed in a 1.5mL tube and centrifuged at 11,000 g to transfer the DNA liquid, approximately contain 2 μ g plasmid DNA or \sim 20 ng/ μ L from the 0.6 mL tube into the 1.5 mL tube.

2.2 Cloning of pDNAs

To perform pDNA cloning, α -select commercially competent *E. coli* cells (Biolone, Australia) were used to transform the synthesised pDNA following the manufacturer's instructions. Each plasmid (5 μ L) was added into 50 μ L competent cells in each chilled tube. The mixtures were incubated on ice for 30 mins and heat shocked at 42°C for 30 secs and returned to ice for 2 mins. pDNAs were isolated using ISOLATE II Plasmid minikit (Biolone, Australia) following the manufacturer's protocol. Bacterial colonies were grown on medium containing ampicillin at 100 μ g/ml. The concentrations of pDNAs were measured using a spectrophotometer (IMPLEN, Germany) at the absorbance length of 260 nm. The isolated DNA was cloned for usage and further analyses.

2.3 Preparation of Mammalian Cell Lines

After the failure of C6/36 to support the growth of PmeHDV [9], we decided to use Vero (African green monkey kidney) cell lines to test the NLSs (Table 2) as Vero cells are robust when manipulated and interferon deficient so they are less likely to reject a plasmid when transfected. Veros were obtained from the frozen stocks at JCU Australia. The cells were cultured in Minimum Essential Modified (MEM) medium supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 1% non-essential amino acids and antibiotics (penicillin (200 U/mL), streptomycin (200 mg/mL), kanamycin (80 mg/mL), polymyxin B (30 U/mL) and 1x amphotericin B (Sigma Aldrich, Australia) in 37°C incubator. At 24 hr prior to

Table 2. NLS sequences inserted in the plasmids in this study

Plasmids	Amino acids (aa) NLS sequences	Ex	Em
pD603-Dasher-VP_NLS	<u>PKKKKKYR</u>	505	525
pD603-Yukon-NS2_NLS	<u>KRDQQKTEKKDDEPPKKKTK</u>	550	563
pD673-Cayenne-NS1_NLS	<u>KKNPELTQFVLVLASMQYVHSNYDKPDRKFI</u>	554	590
pD603-MatchYFP (negative control plasmid)	None	520	542
pD673_Cayenne-PfD DNV **_NLS (positive control plasmid)	<u>RRRRRR</u>	554	590

** *Periplanata fuliginosa* Dengovirus [10]

transfection, cells were seeded in 24 well plates. Viafect™ (Promega, Australia; lipofection reagent) was used per manufacturer's instructions to transfect five different pDNAs into Vero cells.

A selectable marker gene confers a type of reporter gene to determine the success of transfection to introduce the desired gene into a cell. Selectable markers are often antibiotic resistance genes. Ampicillin-r and neomycin-r were fused in our plasmids as selectable markers. Ampicillin-r is considered useful for selectable markers for *E. coli*. Selection of transfected Vero cells was conducted using an analogy of neomycin sulphate, G418 selection antibiotic (Thermo Fisher, Australia) at concentration of 0.5 mg/μl which was added into the cell medium.

2.4 Transfection both a Non-NLS inserted pDNA and NLS-inserted pDNAs into Vero Cells

One day before transfection, Vero cells were plated in 16 wells of a 24 well plates in complete growth medium at 37°C to reach 70-80% confluence. On the day of transfection, pDNA (500ng/well) was diluted per 50 μL of OPTI-I reduced serum MEM (Life Technologies, Australia) and mixed well. The transfection was performed using ViaFect™ (Promega, Australia) at 2 μL per well.

2.5 Cell Visualization Post Transfection

Four days after transfection, growth medium was removed and cells in each well were incubated in 1x PBS for 10 secs and subsequently fixed with acetone for 10 sec. Coverslips with cells were removed from the wells and dried for 10 mins at room temperature. Following this, coverslips with cells stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to A-T rich regions in DNA, were mounted and kept away from light. The presence of nuclear location signals was observed under a fluorescent microscope (Olympus, Japan) using different combination of filter sets of DAPI-blue, FITC-green and TEXAS-red with different excitation (Ex) and emission (Em) wave lengths, namely 358/461, 488/520, and 591/618 nm, respectively.

3. MAIN RESULTS

3.1 Visualization of Protein Sequences of NLSs within Transfected Cells

Transfection of Vero cell cultures with plasmids (Table 2) was assessed by growing on neomycin

containing media. Only cells with the neomycin-resistance plasmids should have survived. The NLS was assessed by the cell's ability to fluoresce at the appropriate wave length (nm). Three fluorochrome filters (FITC-Green, DAPI and Texas Red) were selected as examples detection to show the presence of fluorescence within the infected Vero cells 4 days post-transfection (4dpt).

All transfected cells in our study demonstrated poor to no noticeable translocation of signals in the nuclei even though there was a build-up of fluorescence ringing the outside of the nuclear membrane and in the cytosol. The pictures were similar, so examples are shown (Figs. 2 & 3) of the exposure to the plasmid pD603-Dasher-VP_NLSs (Fig. 2).

The visualization of transfected cells allowed us to identify the location of the NLSs. Ideally, the cell nucleus or cytosol will glow indicating the co-location of the fluorescent signals. There are differences between transfected Vero cells with pD603-Dasher-VP_NLSs and non-transfected Vero cells. In transfected Vero, screening using a single filter failed to show the green fluorescence in nucleus (N) but green granola (foci) was identified in cytosol (c). The merged images between using the two filter sets also showed no fluorescence within the nuclei of the cells as all nuclei were blue, stained with DAPI. Whereas non-transfected Vero cells showed low Dasher green (Fig. 1). The combination the three filters seems light up the fluorescent spots within nuclei in some transfected Vero cells (Fig. 2).

The research on the mechanism of the interplay between hepadensovirus and the host's cells remains at the bioinformatics analysis stage [7]. A major impediment to the study of densoviruses including hepadensovirus is the lack of suitable cell lines that support the replication of the virus to high titre, despite many attempts to *in vitro* propagating some viruses in cell cultures [11-14]. It was reported that C6/36 cell cultures was successfully infected with MrNV but there was no evidence to show complete viral propagation [15]. Success for hepadensovirus was reported only for *P. monodon* hepadensovirus (PmoHDV) in C6/36 [16] but not repeatable for PmeHDV [9]. To date, no experimental investigation on the role of nuclear location signals (NLSs) of PmoHDV during viral infection has occurred.

The NLS assists in the navigation of viruses to the nucleus and leads to further nuclear translocation. The bioinformatical analysis that highlighted many possible NLSs of prawn parvoviruses that have not been recognized before and needed to be tested in experimental systems [7]. Subsequent analyses would

also shed light on functionality. In this study, we transfected the synthetic plasmids (Table 2) into Vero cell cultures hoping to identify their expression and functionality.

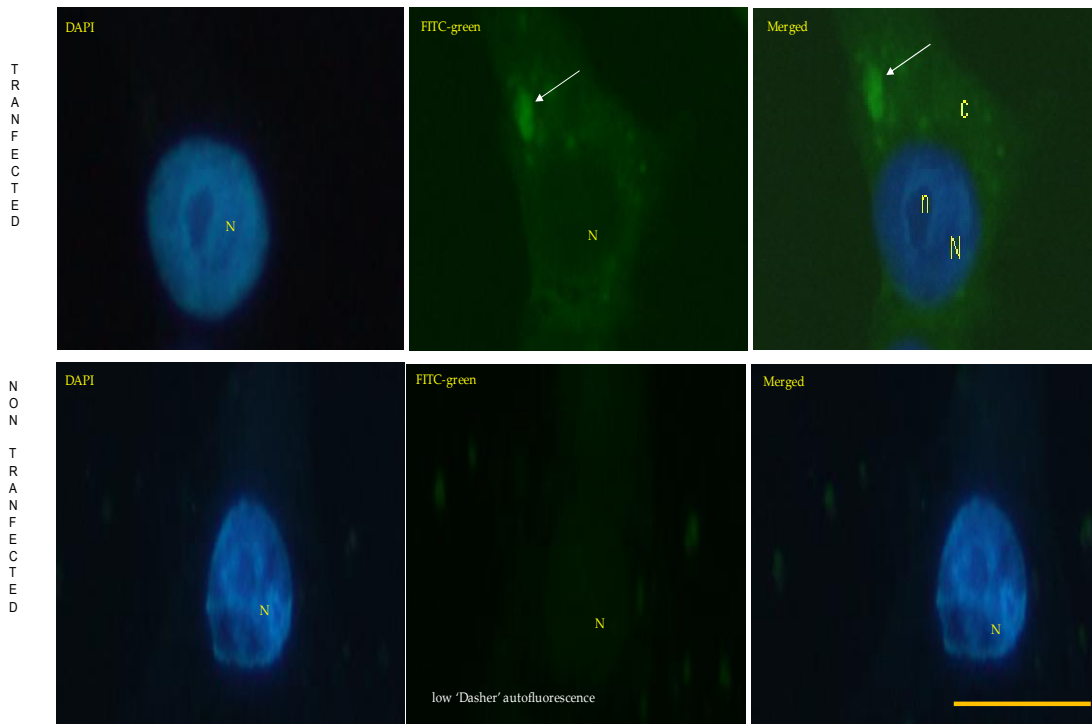


Fig. 1. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS and non-transfected Vero, using combination of different filter sets (Ex358/Em461 (blue); Ex488/Em520 (green); Ex 591/Em618 (red)); N: nucleus; n: nucleolus; c: cytosol; Arrows: green foci. Bars: 20 μ m

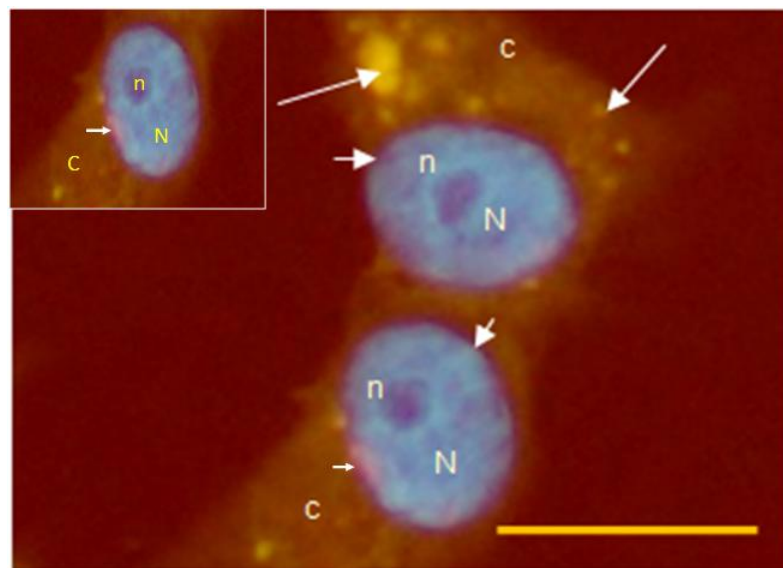


Fig. 2. Transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV, 4 days-post transfection using combination of three filters: Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red); c: cytosol; Long arrows: green foci; Short arrows: red-purple fluorescent. Bar 20 μ m

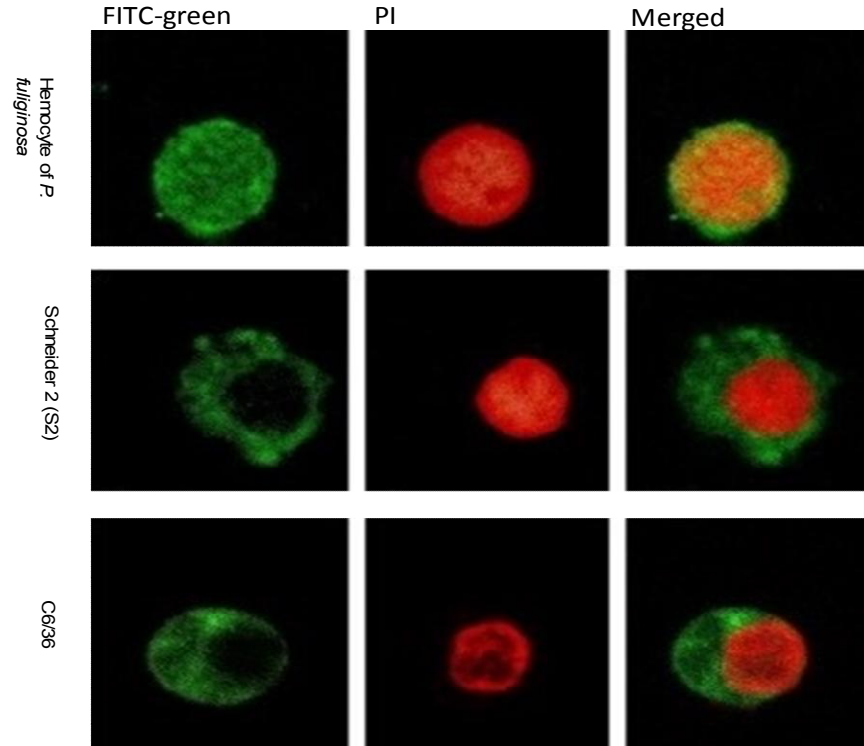


Fig. 3. Expression of NS1 in PfDENV-infected cells (*P. fuliginosa* nymph hemocytes, S2, and C6/36). Cells were counterstained with propidium iodide (PI, red fluorescence) to visualize nucleus morphology, and antibody conjugated fluorescein isothiocyanate (green fluorescence) [10]. Notes: the fluorescent signals are only in the nucleus of the host cockroach haemocytes and not in the insect cell lines (S2 & C636)

Cell transfection and fluorescent analyses have been widely used in previous studies to understand the mechanisms of NLSs during viral infection. As a good example, NS1 *Periplaneta fuliginosa* densovirus (PfDENV) was successfully localized to both cytosol and nucleus of cockroach (*P. fuliginosa*) haemocytes [10]. The NS1 protein was also ectopically (relative to the nucleus) expressed in non-*P. fuliginosa* insect cells such as Schneider line 2 (S2) of *Drosophila melanogaster* and *Aedes albopictus* (C6/36) cell lines, but the NS1 remains outside of the nucleus staying in the cytosol of the non-*P. fuliginosa* insect cells (Fig. 3). We had hoped to see similar results and indeed the fluorescence is outside the nucleus in the Vero cell lines thus our results are identical to the results for the insect cell lines but not successful as in the host cockroach haemocytes (seen in Fig. 3).

Parvovirus DNA replication takes place in the cell nucleus and is strictly dependent on the biochemical activities of the major viral non-structural protein, NS1. NS1 protein is a multifunctional nuclear phosphoprotein which is absolutely required for parvovirus replication both *in vivo* and *in vitro* [17].

Parvoviral NS1 carries out several obligatory roles in virus replication that require NS1 translocation into the nucleus [18-19]. Since the nuclear pores in nuclear envelope have a functional radius of 4.5 nm [20], it is thought that proteins with a molecular mass of less than 67 kDa can passively diffuse through these nuclear pores [20]. NS1 at 61 kDa could theoretically, passively diffuse into the nucleus and therefore in transfection trials, the presence of NS1 should be clearly detected in nucleus.

An insight into the journey of an NLS of a crustacean virus was recently reported. The location of *Macrobrachium rosenbergii* nodavirus capsid protein (MrNVc) was successfully detected in insect cell cultures, *Spodoptera frugiperda* (Sf9) [21]. The fluorescent assay and in a sub-cellular fraction, the MrNVc was shown to enter Sf9 cells and located in the nucleus. Small granules appeared in Sf9 cell cultures incubated with fluorescein-labelled capsid proteins of MrNV virus-like-particles (F-MrNVc VLPs) [21] (Fig. 4), similar to green granola patterns (foci) identified in the cytosol of transfected Vero cell cultures in our study (Figs. 1 & 2).

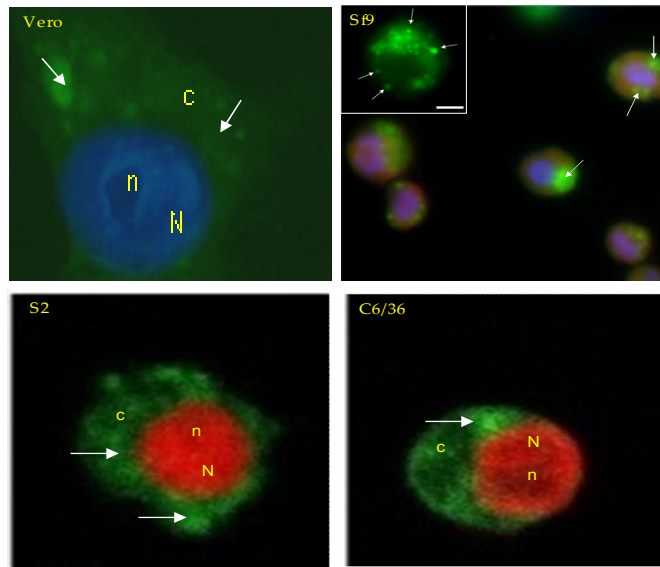


Fig. 4. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV, 4 days-post transfection using combination of three filters: Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red) in our study (top left), with previous transfected cell cultures: Sf9 (top right) [21], S2 (bottom left) and C6/36 (bottom right) [10]; N: nucleus; n: nucleolus; c: cytosol; arrows: green foci

Despite the high virulence and infectivity of for their natural hosts, densoviruses are not known to infect mammals. Previous experiments conducted to infect or transfect vertebrate or vertebrate cells line with densoviruses were unsuccessful [22]. Similarly, there is no report on the successful propagation and transfection of Decapod hepanidensovirus 1 in/into mammalian cells to date. Therefore, an artificial plasmid construct was attempted in this study. Transfecting NLSs in our study failed to show clearly the cellular changes upon the transfection of pDNAs in Vero cell lines even though in the previous study [10], the original host cockroach cells fluoresced. The

results indicate that our techniques may not be sensitive enough to be confident that intranuclear fluorescence is real and therefore not able to test the NLS of PmeHDV.

A recent concept of moonlighting function of importin beta-1 (Imp β -1) in human cells during the cell cycle highlighted the Imp β -1 acting as a nuclear transport receptor during the interphase, when it accumulated at the nuclear envelope (Fig. 5B) [23]. In some of our Vero cells, light fluoresce signals were identified at the nuclear membranes in the merged pictures (see in Figs. 2 & 5).

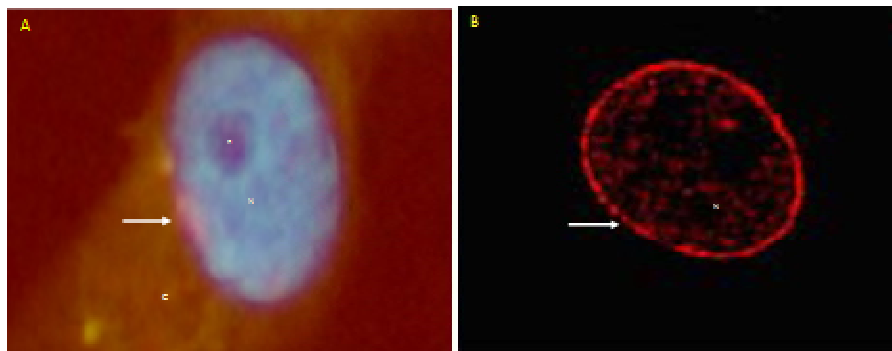


Fig. 5. Comparison between transfected Vero cells with pD603-Dasher-VP_NLS of PmeHDV 4 days-post transfection using combination of three filters (Ex358/Em461 (blue), Ex 488/Em 520 (green), Ex 591/Em618 (red)) in our study and human cells showed Imp β -1 (red) accumulates at the nuclear membrane, encircling the nucleus (blue). α -tubulin stained green during interphase in the cytoskeleton (B) [23]

It is possible that the Vero cell lines might be inappropriate for these NLS but there does seem to be cytoplasmic signals at least, a build-up of fluorescence at the nuclear membrane like the experimental protein is conjugated to importin beta-1 (Imp β -1) [23]. The presence of fluorescent spots

within the cytoplasm as well as at the nuclear membranes of our transfected Vero (Figs. 3 & 6) suggested that some signals, perhaps entered the nuclei. Incubating transfected Vero cells longer (>4 days) before being observed might have improved the results.

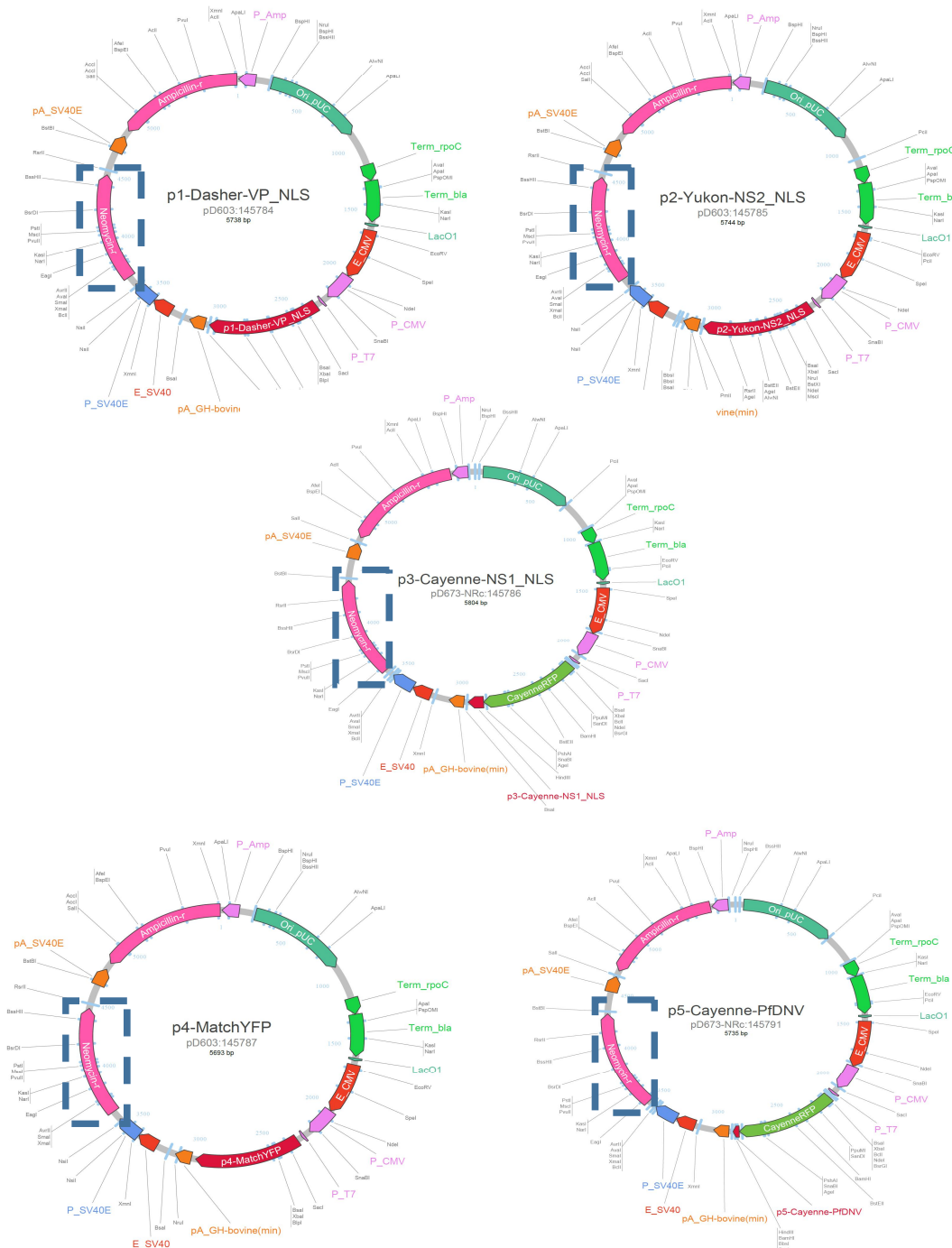


Fig. 6. The map of five constructed plasmids (explanation of abbreviations can be previewed in www.atum.bio)

4. CONCLUSION

Our study found that the nuclear location signals (NLSs) potentially did enter the nuclei, but our fluorescent study was not sensitive enough to be confident in detecting differences in NLS-transfected-cells under different filters. Strong fluorescence appears to be in the cytoplasm of transfected Vero were similar with that of previous study using S2 & C6/36 [10] and that of using Sf9 [21]. Light red-purple at the nuclear membrane of transfected Vero nuclei might be importin/NLSs complex similarly with that was detected in human cancer cells [23]. However, we are not convinced enough to state that the fluorescence's were related to the NLSs of PmeHDV. Therefore, Vero cell lines are not sensitive enough to identify the location of NLSs of PmeHDV. The study of virus-host interaction using proxy cell cultures as models remains a major challenge.

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COMPETING INTERESTS

The authors declare no conflict of interest and the sponsors had no role in the design of this study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

REFERENCES

- Hogarth CA, Calanni S, Jans DA, Loveland KL. Importin α mRNAs have distinct expression profiles during spermatogenesis. *Dev Dyn*. 2006;235(1):253-262.
- Moseley GW, Daniela MR, Michelle AD, Denisse LL, Richard RP, Colin WP, David AJ. Dynein light chain association sequences can facilitate nuclear protein import. *Mol Biol Cell*. 2007;18(8):3204-3213.
- Pryor MJ, Rawlinson SM, Butcher RE, Barton CL, Waterhouse TE, Vasudevan SG, Bardin PG, Wright PJ, Jans DA, Davidson AD. Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. *Traffic*. 2007;8(7):795-807.
- Jans DA, Xiao C, Lam MHC. Nuclear targeting signal recognition: A key control point in nuclear transport? *Bioessays*. 2000;22(6):532-544.
- Cokol M, Nair R, Rost B. Finding nuclear localization signals. *EMBO Rep*. 2000;1(5):411-415.
- Boulikas T. Putative localisation signals (NLS) in protein transcription factors. *J Cell Biochem*. 1994;55(1):32-58.
- Owens L. Bioinformatical analysis of nuclear localisation sequences in penaeid densoviruses. *Mar Genomics*. 2013;12:9-15.
- Chelsky D, Ralph R, Jonak G. Sequence requirements for synthetic peptide mediated translocation to the nucleus. *Mol Cell Biol*. 1989;9(6):2487-2492.
- Syahidah D, Elliman J, Constantinoiu C, Owens L. Mosquito cells (C6/36) fail to support the complete replication of *Penaeus merguensis* hepadensovirus. *J Invertebr Pathol*. 2017;145:31-38.
- Zhou W, Jiamin Z, Yang B, Zhou L, Hu Y. The nuclear localization signal of the NS1 protein is essential for *Periplaneta fuliginosa* densovirus infection. *Virus Res*. 2009;145(1):134-140.
- Sudhakaran R, Parameswaran V, Hameed ASS. *In vitro* replication of *Macrobrachium rosenbergii* nodavirus and extra small virus in C6/36 mosquito cell line. *J Virol Methods*. 2007;146(1-2):112-118.
- Sriton A, Kanthong N, Gangnonngiw W, Sriurairatana S, Ubol S, Flegel TW. Persistent expression of shrimp – virus antigens in two insect cell lines challenged with two shrimp viruses. *Fish Pathology*. 2009;44(2):86-93.
- Gangnonngiw W, Kanthong N, Flegel TW. Successful propagation of shrimp yellow head virus in immortal mosquito cells. *Dis Aquat Organ*. 2010;90(1):77-83.
- Arunrut N, Phromjai J, Gangnonngiw W, Kanthong N, Sriurairatana S, Kiatpathomchai W. *In vitro* cultivation of shrimp Taura syndrome virus (TSV) in a C6/36 mosquito cell line. *J Fish Dis*. 2011;34(10):805-810.
- Hayakijkosol O, Owens L. Non-permissive C6/36 cell culture for the Australian isolate of *Macrobrachium rosenbergii* nodavirus. *J Fish Dis*. 2013;36(4):401-409.
- Madan N, Nambi KSN, Majeed SA, Taju G, Raj NS, Farook MA, Vimal S, Hameed ASS. *In vitro* propagation of hepatopancreatic parvovirus (HPV) of shrimp in C6/36 (*Aedes albopictus*) cell line. *J Invertebr Pathol*. 2013;112(3):229-35.

17. Yang B, Zhang J, Cai D, Li D, Chenm W, Jiang H, Hu Y. Biochemical characterization of *Periplenata fuliginosa* densovirus non-structural protein NS1. *Biochem Biophys Res Commun.* 2006;342(4):1188-196.
18. Raab U, Beckenlehner K, Lowin T, Niller HH, Doyle S, Modrow S. NS1 protein of parvovirus B19 interacts directly with DNA sequences of the p6 promoter and with the cellular transcription factors Sp1/Sp3. *Virology.* 2002; 293(1):86-93.
19. Young PJ, Jensen KT, Burger IR, Pintel DJ, Lorson CI. Minute virus of mice NS1 interacts with the SMN protein and they allocate in novel nuclear bodies induced by parvovirus infection. *J Virol.* 2002;76(8):3892–3904.
20. Paine PI, Moore LC, Horowitz SB. Nuclear envelope permeability. *Nature.* 1975; 254(5496):109-114.
21. Hanapi UM, Yong CH, Goh ZH, Alitheen NB, Yeap SK, Tan WS. Tracking the virus-like particles of *Macrobrachium rosenbergii* nodavirus in insect cells. *Peer J.* 2017;5:e2947.
22. El-Far M, Li Y, Fediére G, Abol-Ela S, Tijssen P. Lack of infection of vertebrate cells by densovirus from the maize worm *Mythimma loreyi* (MIDNV). *Virus Res.* 2004;99(1):17-24.
23. Verrico A, Schininà ME, Di Francesco L, Ilari A, Morea V, Lavia P. Nuclear transport receptors: Moonlighting proteins aberrantly expressed in cancer. *EC Proteomics and Bioinformatics.* 2016;1(1):01-03.