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Identification and characterization of the duck enteritis virus (DEV) US2 gene

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ABSTRACT. The US2 protein has been reported to contribute to duck enteritis virus (DEV) infection; however, its kinetics and localization during infection, and whether it is a component of virion, have not been previously reported. To elucidate the function of DEV US2, *US2* was amplified by polymerase chain reaction (PCR) and inserted into pET-32a(+); this was expressed, the recombinant US2 protein was purified, and a polyclonal antibody generated. In addition, the kinetics and localization of the US2 gene and protein were determined by quantitative real-time fluorescent PCR, ganciclovir (GCV), and cycloheximide (CHX) treatment, western-blot, and indirect immunofluorescence assay. The packaging of US2 into DEV virions was revealed by a protease protection assay. *US2* was found to be transcribed 24 h post-infection (pi) and peaked at 72 h pi; the US2 protein was detected 48 h pi, except in the presence of GCV or CHX. US2 was packed into virions and also localized to the plasma membrane and

cytoplasm in infected cells. The results showed that the DEV *US2* is a late gene, and that its encoding protein could be a tegument component localized mainly in the cytoplasm. This study provides useful data for the further analysis of DEV US2, including an explanation for the genetic conservation among alphaherpesviruses.

Key words: DEV; US2; Tegument; Late gene; Localization

INTRODUCTION

Duck enteritis virus (DEV) is a globular virus that possesses a viral envelope, tegument, and a nucleocapsid. The virus is composed of a linear, double-stranded DNA with a G+C content of 44.89%, and has been completely sequenced by Wu et al recently (Tirath and Sandhu, 2008; Wu et al., 2012). The Chinese virulent DEV strain is a wild-type virulent strain available in GenBank with the accession No. JQ647509.

Homologs of the alphaherpesvirus US2 are found in herpes simplex virus-2 (HSV-2), canine herpesvirus (CHV), bovine herpesvirus-1 (BHV-1), duck enteritis virus (DEV), Marek's disease virus (MDV), pseudorabies virus (PRV), and equine herpesvirus-1 (EHV-1), among others (Cantello et al., 1991; Haanes and Tomlinson, 1998; Jiang et al., 1998; Belknap et al., 1999; Meindl and Osterrieder, 1999; Ben-Arieh et al., 2001; Clase et al., 2003; Gao et al., 2012b). The properties of US2 have not been extensively studied, despite the fact that it is conserved among alphaherpesviruses (Clase et al., 2003). Duck plague (DP), an acute and contagious infectious disease affecting ducks, swans, and geese, caused by the duck enteritis virus, is responsible for significant losses in agricultural industries (Tirath and Sandhu, 2008; Wu et al., 2012).

Recent studies have suggested that US2 is involved in the penetration and cell-to-cell spread of DEV in susceptible cells (Wei et al., 2013). It is conceivable that US2 plays an important role in the spread of virus; however, the characteristics of US2 remain unclear. In this study, a prokaryotic expression vector was constructed, US2 was expressed in a prokaryotic system, recombinant US2 protein was purified, and a polyclonal antibody was generated to analyze the transcriptional level of the DEV *US2* gene and the intracellular localization of the US2 protein in infected cells. Additionally, ganciclovir (GCV) and cycloheximide (CHX) treatment was used to classify the kinetics of *US2* gene expression (Wu et al., 2011). The transcriptional level and western blot of US2 in infected cells was also used to confirm the kinetics of US2. Finally, in order to determine if the DEV US2 protein was packaged into virions, extracellular virions were purified and subjected to a protease protection assay. The goal of these experiments was to functionally elucidate the US2 gene and protein; these findings may provide more information facilitating further exploration of the molecular mechanism of US2 recruitment.

MATERIAL AND METHODS

Virus, cells, serum, enzymes, and plasmids

The CHv-DEV strain has been previously isolated and identified in our laboratory (Cheng et al., 2006). Duck embryo fibroblast (DEF) monolayer cells were cultured in Eagle's minimal essential medium (MEM) (Gibco, Life technologies, Burlington, ON, CA) supplemented with 10%

fetal bovine serum at 37°C in a 5% CO_2 environment. MEM was supplemented with 2% FBS when cell monolayers were incubated with DEV. Both the CHv-DEV strain and rabbit anti-DEV IgG were obtained from the Key Laboratory of Animal Disease and Human Health of Sichuan Province. All enzymes used in the cloning procedures were purchased from TaKaRa Bio Inc. (Dalian, China). In addition, *Escherichia coli* DH5α (Tiangen, Beijing, China), *E. coli* BL21 (DE3) (Tiangen), pMD18-T/β-actin vector, and the pET-32a(+) expression vector (Novagen, Shanghai, China) were stored in our laboratory.

PCR amplification and plasmid construction

The US2 open reading frame of DEV (GenBank accession No. EU195086), including the methionine start codon, was amplified from CHv-DEV by PCR, using primers containing the *Eco*RI and *Hin*dIII sites, as previously described (Wu, 2011). The primers were designed by Oligo6 as follows: (P1) forward primer: 5'-G^AATTCATGGGTGTGGTTATATTGT-3' and (P2) reverse primer: 5'-A^AGCTTCTAACAGGCTATACAAC-3'. The PCR products were digested with *Eco*RI and *Hin*dIII (TaKaRa Bio Inc., Dalian, China), and ligated to similarly digested pMD-18T (TaKaRa Bio Inc., Dalian, China) and pET-32a(+). All constructs were confirmed by DNA sequencing (Invitrogen, Carlsbad, CA, USA).

Expression and purification of recombinant US2 protein

The recombinant expression plasmid pET-32a(+)/US2 was transformed into *E. coli* BL21; the bacterial cell production was induced using optimized amounts of isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. The resulting recombinant US2 protein was then purified as previously described (Xie et al., 2010). Briefly, the cells were centrifuged at 10,000 rpm for 10 min, resuspended in 20 mM Tris buffer (pH 8.0) containing lysozyme (0.1 mg/mL), and subsequently incubated at -20°C overnight. The cell lysate was sonicated on ice for 5 min, at an amplitude of 30% and a pulse frequency of 30 s, using an ultrasonic instrument (ultrasomic processor-500, Autoscience instrument Co., Ltd., Tian Jing, China). The sonicated lysate was centrifuged at 12,000 rpm for 10 min, and the supernatant and pellets were collected for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results indicated that the recombinant US2 protein had formed inclusion bodies. The pellets were then resuspended in 20 mL urea washing buffer (6 M urea, 50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100; pH 8.0) under constant stirring for 10 min, and subsequently centrifuged at 12,000 rpm for 10 min at 4°C. The above steps were repeated six times in order to release the trapped protein. The suspension was finally centrifuged at 12,000 rpm for 10 min at 4°C, and resuspended in denaturing buffer containing 8 M urea, prior to SDS-PAGE analysis.

Identification of recombinant US2 protein by western blot analysis

The reactivity and specificity of the recombinant US2 protein was determined by western blot analyses using the purified recombinant US2 protein, as described previously (Towbin et al., 1992). The purified US2 protein was loaded onto a 12% SDS-PAGE and transferred by electroblotting onto a polyvinylidene difluoride (PVDF) membrane (Millipore) according to the manufacturer protocols. The membranes were incubated for 1 h with primary rabbit anti-DEV IgG,

washed thrice with PBST, and finally incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary IgG (1:5000) for 30 min at 37°C. The membranes were then washed thrice with PBST, and treated with 3,3'-diaminobenzidine (DAB) (Zhongshan Co. Ltd., Beijing, China) in 0.1% H_2O_2.

Generation of rabbit anti-US2 IgG

Rabbit anti-US2 polyclonal antibodies were prepared as previously described (Xiang et al., 2010; Wu et al., 2011). Briefly, male New Zealand white rabbits were immunized intradermally with a mixture of 0.5 mg purified US2 protein and an equal amount of complete Freund's adjuvant (Sigma-Aldrich, Shanghai, China). Two weeks later, 0.75 mg of the purified US2 protein and an equal amount of Freund's incomplete adjuvant were administered for secondary immunity. The rabbits were boosted subcutaneously with 1.0 mg each of purified US2 protein and incomplete Freund's adjuvant at 1 week intervals. The rabbits were injected intravenously with 0.1 mg purified US2 protein after 7 days, and the serum was subsequently collected after 17 days. Pre-immune serum was obtained from non-vaccinated healthy rabbits as a control.

Agar diffusion reactions were used to detect the antiserum titer (Lu et al., 2010; Wu et al., 2011). Briefly, 1 g agar was dissolved in 100 mL 0.85% sodium chloride. The mixture was heated, cooled to 55°C, and then poured into plates to a thickness of 2 mm. The agar was perforated with holes (3 mm diameter) that could hold approximately 100 µL of solution. Forty microliter each of the pre-immune serum, mock, 1:2, 1:4, 1:8, 1:16, and 1:32 diluted antiserum were added into the peripheral wells. Finally, 20 µL of purified US2 protein was added to the central well. The plate was incubated at 37°C for 10 h before observation.

The rabbit IgG fraction was precipitated from the harvested rabbit polyclonal antiserum by ammonium sulfate precipitation and High-Q anion-exchange chromatography, according to the manufacturer protocols (McGuire et al., 1996).

US2 expression in infected cells

The US2 expression was detected by western blot as previously described (Clase et al., 2003). Briefly, confluent monolayers of DEFs were infected and mock-infected with DEV, and harvested at 12, 24, 36, 48, 60, 72, and 84 h post infection (pi) to determine the level of US2 expression in infected cells. At the indicated times, the medium was removed and the cells washed thrice with PBS. The cells were scraped into 40 µL PBS and transferred to a 1.5 mL microfuge tube containing 10 µL SDS-PAGE sample buffer. Aliquots were heated to 100°C for 10 min, electrophoresed through 12% SDS-PAGE gels, and analyzed by western blot using polyclonal antiserum against US2 (Clase et al., 2003; Xiang et al., 2010). In this case, the purified DEV US2 IgG was used as the primary antibody for dynamic expression analyses.

Indirect immunofluorescence assay

Indirect immunofluorescent assay was performed as previously described (Clase et al., 2003; Zhao et al., 2008; Shen et al., 2009; Kang and Banfield, 2010; Xing et al., 2011). DEF cells were seeded onto glass coverslips and grown to a confluence of 30-40% prior to mock infection or infection with DEV in 6-well plates. The cells were washed with PBS 39, 49.5, 60, 72.5, and 84 h pi,

and subsequently fixed with 4% paraformaldehyde-PBS for 30 min at room temperature. The cells were rinsed with PBS and permeabilized in PBS containing 0.1% Triton X-100 for 10 min at room temperature. The cells were then washed with PBS, blocked for 1h in PBS containing 5% BSA at 37°C, and incubated with purified US2 antiserum or pre-immune serum (1:50) at 4°C overnight. The cells were washed three times for 10 min in PBS, and treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Zhongshan Co. Ltd., Shanghai, China) for 45 min at 37°C. The cell nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) counter-staining (5 µg/mL; Beyotime Institute of Biotechnology, Shanghai, China) as previously described (Miller et al., 2000). Coverslips were mounted on glass slides, and the images were captured using a fluorescence microscope imaging system (Nikon 80; Nikon, Tokyo, Japan).

Protease treatment of purified virions

Virions were purified using previously described procedures (Clase et al., 2003; Lyman et al., 2006; Xie et al., 2009) by ultra-centrifugation (Beckman LE-80K). Briefly, the virions were treated with proteinase K (10 µg/mL) in the presence and absence of SDS (1%). Four experimental samples were prepared: (1) 20 µL virion suspension with 10 µL SDS and 20 µL proteinase K; (2) 20 µL virion with 10 µL SDS and 20 µL PBS; (3) 20 µL virions with 20 µL proteinase K and 10 µL PBS; and (4) 50 µL PBS. Phenylmethanesulfonyl fluoride (PMSF) was added to each sample after 1 h incubation at room temperature, to inhibit further reactions. The samples were analyzed by SDS-PAGE and transferred to a PVDF membrane for western blot detection of US2 and UL51.

Transcriptional levels of the *US2* gene

The DEV CHv strain was propagated in DEFs. The levels of the mRNA transcripts of *US2* were determined by a rapid real-time quantitative polymerase chain reaction (qRT-PCR) using an Icycler IQ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) coupled with SYBR Green chemistry. Total RNA was extracted from DEV infected DEFs at different times: 12, 24, 36, 48, 60, 72, 84, and 96 h pi using the Total RNA Isolation kit (TaKaRa Bio Inc.); the RNA was reverse transcribed to cDNA. The primers used for qRT-PCR were ΔUS2 F (5'-AGACGGTTCCGAAAGTACAG-3') and US2 R (5'-TCGGCAGCACCAATAATCC-3'), β-actin F (5'-CCGGGCATCGCTGACA-3') and β-actin R (5'-GGATTCATCATACTCCTGCTTGCT-3'). The *in vitro* transcriptional level of the DEV *US2* gene was detected by a previously described qRT-PCR process (Lian et al., 2010). The optimized 20 µL real-time PCR mixture comprised 1 µL (10 pM) of each primer, 1 µL DNA template, 10 µL SYBR Green I Mix, and 7 µL autoclaved double-filtered nanopure water. The template was replaced with water for no-template controls. The qRT-PCR program consisted of an initial 5 min denaturation step at 95°C, followed by 45 cycles of denaturation (94°C, 30 s), annealing (61°C, 30 s), and extension (72°C, 30 s). The relative fluorescence and melt curve was measured. The relative amount of the *US2* mRNA expression was measured by the $2^{-\Delta\Delta Ct}$ method. Each reaction of DEV *US2* and β-*actin* was optimized in triplicate based on their primers (Qing et al., 2010).

Ganciclovir and cycloheximide treatment

The samples were treated with GCV and CHX as previously described (Huang and Wu, 2004; Luo et al., 2012). Briefly, confluent monolayers of DEF cells growing in 25 cm^2 cell culture

flasks were treated with GCV and CHX. The cell culture medium was replaced with MEM-10% FBS with or without GCV (KeLun, Hu Nan, China) or CHX (Sigma-Aldrich). The cells were infected with DEV and incubated in the presence or absence of 300 μg/mL of GCV or 100 μg/mL CHX for 36 h. Total RNA was extracted from the cell lysates and reverse transcribed using the PrimeScript™ RT reagent Kit (TaKaRa). The ΔUS2 and β-actin primers were used to detect the cDNA templates by normal PCR.

RESULTS

Expression and purification of recombinant US2 protein

The recombinant plasmid pET-32a(+)/US2 expressed a considerable amount of a 46 kDa protein, while the same band was absent when induced from the empty pET32a(+) control vector (Figure 1A). The recombinant US2 protein was purified in washing buffer by continuous stirring, separated by SDS-PAGE, and analyzed by staining with Coomassie brilliant blue. This allowed for the visualization of a high purity US2 protein band, compared to the BSA controls (Figure 1B).

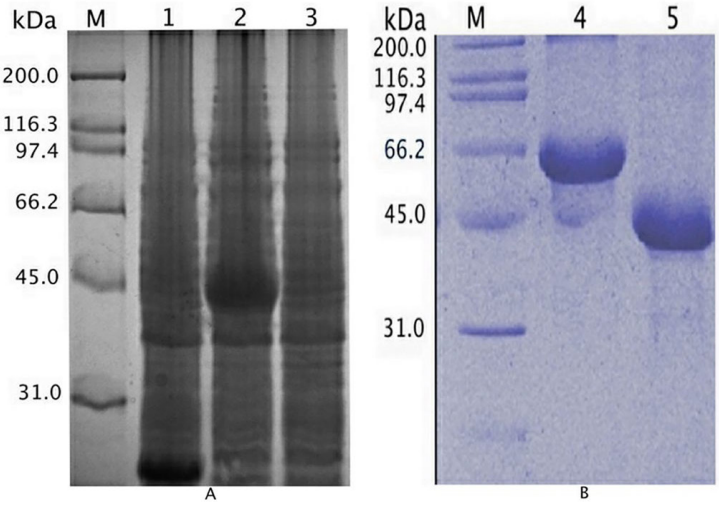

Figure 1. Analysis of the expression and purification of fusion protein DEV US2 by SDS-PAGE. **A.** *Lane M* = protein marker; *lane 1* = the total proteins of cells harboring plasmid pET-32a(+) induced by IPTG treatment; *lane 2* = the total proteins of cells harboring plasmid pET-32a(+)/US2 induced by IPTG; *lane 3* = the total proteins of cells harboring plasmid pET-32a(+)/US2 without IPTG induction. **B.** *Lane 4* = bovine serum albumin (BSA) standard (1 mg/mL); *lane 5* = the purified fusion US2 protein.

Identification of US2 by western blot and agar diffusion reaction

Rabbit polyclonal antiserum against DEV was used in the western clot identification of the recombinant US2 protein. The estimated molecular mass of the recombinant DEV US2 protein (determined by SDS-PAGE) was approximately 46 kDa (Figure 2A). The agar diffusion reaction precipitating line indicated that the largest positive dilution of rabbit anti-US2 antiserum was 1:16 (Figure 2B). Pre-immune serum, which was used as a negative control, showed no antigen-antibody complexes in both SDS-PAGE and agar diffusion assays.

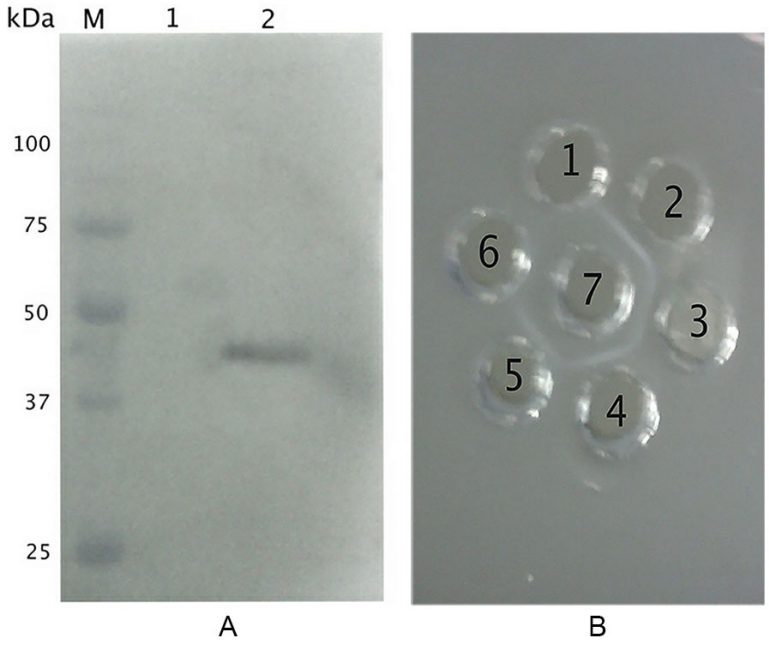

Figure 2. Identification of DEV US2 protein and analysis of the antiserum titer by agar diffusion. **A.** Recombinant US2 expression was analyzed by western blot using rabbit anti-DEV IgG. **B.** Agar diffusion reaction was designed to determine the specificity and sensitivity of anti-US2 serum, and the results of the agar diffusion reaction of the anti-US2 serum with purified US2 protein suggested that the largest dilution was 1:16. *Lane M* = protein marker; *lane 1* = western blot of the total proteins extracted from cells harboring the plasmid pET-32a(+)/US2 without IPTG induction; *lane 2* = western blot of the total proteins of cells harboring pET-32a(+)/US2 with IPTG induction; 1, rabbit pre-serum; 2, 2-fold diluted antiserum; 3, 4-fold diluted antiserum; 4, 8-fold diluted antiserum; 5, 16-fold diluted antiserum; 6, 32-fold diluted antiserum; and 7, purified US2 protein.

Kinetics of DEV US2

The *US2* gene expression in the presence of GCV and CHX was analyzed. US2 expression was not observed when cells were treated with GCV or CHX, while the DEV-infected cells without DNA or protein inhibitors showed a US2 signal (Figure 3A). The transcriptional level of DEV US2 in non-inhibited cells (Figure 3B) revealed that *US2* mRNA expression was relatively low 24-36 h pi, increased after 48 h pi, peaked at 72 h pi, and then decreased. In addition, the US2 protein was first detected in cell lysates by western blot 48 h after infection (Figure 4). Taken together, this data suggests that the US2 protein is expressed during late kinetics.

Protease protection assay

Treated virions were subjected to SDS-PAGE, and US2 was detected by western blot. As shown in Figure 5A (lane 1), US2 protein was susceptible to digestion when virions were treated with both SDS and protease K, and remained unaffected when treated with protease K, SDS, or PBS alone (lanes 2 to 4). UL51, a known tegument protein, was used as a control (Figure 5B).

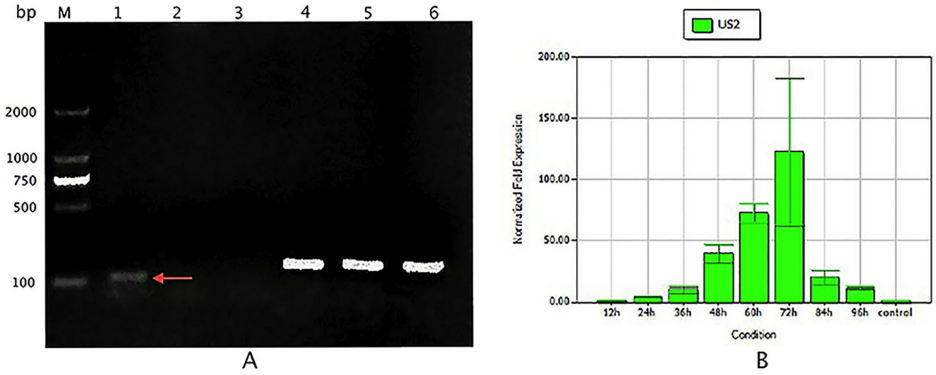

Figure 3. Results of the nucleic acid inhibition and transcriptional expression of the *US2* gene. **A.** *Lane M* = DNA marker. DEF was infected with DEV in the presence of 300 μg/mL ganciclovir or 100 μg/mL cycloheximide and the total RNA of infected cells was harvested at 36 h post infection. RNA was reverse transcribed into cDNA and detected by PCR using primers specific for the *US2* and β-*actin* genes. *Lanes 1* to *3* = PCR using △US2 gene primers on templates from DEV-infected DEF cells in the presence of PBS, GCV, and CHX respectively. *Lanes 4* to *6* = the results of PCR using β-actin primers (templates were identical to the ones used on lanes 1-3). **B.** Relative content of the *US2* mRNA was calculated at 12, 24, 36, 48, 60, 72, 84, and 96 h pi using the $2^{\Delta\Delta Ct}$ method.

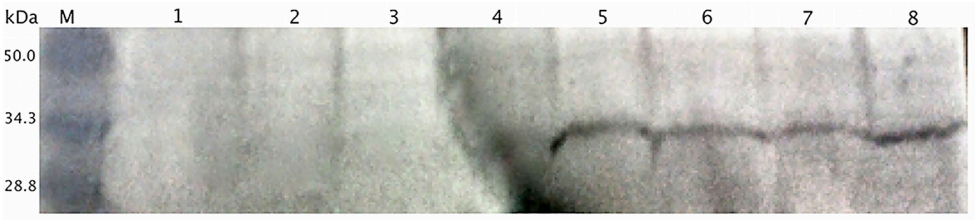

Figure. 4. Western blot analysis of *US2* expression in DEV infected cells. *Lane M* = marker; *lane 1* = mock infection; *lanes 2-8* = times post-infection (12, 24, 36, 48, 60, 72, 84 h pi). The US2 protein was first detected by western blot 48 h after infection, and the specific band corresponded to the expected molecular weight of DEV US2 (~30 kDa).

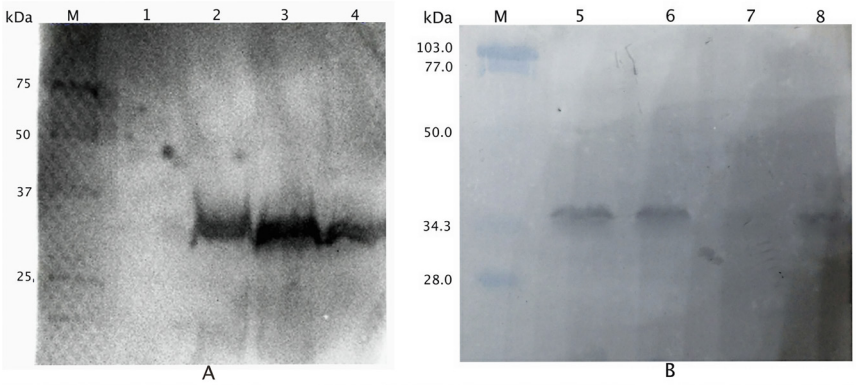

Figure 5. Western blot of purified virus analyzed for US2 and UL51 through protease protection assay. *Lane M* = marker; *lane 1* = DEV virions treated with SDS and Protease K; *lane 2* = DEV virions treated with protease K alone; *lane 3* = DEV virions treated with SDS; *lane 4* = DEV virions treated with PBS alone as a control; *lane 5* = DEV virions treated with protease K alone; *lane 6* = DEV virions treated with SDS; *lane 7* = DEV virions in the presence of both SDS and protease K; *lane 8* = DEV virions treated with PBS alone as a control.

Localization of the US2 protein

US2 was sporadically distributed on the plasma membrane (Figure 6) at 39 h pi and 49.5 h pi US2 was localized in the cytoplasm of infected cells at 60 h pi (Figure 7A); the fluorescence was enhanced and localized mainly in the cytoplasm near the nucleus at 84 h pi (Figure 7B).

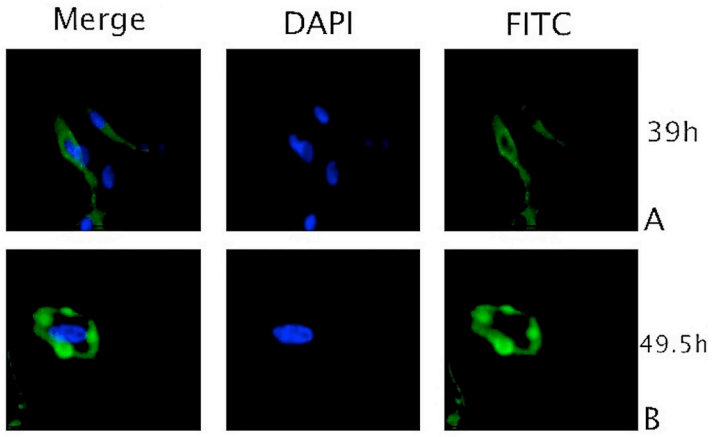

Figure 6. Intracellular localization of US2. DEV-infected cells were fixed at 39 h pi and 49.5 h pi. The samples were incubated with anti-US2 IgG and subsequently stained with a FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue).

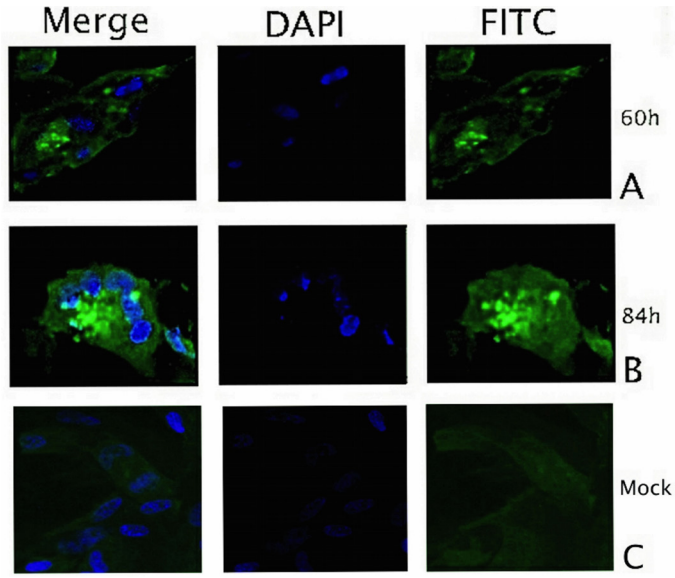

Figure 7. Intracellular localization of US2. DEV-infected cells were fixed at 60 h pi and 84 h pi. The samples were incubated with anti-US2 serum and subsequently stained with a FITC-conjugated secondary antibody. Nuclei were counterstained with DAPI (blue). C represents a sample from mock-infected DEF, which was processed and analyzed as a control.

DISCUSSION

This report presents the detailed characterization of the DEV US2 protein. Unlike PRV US2, expression of DEV US2 is sensitive to the DNA synthesis inhibitor ganciclovir (GCV), which suggests that DEV *US2* is a late gene. DNA synthesis in herpesvirus-infected cells is significantly reduced by ganciclovir treatment, whereas ganciclovir had little effect on DNA synthesis in mock cells (St Clair et al., 1987). Herpesvirus genes are grouped into three types (α, β, and γ genes) according to their transcription kinetics (Workman et al., 1988; Dunn et al., 2003; van Beurden et al., 2013). Immediate-early genes (IE/α genes) are the first viral genes expressed, early genes (E/β genes) are expressed prior to viral DNA replication, and late genes (L/γ genes) are dependent on viral replication. We examined the synthesis of US2 in the presence of GCV, an inhibitor of viral DNA synthesis or CHX, an inhibitor of protein synthesis, to determine the kinetics of US2. US2 was seen to be affected by both GCV and CHX (Figure 3A), indicating that US2 is a late gene dependent on viral replication for its expression.

Transcription of *DEV* US2 mRNA was at a low level from 24 to 36 h pi (Figure 3B), and increased 48 h pi, with a peak at 72 h pi. In addition, the DEV US2 protein was detectable by western blot at 48 h pi. The results of transcriptional level and dynamic expression analyses confirmed that *US2* is a late gene (Geballe et al., 1986).

IF assay revealed the presence of DEV US2 in the plasma membrane and cytoplasm adjacent to the nucleus of infected cells. We determined the location of US2 at different times from 39 h to 84 h pi to further elucidate the dynamic process of US2 protein in cell. Wei et al. (2013) revealed a time point during the infection when US2 was localized in the cytoplasm; the results of our study agrees with this observation. In addition, the dynamic expression process of US2 was highlighted in this study. In brief, this study revealed the specific dynamic characteristics of US2 (from the CHv-DEV virulent strain).

Similarly, the EHV-1 and PRV US2 proteins were also localized on the plasma membrane despite lacking an obvious signal sequence or transmembrane domain. In contrast, HSV-2 US2 was shown to be localized in the cytoplasm and discrete granules within and at the periphery of the nucleus, instead of the plasma membrane. Our results suggest a dynamic change in the intracellular localization of US2 during the course of DEV infection; specifically, US2 was observed to be localized at the plasma membrane despite lacking a classical N-terminal signal sequence or a putative transmembrane domain (Gao, 2012a). However, a C-terminal CAAX motif, which is a common site for protein prenylation and is associated with plasma membrane localization as in PRV US2, was identified (Jung and Desrosiers, 1991; Kato et al., 1992; Kang and Banfield, 2010). Plasma membrane association is promoted by a series of three closely linked posttranslational modification steps signaled by the consensus carboxyl-terminal CAAX motif (C = cysteine; A = any aliphatic amino acid; X = any amino acid)(Kang and Banfield, 2010). Accordingly, we suspect that the plasma membrane association may be driven by the CAAX motif, as it could provide a site for lipid modification, thereby facilitating its association with the plasma membrane.

Protease protection assays indicated that US2 is a component of the DEV virion, which is only digested by protease K when the lipid envelope is treated by SDS; therefore, we concluded that the US2 protein could be a tegument component.

In summary, DEV US2 is a late gene expressing a ~30 kDa protein, which could be a tegument component that is mainly localized in the cytoplasm. Additionally, the US2 protein was also associated with membranes although it has no obvious N-terminal signal sequence or a transmembrane domain.

Conflicts of interest

The authors declare no conflict of interest.

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