Acta virologica **56**: 293 – 304, 2012 doi:10.4149/av_2012_04_293

Prokaryotic expression and characteristics of duck enteritis virus UL29 gene

A. CHENG[1,2,3,§], S. ZHANG[1,3,§], X. ZHANG[1,3,§], M. WANG[1,2,3,*], D. ZHU[2,3], R. JIA[1,2,3], X. CHEN[2,3]

[1]Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Wenjiang, Chengdu city, Sichuan, P. R.China; [2]Avian Disease Research Center, College of Veterinary Medicine of Sichuan Agricultural University, Ya′an, Sichuan, P. R. China; [3]Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Wenjiang, Chengdu city, Sichuan, P. R. China

Received April 16, 2012; accepted October 29, 2012

Summary. – Duck enteritis virus (DEV) causes a contagious, acute and highly lethal disease in all ages of birds from the order *Anseriformes*. DEV leads to heavy economic losses to the commercial duck industry due to its high mortality rate and decrease in egg production. With development of molecular biology, more information about DEV genes is reported, nonetheless little information is known about DEV UL29 gene and its product major DNA-binding protein or infected-cell protein 8 (ICP8). The sequence characteristics of DEV UL29 gene was initially showed in our article. Phylogenetic tree analysis provided useful proof that DEV belongs to the subfamily *Alphaherpesvirinae*. The predicted characteristics of ICP8 amino acid sequence showed that ICP8 possesses good immunogenicity and is a hydrophobic protein. These findings correlate with the experimental results that ICP8 protein forms inclusion bodies in the prokaryotic expression system. By immunofluorescence we have identified ICP8 as nuclear protein. All the fundamental data in this article contribute to understanding the functions of DEV UL29 gene and its product ICP8.

Keywords: duck enteritis virus; UL29 gene; infected-cell protein 8

Introduction

Duck enteritis virus (DEV), which causes a contagious, acute and highly lethal disease in all ages of birds from the order *Anseriformes* (ducks, geese and swans), is one of the most destructive diseases to waterfowl in the world (Sandhu and Metwally, 2008; Montali *et al*., 1976; Proctor, 1976; Davison *et al*., 1993). This disease was firstly discovered in Netherlands in 1923 and in 1959 in China (Baudet, 1923; Huang *et al*., 1980). Tons of ducks are raised every year, in Sichuan province of China and many of them are infected with DEV. Due to its high mortality rate and decrease in duck egg production, DEV causes heavy economic losses in the commercial duck industry (Sandhu and Metwally, 2008).

DEV (the family *Herpesviridae*) and its virion is composed of linear, double-stranded DNA genome, the capsid with icosahedral symmetry and the envelope (Fauquet *et al*., 2005; Gardner *et al*., 1993). The research, in the last years, is mainly focused on the aspects of etiology, pathology, immunology, diagnostics, prevention and treatment. Many important achievements on these aspects were gained (Guo *et al*., 2009; Qi *et al*., 2008; Cheng *et al*., 2008). However, with the development of molecular biology, more information about DEV genes, like UL31, UL38, UL51, and UL53, are reported (Xiang *et al*., 2010; Xie *et al*., 2010; Shen *et al*., 2010; Zhang *et al*., 2010a). Only little information about DEV UL29 is known at present.

DEV UL29 gene encodes major DNA-binding protein or infected-cell protein 8 (ICP8). Analysis of the conserved domains by NCBI showed that DEV UL29 gene is a highly conserved gene among the family *Herpesviridae*. We assumed that DEV UL29 gene has the same characteristics

*Corresponding author. E-mail: mshwang@163.com; phone: +86-835-2885774. §Contributed equally to this work.

Abbreviations: DEV = duck enteritis virus; ICP8 = major DNA-binding protein or infected-cell protein 8; IPTG = isopropyl-β-D-thiogalactopyranoside; p.i. = post infection

as UL29 gene of other herpesviruses. For herpes simplex virus, ICP8, a 130 kDa polypeptide, is expressed as a β or delayed-early-gene product and one of seven virus-encoded proteins required for replication of the herpes simplex virus genome (Littler *et al*., 1983; Powell *et al*., 1981; Challberg, 1986; Conley *et al*., 1981). According to the previous research, ICP8 has abilities to bind DNA *in vitro* and *in vivo*, down-regulate the expression of viral genes from parental genomes, localize in the cell nucleus, stimulate late-gene expression from progeny templates, and promote organization of nuclear structures involved in viral and cellular DNA replication (Bayliss *et al*., 1975; Knipe and Spang, 1982; Gao and Knipe, 1991, 1992; Godowski and Knipe, 1986).

To date, there is little information reported on characteristics and functions of DEV UL29 gene. We have analyzed sequence characteristics by bioinformatics software, prepared two recombinant plasmids, used expression in prokaryotic system, produced polyclonal antibody of DEV UL29 gene and analyzed subcellular localization of DEV ICP8. All our data are useful to understand the characteristics and classification of DEV in the family *Herpesviridae* and functions of DEV ICP8.

Materials and Methods

DEV UL29 gene characterization by bioinformatics software. The conserved domains of DEV UL29 (GenBank Acc. No. EF643566) gene were analyzed through NCBI, and the UL29 gene reference sequences of other herpesviruses were searched by BLASTn (Table 1). All the reference nucleotide sequences were transformed into amino acid sequences by DNASTAR6.0 software (DNASTAR, Inc., USA), which were then used to construct the phylogenetic tree by DNASTAR6.0 and MEGA software (DNASTAR, Inc., USA). The antigenicity prediction of DEV ICP8 was analyzed by DNASTAR6.0, the hydrophobicity was predicted by ExPASy (http://cn.expasy.org/tools/protscale.html), the secondary structure of DEV ICP8 was analyzed by PHYRE (http://www.sbg.bio.ic.ac.uk/servers/phyre), the phosphorylation site prediction and glycosylation site prediction were analyzed by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc) websites. Eventually, the 3D structure prediction of DEV ICP8 was predicted by Protein data bank (http://www.pdb.org/pdb/) (Zhang *et al*., 2010a).

pMD18-UL29 plasmid construction. Set of primers was designed from DEV UL29 gene by Primer5.0 (PREMIER Biosoft, USA) and Oligo6.0 software (Molecular Biology Insights, Inc., USA). The forward primer (P1): 5′-GGATCCATGGAAGGCGCTGGGAAGA-3′ and the reverse primer (P2) 5′-CTCGAGTTAAAGCATATCAACAGAAAGAGCAGTT-3′ (TaKaRa, Japan) contained the *BamH*I and *Xho*I restriction sites (underlined). PCR products were purified by TIANgel Midi Purification Kit (TianGen, Germany). The purified products were then cloned into pMD18-T plasmid (TaKaRa, Japan) according to the manufacturer′s instructions of T4 DNA ligase (Invitrogen, USA). The recombinant plasmid was transformed into *Escherichia coli* DH5α competent cells (TaKaRa, Japan), and the positive recombinant plasmids pMD18-UL29 were selected on the ampicilline/isopropyl-β-D-thiogalactopyranoside (IPTG)/X-gal agar plate (Xie *et al*., 2010). The positive recombinant plasmids were verified by colony PCR, restriction digestion (*Bam*HI and *Xho*I) and sequencing.

*pET32-UL29 expression plasmid construction***.** The positive plasmid pMD18-UL29 and plasmid pET-32a (+) (Novagen, Germany) were digested by restriction enzymes (*Bam*HI and *Xho*I). The fragments were gel excised and ligated with DNA ligation kit 2.0 (TaKaRa, Japan) to construct the recombinant prokaryotic expression plasmid pET32-UL29. The positive expression plasmids were verified by colony PCR, restriction enzyme digestion and sequencing (Lian *et al*., 2010).

DEV ICP8 identification and its prokaryotic expression. The positive expression plasmid was transformed into *Escherichia coli* Rosetta (DE3) competent cells (Novagen, Germany). A single positive colony was inoculated into 6 ml of LB medium with ampicilline (100 μg/ml) and grew overnight. The culture was inoculated into fresh LB medium with ampicilline (100 μg/ml) with the ratio 1:100, and then cultivated at 37°C for 2 hrs. When the A_{600} was 0.6, culture was induced by 0.2 mmol/l of IPTG (Novagen) at 37°C for 4 hrs. As the negative controls we used the uninduced and induced culture with empty vector (pET32) and uninduced culture with recombinant plasmid. All the samples were boiled with 10×SDS buffer for 10 min and centrifuged at 12000 rpm for 10 min. With the purpose of analyzing the solubility of DEV ICP8, 200 ml of induced culture with pET32-UL29 was collected and centrifuged at 12000 rpm for 15 min. The pellets were resuspended in 20 mmol/l Tris-HCl buffer with 0.1 mg/ml lysozyme, frozen overnight at -20°C, and disrupted by ultrasonication. After this procedure, the supernatants and pellets were collected. Aliquot of sample was boiled with 10×SDS buffer for 10 min and separated by SDS-PAGE in order to analyze the expression and solubility of DEV ICP8.

The same samples were subjected to Western blot analysis with anti-DEV antibody, to prove that recombinant ICP8 is a viral protein. Western blot analysis was done according to standard procedures (Towbin *et al*., 1979). Briefly, the PVDF membrane was blocked with 10% skimmed milk and incubated with rabbit anti-DEV polyclonal antibody for 1 hr, while the negative control was incubated with negative rabbit serum. The PVDF membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen, USA) with dilution 1:5,000. After washing the PVDF membrane, the diamino benzidine was used for visualization.

Optimalization of bacterial induction. Many factors affect the prokaryotic expression, including temperatures, induction durations and IPTG concentrations (Zhang *et al*., 2010b). In order to optimize the prokaryotic expression, three experiments were carried out. First, for optimizing the temperature, the bacterial cultures were induced with 0.2 mmol/l of IPTG and cultivated 4

Table 1. DEV UL29 gene and 29 reference sequences of other herpesviruses

Abbreviation	Virus name	Genbank Acc. No.	Subfamily
DEV	Duck enteritis virus	EF643566	*not classified Alphaherpesvirinae*
CeHV-2	Cecropithecine herpesvirus 2	AY714813.1	*Alphaherpesvirinae*
CeHV-16	Cecropithecine herpesvirus16	NC_007653.1	*Alphaherpesvirinae*
MeHV-1	Meleagrid herpesvirus 1	AF282130.1	*Alphaherpesvirinae*
HHV-1	Human herpesvirus 1	NC_001806.1	*Alphaherpesvirinae*
HHV-2	Human herpesvirus 2	NC_001798.1	*Alphaherpesvirinae*
HHV-3	Human herpesvirus 3	DQ674250.1	*Alphaherpesvirinae*
BHV-1	Bovine herpesvirus 1	X94677.1	*Alphaherpesvirinae*
BHV-5	Bovine herpesvirus 5	NC_005261.2	*Alphaherpesvirinae*
EHV-1	Equine herpesvirus 1	AY464052.1	*Alphaherpesvirinae*
EHV-4	Equid herpesvirus 4	AF030027.1	*Alphaherpesvirinae*
PsHV-1	Psittacid herpesvirus 1	NC_005264.1	*Alphaherpesvirinae*
SHV-1	Suid herpesvirus 1	NC_006151.1	*Alphaherpesvirinae*
McHV-1	Macacine herpesvirus 1	NC_004812.1	*Alphaherpesvirinae*
GaHV-2	Gallid herpesvirus 2	EF523390.1	*Alphaherpesvirinae*
GaHV-3	Gallid herpesvirus 3	NC_002577.1	*Alphaherpesvirinae*
GaHV-1	Gallid herpesvirus 1	NC_006623.1	*Alphaherpesvirinae*
PaHV-2	Papiine herpesvirus 2	NC_007653.1	*Alphaherpesvirinae*
SaHV-1	Saimiriine herpesvirus 1	NC_014567.1	*Alphaherpesvirinae*
FHV-1	Felid herpesvirus 1	FJ478159.2	*Gammaherpesvirinae*
HHV-8	Human herpesvirus 8	Nc_009333.1	*Gammaherpesvirinae*
MHV-4	Murid herpesvirus 4	AF105037.1	*Gammaherpesvirinae*
HHV-4	Human herpesvirus 4	DQ279927.1	*Gammaherpesvirinae*
OvHV-2	Ovine herpesvirus 2	AY839756.1	*Betaherpesvirinae*
HHV-5	Human herpesvirus 5	NC_006273.2	*Betaherpesvirinae*
McHV-3	Macacine erpesvirus 3	NC_006150.1	*Betaherpesvirinae*
THV-1	Tupaiid herpesvirus 1	NC_002794.1	*Betaherpesvirinae*
HHV-7	Human herpesvirus 7	AF037218.1	*Betaherpesvirinae*
CavHV-2	Caviid herpesvirus 2	NC_011587.1	*Betaherpesvirinae*
PHV-2	Panine herpesvirus 2	NC_003521.1	

hrs at three different temperatures (23, 30, and 37°C). Second, in order to optimize the induction duration, the bacterial cultures were induced with 0.2 mmol/l of IPTG at the optimal temperature and cultivated in six different intervals (1, 2, 3, 4, 5, and 6 hrs). At last, the IPTG concentrations were also optimized under the optimal temperature and induction duration. Seven IPTG concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/l) were done. All the samples were collected and analyzed by SDS-PAGE.

DEV ICP8 purification and preparation of anti-ICP8 polyclonal antibody. In order to prepare the purified DEV ICP8, the fraction with inclusion bodies was collected and resuspended in buffer (4 mol/l urea, 50 mmol/l pH 8.0 Tris, 1 mmol/l EDTA, 150 mmol/l NaCl and 0.1% Triton X-100) and washed five times. The pellet was dissolved in 8 mol/l of urea. The urea was removed by the gradient dialysis. Finally, the initially purified recombinant His-tagged ICP8 was again purified by immobilized metal affinity chromatography on Ni^{2+}-NTA affinity resin (Cai *et al.*, 2009). To produce anti-DEV ICP8 polyclonal antibody, 6 healthy white rabbits were immunized intradermally with a 1.0 mg of ICP8 with an equal volume of complete Freund's adjuvant (Promega, USA). One week later, the rabbits were immunized intramuscularly with 0.5 mg of ICP8 with complete Freund′s adjuvant. This procedure was repeated in one-week interval. After the last immunization, two weeks later, the antiserum was collected from the carotid artery and stored at -70°C (Pan *et al.*, 2010). The antiserum was firstly extracted by saturated ammonium sulfate, and then purified by the DEAE-Sepharose column (Bio-Rad, USA) (Temponi *et al.*, 1989).

Intracellular localization of DEV ICP8. Duck embryo fibroblasts (DEF) were seeded in six-well plates with coverslips. The cells grew into monolayer and were infected with DEV. The coverslips were collected at different time points, such as 8, 24, 36, 48, and 54 hrs post infection (p.i.) and fixed with 4% paraformaldehyde at 4°C for 16 hrs. After washing three times, the cells were permeabilized with PBS with 2% Triton X-100. The cells were blocked with 5% BSA at 37°C for 1 hr. After washing three times with PBS, the cover slips were incubated with the purified rabbit anti-ICP8 polyclonal antibodies (1:200 dilution) at 37°C for 1 hr. The negative control was incubated with serum of the healthy white rabbit. The coverslips were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (1:100 dilution) (Sino-American Biotechnology Co., China) at 37°C for 1 hr. Eventually, 4, 6-Diamidino-2-phenylindole (DAPI, Sigma) staining was used to visualize the cell nuclei. Bio-Rad MRC 1024 imaging system was used for visualization (Shen *et al.*, 2009).

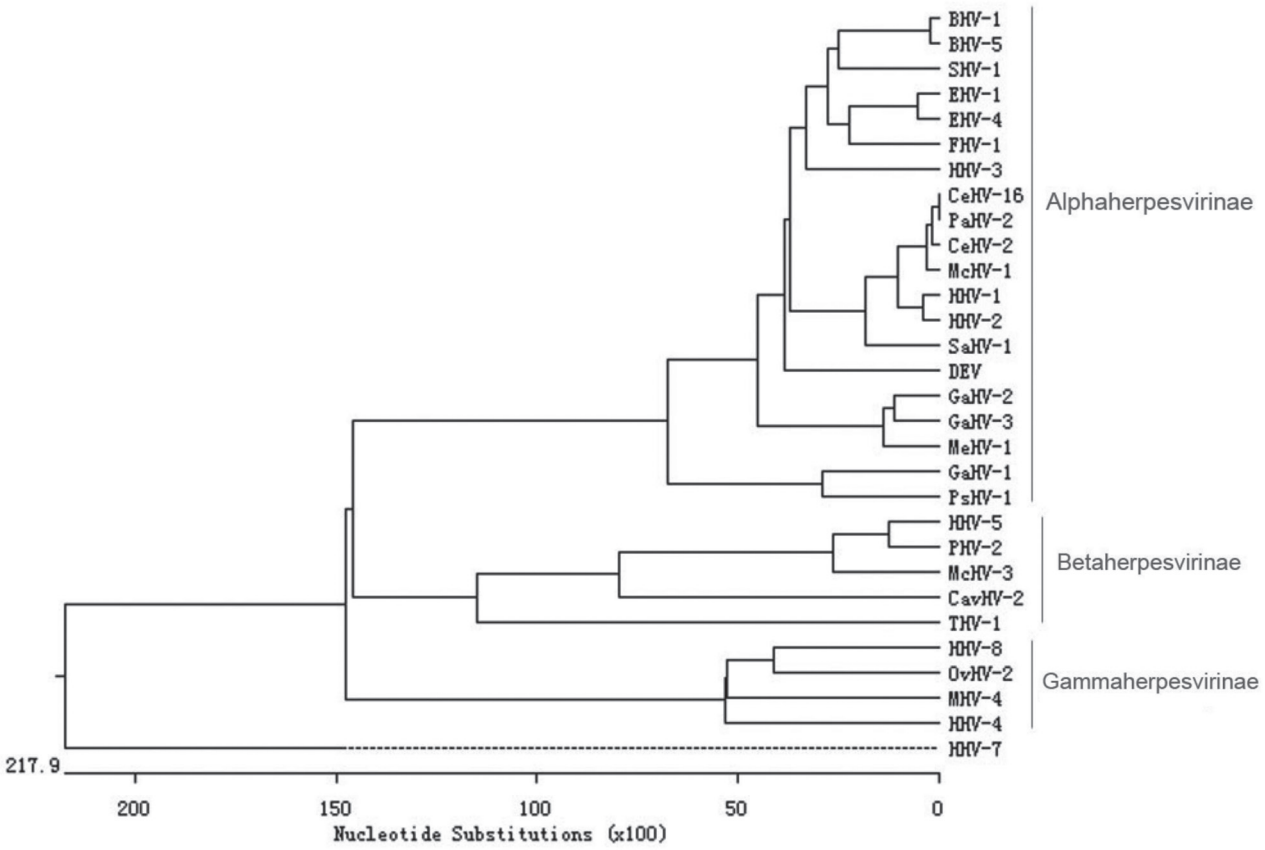

Fig. 1

Phylogenetic tree of the ICP8 amino acid sequence encoded by UL29 gene of DEV and other reference sequences

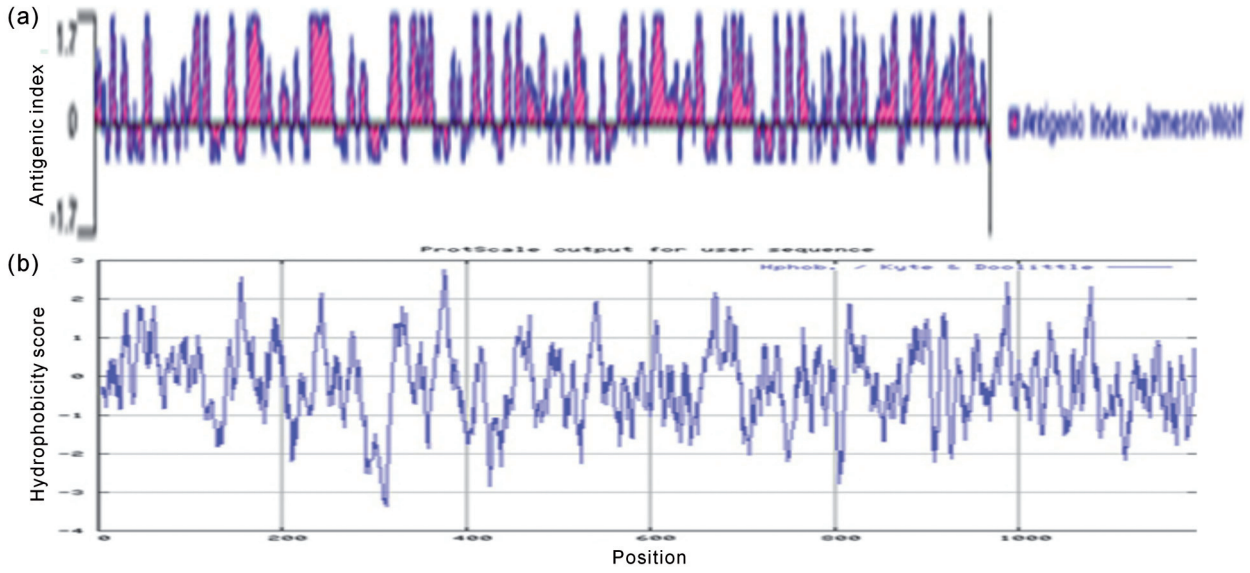

Fig. 2

Antigenicity and hydrophobicity prediction

Antigenicity (a) and hydrophobicity (b) prediction of the amino acid sequence encoded by DEV UL29 gene.

Results

Sequence characteristics of DEV UL29 gene analyzed by bioinformatics software

Analysis of NCBI conserved domains revealed that the amino acid sequence of DEV ICP8 is highly conservative. Twenty nine reference sequences of the UL29 gene (Table 1) were obtained from NCBI BLASTn. All the reference sequences were translated into amino acid sequences by DNASTAR6.0 software. They were used to construct phylogenetic tree by MEGA software. DEV ICP8 gene has highly genetic relationship with that of Gallid herpesvirus 2, Gallid herpesvirus 3, and Meleagrid herpesvirus 1, which are all members of the subfamily *Alphaherpesvirinae* (Fig. 1). DEV ICP8 has higher similarity with that of the subfamily *Alphaherpesvirinae*, especially with the bird herpesviruses, than that of the subfamily *Gammaherpesvirinae* or *Betaherpesvirinae*.

The antigenicity of DEV ICP8 was predicted by DNASTAR6.0 (Protean). The antigenicity of DEV ICP8 was uniformly distributed along the whole polypeptide chain of ICP8 (Fig. 2a), therefore the DEV ICP8 was presumed to have good antigenicity. The hydrophilicity analysis of DEV ICP8 showed that the hydrophobicity maximum of this protein is 2.756 and the minimum is -3.378 (Fig. 2b). The hydrophilic region was shorter than the hydrophobic region. Based on the above results, DEV ICP8 was predicted as a hydrophobic protein. The predicted result of secondary protein structure demonstrated that ICP8 has 38% of random coil, 49% of α-helix and 13% of β-strand structures.

The predicted results of the phosphorylation and N-glycosylation sites revealed 5 potential N-glycosylation sites (Fig. 3b) and 51 potential phosphorylation sites including

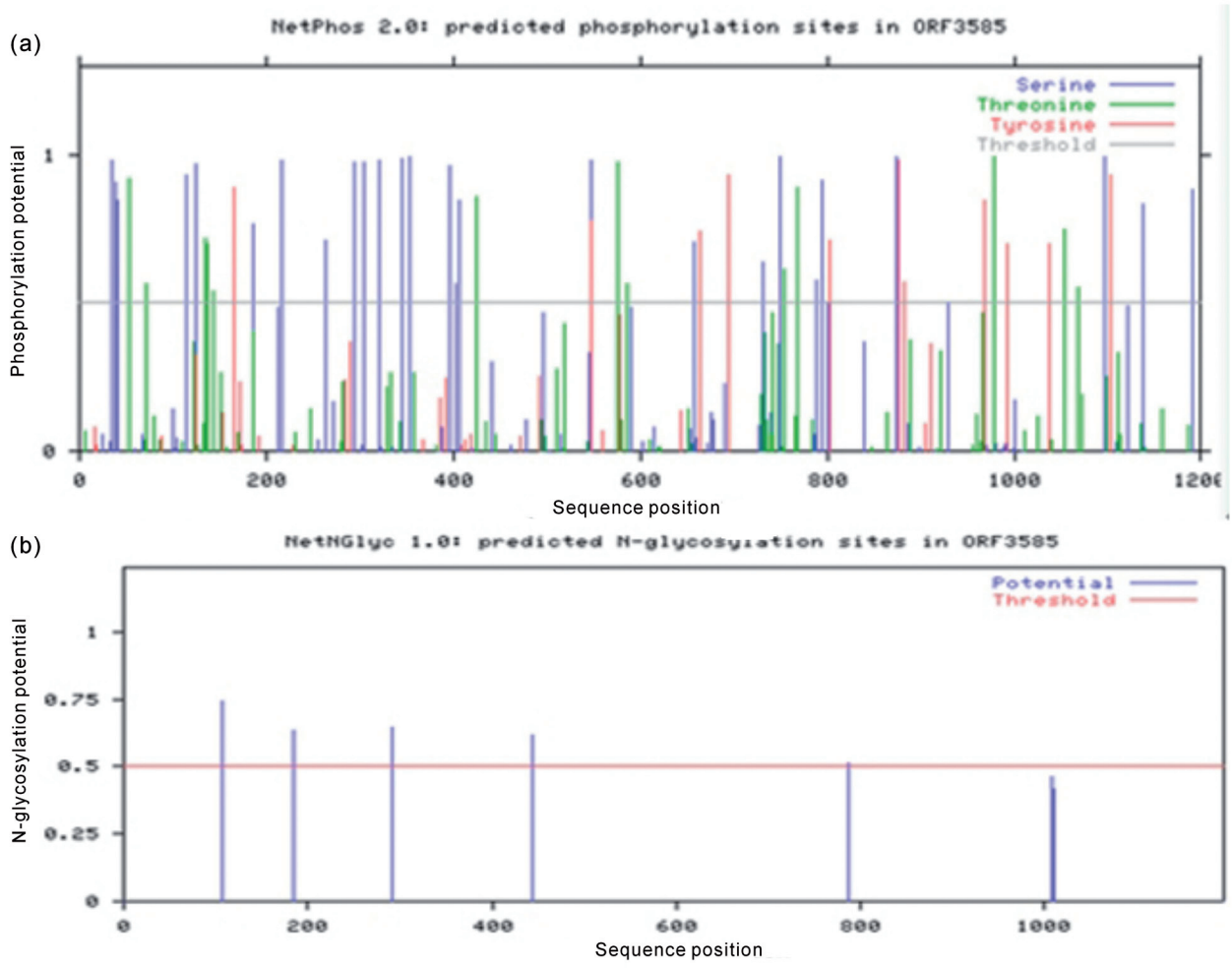

Fig. 3

Phosphorylation and N-glycosylation site prediction

Phosphorylation (a) and N-glycosylation (b) site prediction of the amino acid sequence encoded by DEV UL29 gene.

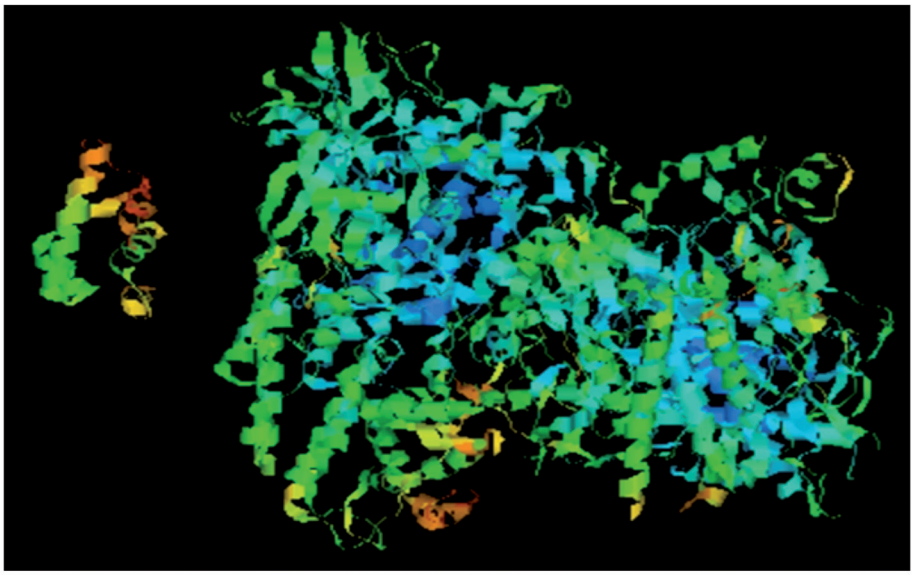

Fig. 4

3D structure prediction of the ICP8 protein encoded by DEV UL29 gene

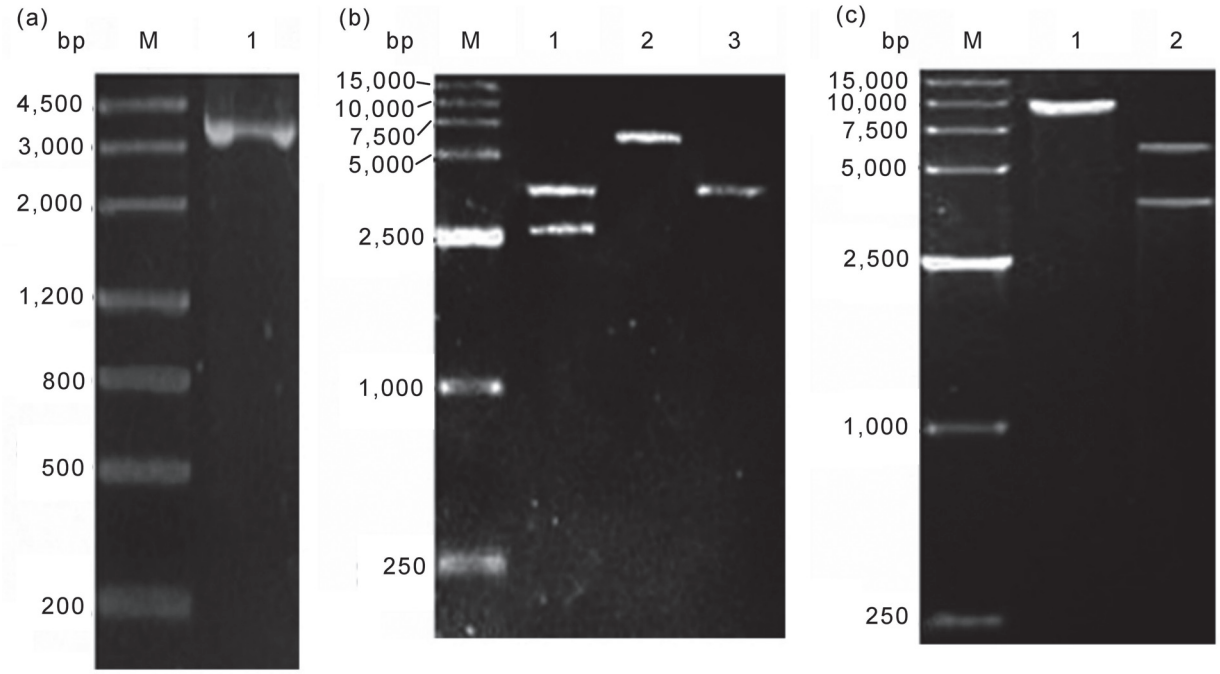

Fig. 5

Verification of the pMD18-UL29 plasmid and expression plasmid pET32-UL29

PCR amplification of DEV UL29 gene (a). Verification of the recombinant plasmid pMD18-UL29 (lane 2) by restriction analysis with *Bam*HI and *Xho*l (lane 1) and PCR amplification (lane 3) (b). Verification of the recombinant expression plasmid pET32-UL29 (lane 1) by restriction analysis with *Bam*HI and *Xho*I (lane 2) (c). M: DNA Marker III (a) or DNA marker-DL15000 (b, c).

27 Ser phosphorylation sites, 13 Thr phosphorylation sites and 11 Tyr phosphorylation sites (Fig. 3a). The potential N-glycosylation site was very important for protein characteristics such as antigenic determinants, electric charge property and heat stability. Therefore these predictions will provide useful information to understand the properties of

DEV ICP8. In order to better understand the structure and function of DEV ICP8, the homologous 3D structure was analyzed (Fig. 4).

Construction of the pMD18-UL29 plasmid and expression plasmid pET32-UL29

A pair of primers (P1 and P2) were designed to amplify DEV UL29 gene, and the amplified band correlated with the theoretical nucleic acid length of UL29 gene (3585 bp) (Fig. 5a). PCR product was after excision and purification ligated into pMD18-T vector by T4 DNA ligase. The recombinant clone plasmids were analyzed by colony PCR. The PCR results revealed a specific band of approximately 3600 bp, which is in correlation with the theoretical nucleic acid length of UL29 gene (Fig. 5b, lane 3). The plasmid pMD18-T/UL29 was verified by electrophoresis (6300 bp, Fig. 5b, lane 2) and *Bam*HI and *Xhol*I digestion. Enzyme digestion showed two bands of about 2700 bp and 3600 bp, which correlated with the nucleic acid length of pMD18-T vector and DEV UL29 gene (Fig. 5b, lane 1), respectively. The positive recombinant plasmids were verified by sequencing.

The recombinant plasmid pMD18-UL29 and pET-32a (+) expression vector were digested by *Bam*HI and *Xhol*I, and then ligated to construct the recombinant expression plasmid pET32-UL29. The recombinant expression plasmid was analyzed by electrophoresis (9500 bp, Fig. 5c, lane 1) and *Bam*HI and *Xhol*I digestion. After enzyme digestion with

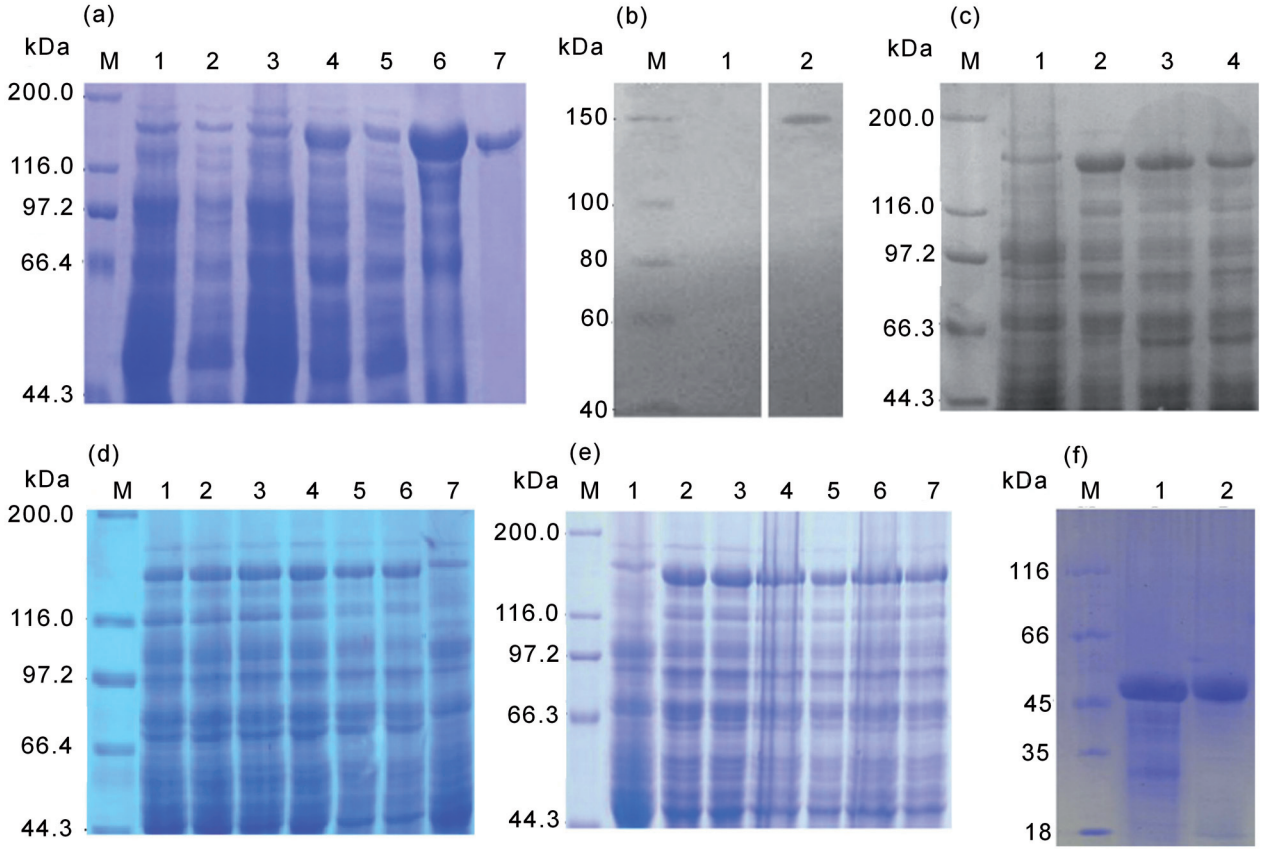

Fig. 6

Prokaryotic expression of DEV ICP8 and polyclonal anti-ICP8 antibody

Prokaryotic expression of the recombinant plasmid pET32-UL29 (a). Uninduced (lane 1) and induced (lane 2) culture transformed with pET32a (+). Uninduced (lane 3) and induced (lane 4) culture transformed with pET32-UL29. IPTG induced supernatant (lane 5) and inclusion bodies (lane 6) of recombinant protein. Purified UL29 protein (lane 7). Identification of the recombinant protein by Western blot analysis incubated with the rabbit pre-immune serum (lane 1) and with rabbit anti-DEV positive serum (lane 2) (b). Optimalization of induction temperature (c). Uninduced culture (lane 1) and culture induced at 23°C (lane 2), 30°C (lane 3), and 37°C (lane 4). Optimalization of induction duration (d). Uninduced culture (lane 7) and culture induced for 1–6 hrs (lanes 1–6). Optimalization of IPTG concentration (e). Cultures were induced by: 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/l of IPTG (lanes 1–7). The purified rabbit anti-ICP8 IgG analyzed by SDS-PAGE (f). The rabbit anti-ICP8 IgG was precipitated by saturated ammonium sulfate (lane 1) following by the DEAE-Sepharose column purification (lane 2). M: Protein molecular mass marker.

*Bam*HI and *Xhol*I, we obtained two bands of about 5900 bp and 3600 bp (Fig. 5c, lane 2), which were in correlation with the nucleic acid length of pET-32a (+) expression vector and DEV UL29 gene. Positive recombinant expression plasmids were verified by sequencing.

Prokaryotic expression of DEV ICP8

The positive recombinant expression plasmid pET32-UL29 was transformed into *E. coli* Rosetta (DE3). The bacteria transformed by pET32-UL29 were induced by IPTG and separated on SDS-PAGE gel, which revealed a specific band of about 148 kDa (Fig. 6a, lane 4). However, culture with plasmid pET32a (+) either induced or uninduced by IPTG, and uninduced culture with plasmid pET32-UL29, didn't show any specific band (Fig. 6a, lanes 1, 2, 3). The induced culture was collected and ultrasonicated. The DEV ICP8 was identified in insoluble protein fraction (Fig. 6a, lanes 5, 6), which showed that the ICP8 protein formed inclusion bodies in the prokaryotic expression system.

Western blot analysis was used to identify whether the expressed protein is one of the DEV proteins. The recombinant protein specifically reacted with rabbit anti-DEV positive serum and revealed a single and specific band in correlation with the theoretical molecular mass of 148 kDa of ICP8 (Fig. 6b, lane 2), in comparison to negative control incubated with rabbit pre-immune serum, where no band was visible (Fig. 6b, lane 1).

With the purpose of optimizing the expression conditions, such as induction temperature, induction duration and IPTG concentration, we estimated optimal temperature as 23°C (Fig. 6c), the induction duration as 3hrs (Fig. 6d), and the optimal IPTG concentration as 0.2 mmol/l (Fig. 6e).

Purification of DEV ICP8 and preparation of the purified anti-ICP8 polyclonal antibody

The recombinant His-tagged ICP8 was purified by immobilized metal affinity chromatography on Ni^{2+}-NTA affinity resin. The purified ICP8 protein was analyzed by SDS-PAGE where only one distinct band with molecular mass of 148 kDa was visible (Fig. 6a, lane 7). The purified recombinant His-tagged ICP8 was used to produce the polyclonal antibody. After collecting immune rabbit serum, the positive serum was purified with saturated ammonium sulfate (Fig. 6f, lane 1) following by purification with DEAE-Sepharose column (Fig. 6f, lane 2).

Intracellular localization of DEV ICP8

Compared to negative control with no specific fluorescence (Fig. 7, mock), the specific dispersed green fluorescence could be seen in precursor repetitive region of the nucleus 8 hrs p.i. ICP8 expression increased and proteins formed clusters and accumulated in nucleus or around nuclear membrane 24 and 36 hrs p.i. (Figs. 7 and 8). After 48 hrs p.i., the specific green fluorescence was found only in nucleus (Fig. 7, 48 hrs p.i.). After 54 hrs p.i. the specific green fluorescence decreased and dispersed, and the cells and nuclei began to collapse (Fig. 7, 54 hrs p.i.).

Discussion

Analysis of conserved domains of DEV UL29 gene revealed a conserved gene. The UL29 genes of other 29 reference herpesviruses were used to construct the phylogenetic tree. It classified DEV as a member of *Alphaherpesvirinae* subfamily, and DEV ICP8 as highly similar to ICP8 proteins of other members of this subfamily. Because of the high similarity, the characteristics and functions of other ICP8 proteins of the subfamily *Alphaherpesvirinae* could provide useful information for the research of DEV ICP8.

Bioinformatic analysis was very important to understand the immunogenic property of DEV ICP8. B-cell epitopes are specifically recognized by B-cell receptor or antibody. There are three characteristics of B-cell epitopes: localization at the surface of proteantigen, easy connection with B-cell antigen receptors or antibody and flexibility in binding to antigen receptor or antibody (Sun *et al.*, 2008). The proteantigen amino acid residues are hydrophilic and hydrophobic. The hydrophilic amino acids are located at the surface of proteantigen. The domains with hydrophilic amino acids, in which the surface charge polarity reached the maximum value, are always the sites such as the protein epitopes, antigen-antibody and ligand-acceptor sites (Zhang *et al.*, 2010b). The relationship between secondary structure and distribution of the epitopes was very close. Alpha-helix domains, which contain hydrogen bond to maintain the structure, do not change their conformation easily upon antibody binding. On the contrary the β-strands and random coil domains always located at the surface of protein, easily change their conformation upon antibody binding and have higher possibility to become epitopes (Zhang *et al.*, 2010c; Sun *et al.*, 2008).

By antigenicity prediction software we showed that ICP8 is a good antigen, because the epitopes are distributed in the whole amino acid sequence. The results of hydrophobicity analysis showed that the hydrophilic region was shorter than hydrophobic region. Based on this information and the fact that in prokaryotic expression system ICP8 formed inclusion bodies, we assumed that ICP8 really is hydrophobic protein. The predicted results of secondary structure showed that flexibility domains, the random coil (38%) and β-strand (13%), occupy 51% of the ICP8 protein. This makes ICP8 very flexible, so it can easily change its conformation. Thanks to those attributes, ICP8 has very good immunogenicity. The predicted results of glycosylation and phosphorylation sites

showed that the ICP8 has 5 potential N-glycosylation sites and 51 potential phosphorylation sites, what is very important for antigenic determination, protein electric charge and enzymatic properties. The predicted 3D structure of ICP8 showed high similarity with other ICP8 proteins.

In order to prepare the anti-ICP8 polyclonal antibody, the recombinant plasmid pMD18-UL29 and recombinant expression plasmid pET32-UL29 were constructed. Recombinant protein migrated as specific band of about 148 kDa, which was in correlation with the theoretical molecular mass of His-tagged ICP8. The ICP8 protein molecular mass was about 131.2 kDa and that of His-tagged protein was approximately 20 kDa. We had to optimize the expression conditions carefully, because the recombinant protein formed inclusion bodies. The optimal expression conditions were at 23°C for 3 hrs with 0.2 mmol/l of IPTG. Western blot analysis with rabbit anti-DEV IgG was used to identify ICP8 as a DEV protein. Twice purified recombinant protein possessing good antigenicity was used for immunizations. After immunization of the rabbits, immune sera were collected and purified by the DEAE-Sepharose column.

The intracellular localization of the protein was the basis for understanding the functions of the protein. In recent

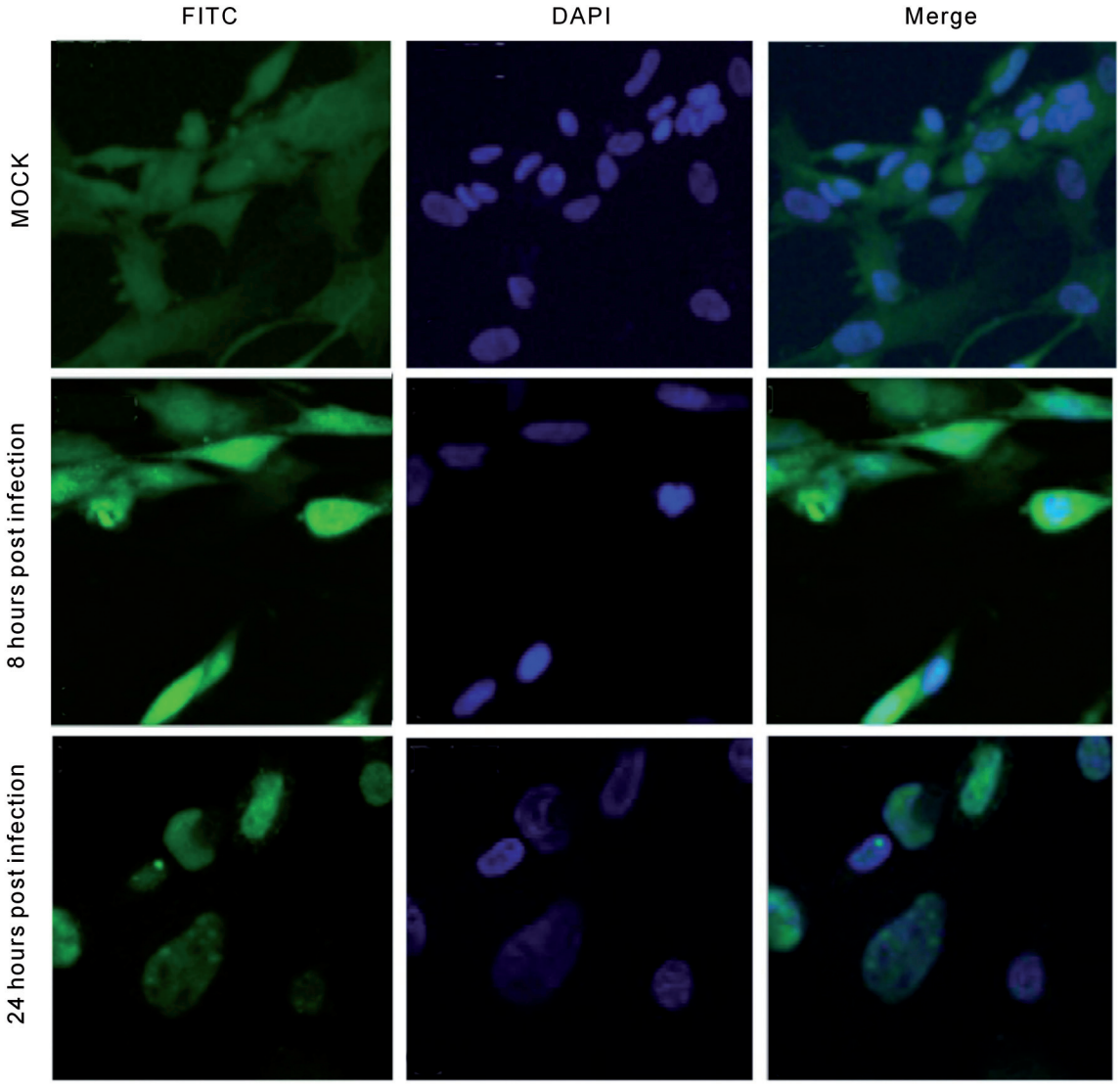

Fig. 7

Subcellular localization of DEV ICP8

Subcellular localization of DEV ICP8 at different time points after infection (8 and 24 hrs p.i.) stained with the rabbit anti-ICP8 polyclonal antibody, which was recognized by the goat anti-rabbit IgG/FITC, and DAPI for nucleus visualization, compared to uninfected cells (mock). Magnification 400×.

years, there were reports that the ICP8 encoded by herpes simplex virus localizes to the cell nucleus independently of other viral proteins (Gao and Knipe, 1992). The protein encoded by UL29 gene of the bovine herpesvirus 5 was localized at the cell nucleus (Delhon *et al.*, 2003).

In our study of subcellular localization of the DEV ICP8, we demonstrated the apparent punctiform fluorescence in cytoplasm 8 hrs p.i. as UL29 gene began to express. Later, the apparent punctiform fluorescence could be seen in the nucleus 24 and 36 hrs p.i., and then it dispersed in to the cytoplasm 54 hrs p.i. Changes in the subcellular localization may be due to the place of synthesis (cytoplasm) and place of function (nucleus). Nuclear localization demonstrates the function of ICP8 in DEV replication. This result was consistent with that ICP8 role in organizing DNA replication proteins in the cell nucleus. ICP8 may provide a paradigm of proteins that interact with the structural framework of the cell nucleus and play role in defining the intranuclear localization of DNA replication proteins (De and Knipe, 1988; Gao and Knipe, 1993).

This article provided some foundational and useful data to understand the functions of DEV ICP8.

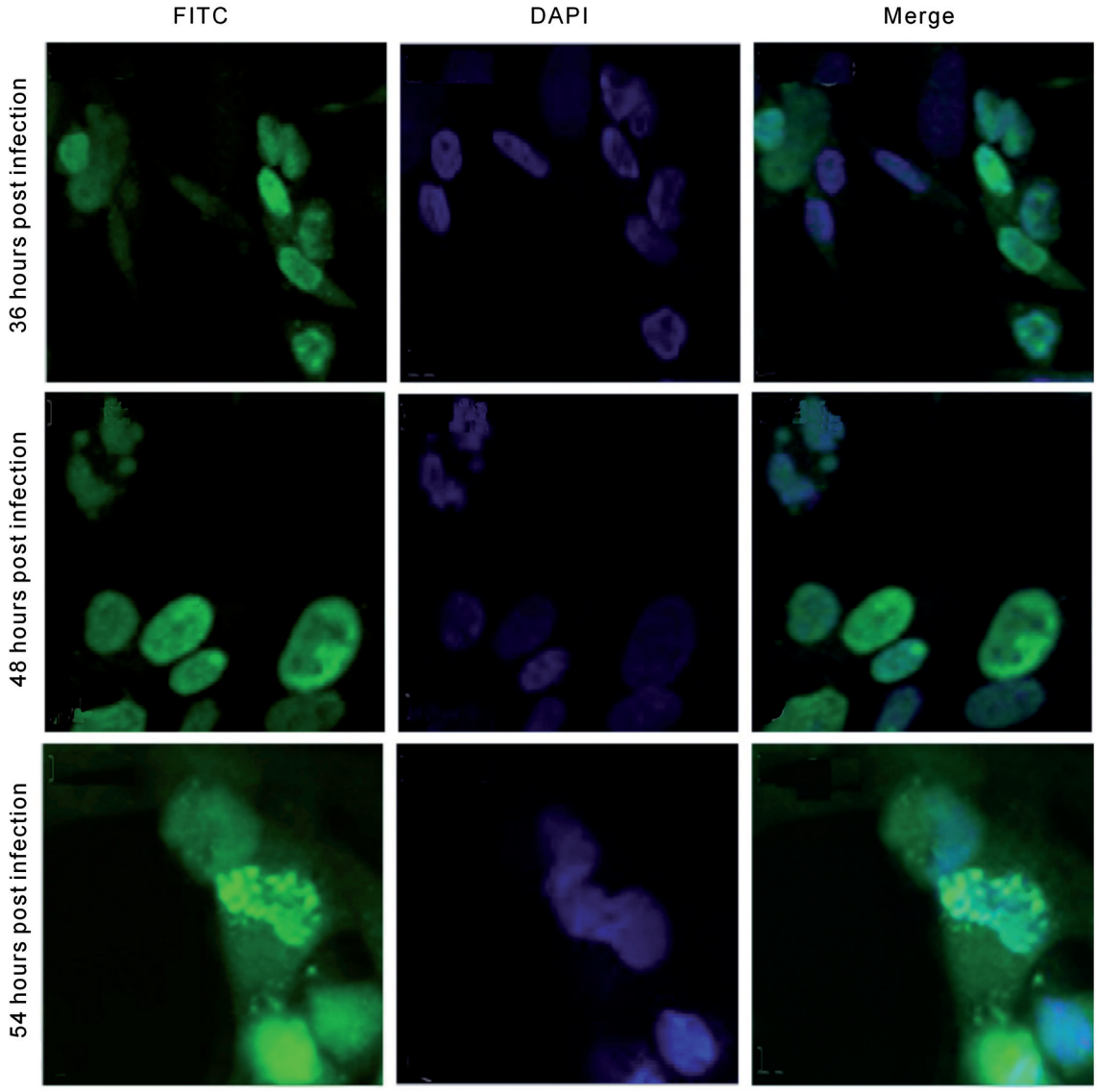

Fig. 8

Subcellular localization of DEV ICP8

Subcellular localization of DEV ICP8 at different time points after infection (36, 48, and 54 hrs p.i.) stained with the rabbit anti-ICP8 polyclonal antibody, which was recognized by the goat anti-rabbit IgG/FITC, and DAPI for nucleus visualization, compared to uninfected cells (mock). Magnification 400×.

Acknowledgement. The research was supported by Changjiang Scholars and Innovative Research Team in University (PCSIRT0848) and China Agricultural Research System (CARS-43-8).

References

Baudet AE (1923): Mortality in ducks in the Netherlands caused by a filterable virus: oteins in infected cells and in the virus structure. Virology 68, 124–134.

Bayliss GJ, Marsden HS, Hay J (1975): Herpes simplex virus proteins: DNA-binding proteins in infected cells and in the virus structure. Virology 68, 124–134. http://dx.doi.org/10.1016/0042-6822(75)90154-3

Cai MS, Cheng AC, Wang MS, Zhao LC, Zhu DK, Luo QH, Liu F, Chen XY (2009): His6-tagged UL35 protein of duck plague virus: expression, purification, and production of polyclonal antibody. Intervirology 52, 141–151. http://dx.doi.org/10.1159/000221833

Challberg MD (1986): A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA 83, 9094–9098. http://dx.doi.org/10.1073/pnas.83.23.9094

Cheng AC, Liao YH, Zhu DK, Ming SW, Luo QH, Jia RY, Chen XY(2008): Development and application of indirect in situ polymerase chain reaction for detecting duck enteritis virus in paraffin sections. Sci. Agric. Sinica 41, 4365–4371 (in Chinese).

Conley AJ, Knipe DM, Jones PC, Roizman B (1981): Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of T polypeptides. J. Virol. 37, 191–206.

Davison S, Converse KA, Hamir AN, Eckroade RJ (1993): Duck viral enteritis in Muscovy ducks in Pennsylvania. Avian Dis. 37, 1142–1146. http://dx.doi.org/10.2307/1591927

De Bruyn Kops A, Knipe DM (1988): Formation of DNA replication structures in herpes virus-infected cells requires viral DNA binding protein. Cell 55, 857–868. http://dx.doi.org/10.1016/0092-8674(88)90141-9

Delhon G, Moraes MP, Liu Z, Afonso CL, Flores EF, Weiblen R, Kutish GF, Rock DL (2003): Genome of bovine herpesvirus 5. J. Virol. 77, 10339–10347. http://dx.doi.org/10.1128/JVI.77.19.10339-10347.2003

Fauquet CM, Mayo MA, Maniloff J (2005): Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, California, pp.2482–2487.

Gao M, Knipe DM (1991): Potential role for herpes simplex virus DNA replication protein in stimulation of late gene expression. J. Virol. 65, 2666–2675.

Gao M, Knipe DM (1993): Intragenic complementation of herpes simplex virus ICP8 DNA-binding protein mutants. J. Virol. 67, 876–885.

Gardner R, Wilkerson J, Johnson JC (1993): Molecular characterization of the DNA of Anatid herpesvirus 1. Intervirology 36, 99–112.

Godowski PJ, Knipe DM (1986): Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA 83, 256–260. http://dx.doi.org/10.1073/pnas.83.2.256

Guo YF, Shen CJ, Cheng AC, Wang MS, Chen S, Zhang N, Zhou Y(2009): Anatid herpesvirus 1 virulent strain induces syncytium and apoptosis in duck embryo fibroblast cultures. Vet. Microbiol. 138, 258–265. http://dx.doi.org/10.1016/j.vetmic.2009.04.006

Huang YX, Ou SS, Qi RL(1980): Investigation on duck plague virus. J. South China Agric. Univ. 1, 21–26 (in Chinese).

Knipe DM, Spang AE (1982): Definition of a series of stages in the association of two herpesvirus proteins with the cell nucleus. J. Virol. 43, 314–324.

Lian B, Xu C, Cheng AC, Wang MS, Zhu DK, Luo QH, Jia RY, Bi FJ, Chen ZL, Zhou Y, Yang ZX, Chen XY (2010): Identification and characterization of duck plague virus glycoprotein C gene and gene product. Virol. J. 7, 349. http://dx.doi.org/10.1186/1743-422X-7-349

Littler E, Purifoy D, Minson A, Powell KL (1983): Herpes simplex virus nonstructural proteins. III. Function of the major DNA-binding protein. J. Gen. Virol. 64, 983–995. http://dx.doi.org/10.1099/0022-1317-64-5-983

Montali RJ, Bush M, Greenwell GA (1976): An epornitic of duck viral enteritis in a zoological park. J. Am. Vet. Med. Assoc. 169, 954–958.

Pan WW, Ren XM, Guo H, Ding Q, Zheng AC (2010): Expression, purification of herpes simplex virus type 1 UL4 protein, and production and characterization of UL4 polyclonal antibody. J. Virol. Methods 163, 465–469. http://dx.doi.org/10.1016/j.jviromet.2009.11.015

Powell KL, Littler E, Purifoy DJ(1981): Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. 39, 894–902.

Proctor SJ (1976): Pathogenesis of duck plague in the bursa of Fabricius, thymus, and spleen. Am. J. Vet. Res. 37, 427–431.

Qi XF, Yang XY, Cheng AC, Wang MS, Zhu DK, Jia RY (2008): Quantitative analysis of virulent duck enteritis virus loads in experimentally infected ducklings. Avian Dis. 52, 338–344. http://dx.doi.org/10.1637/8120-100207-ResNote.1

Sandhu TS, Metwally SA (2008): Duck virus enteritis (Duck Plague). In Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE (Ed.): Diseases of Poultry. 2th ed. Oxford, Blackwell Publishing, pp. 384–393.

Shen CJ, Cheng AC, Wang MS, Sun KF, Jia RY, Sun T, Zhang N, Zhu DK, Luo QH, Zhou Y, Chen XY (2010): Development and evaluation of an immunochromatographic strip test based on the recombinant UL51 protein for detecting antibody against duck enteritis virus. Virol. J. 7, 268. http://dx.doi.org/10.1186/1743-422X-7-268

Shen CJ, Guo YF, Cheng AC, Wang MS, Zhou Y, Lin D, Xin HY, Zhang N (2009): Characterization of subcellular localization of duck enteritis virus UL51 protein. Virol. J. 6, 92. http://dx.doi.org/10.1186/1743-422X-6-92

Sun T, Cheng AC, Wang MS, Guo YF, Jia RY, Shen CJ (2008): Prediction of epitopes on B-cell of UL6 gene of duck en-

teritis virus and prokaryotic expression of major antigen determinant sequence. Chinese Vet. Sci. 38, 939–945 (in Chinese).

Temponi M, Kageshita T, Perosa F, Ono R, Okada H, Ferrone S (1989): Purification of murine IgG monoclonal antibodies by precipitation with caprylic acid: comparison with other methods of purification. Hybridoma 8, 85–95. http://dx.doi.org/10.1089/hyb.1989.8.85

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354. http://dx.doi.org/10.1073/pnas.76.9.4350

Xiang J, Ma GP, Zhang SC, Cheng AC, Wang M,S Zhu DK, Jia RY, Luo QH, Chen ZL, Chen XY (2010): Expression and intracellular localization of duck enteritis virus pUL38 protein. Virol. J. 7,162. http://dx.doi.org/10.1186/1743-422X-7-162

Xie W, Cheng AC, Wang MS, Chang H, Zhu DK, Luo QH (2010): Molecular cloning and characterization of the UL31 gene from duck enteritis virus. Mol. Biol. Rep. 37, 1495–1503. http://dx.doi.org/10.1007/s11033-009-9546-y

Zhang SC, Cheng AC, Wang MS, Xiang J, Zhu DK, Jia RY, Luo QH, Cui HM, Zhou Y, Wang Y, Xu ZW, Chen ZL, Chen XY, Wang XY (2010a): Bioinformatics Analysis and characteristics of glycoprotein K encoded by the newly identified UL53 gene of duck enteritis virus. IEEE, The 4th International Conference on Bioinformatics and Biomedical Engineering (Icbbe2010), Chengdu, China, June 18–20.

Zhang SC, Ma GP, Xiang J, Cheng AC, Wang MS, Zhu DK, Jia RY, Luo QH, Chen ZL, Chen XY (2010b): Expressing gK gene of duck enteritis virus guided by bioinformatics and its applied prospect in diagnosis. Virol. J. 7, 168. http://dx.doi.org/10.1186/1743-422X-7-168