Prokaryotic expression and characteristics of duck enteritis virus UL29 gene

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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...
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; Chalberg, 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’-CTCGAGTTAAAGCATCGGAGGCAGTT-3’ (TaKaRa, Japan) contained the BamHI and XhoI 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 ampicillin/isooseryl-β-D-thiogalactopyranoside (IPTG)/X-gal agar plate (Xie et al., 2010). The positive recombinant plasmids were verified by colony PCR, restriction digestion (BamHI and XhoI) and sequencing.

**pET32-UL29 expression plasmid construction.** The positive plasmid pMD18-UL29 and plasmid pET-32a (+) (Novagen, Germany) were digested by restriction enzymes (BamHI and XhoI). The fragments were gel excised and ligated with DNA ligation kit (TaKaRa, Japan), and the positive recombinant plasmids 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 ampicillin (100 μg/ml) and grew overnight. The culture was inoculated into fresh LB medium with ampicillin (100 μg/ml) with the ratio 1:100, and then cultivated at 37°C for 2 hrs. When the A600 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.

**Optimization 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
with complete Freund’s adjuvant. This procedure was repeated in the rabbits were immunized intramuscularly with 0.5 mg of ICP8 of complete Freund’s adjuvant (Promega, USA). One week later, nized intradermally with a 1.0 mg of ICP8 with an equal volume ICP8 polyclonal antibody, 6 healthy white rabbits were immu-

The initially purified recombinant His-tagged ICP8 was again purified by immobilized metal affinity chromatography dialysis. Finally, the initially purified recombinant His-tagged ICP8 dissolved in 8 mol/l of urea. The urea was removed by the gradient NaCl and 0.1% Triton X-100) and washed five times. The pellet was (4 mol/l urea, 50 mmol/l pH 8.0 Tris, 1 mmol/l EDTA, 150 mmol/l Ni2+-NTA affinity resin (Cai et al, 2009). To produce anti-DEV ICIP8 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 Ni2+-NTA affinity resin (Cai et al, 2009). To produce anti-DEV ICIP8 polyclonal antibody, 6 healthy white rabbits were immu-

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Virus name</th>
<th>Genbank Acc. No.</th>
<th>Subfamily</th>
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<td>not classified</td>
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<td>NC_003521.1</td>
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</table>

hs 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 Ni2+-NTA affinity resin (Cai et al, 2009). To produce anti-DEV ICIP8 polyclonal antibody, 6 healthy white rabbits were immunized intraderrmally 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 coverslips 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...

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**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 BamHI and XhoI (lane 1) and PCR amplification (lane 3) (b). Verification of the recombinant expression plasmid pET32-UL29 (lane 1) by restriction analysis with BamHI and XhoI (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 BamHI and XhoII 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 BamHI and XhoII, 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 BamHI and XhoII digestion. After enzyme digestion with

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**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). Optimization 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). Optimization of induction duration (d). Uninduced culture (lane 7) and culture induced for 1–6 hrs (lanes 1–6). Optimization 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.
BamHI and XhoI, 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 by 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 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 such as induction temperature, induction duration and IPTG concentration, we estimated optimal temperature as 23°C such as induction temperature, induction duration and IPTG concentration, we estimated optimal temperature as 23°C.

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 Ni²⁺-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 are very 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).

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.

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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 are very useful information for the research of DEV ICP8.
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

![Subcellular localization of DEV ICP8](image)

**Fig. 7**

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.
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References


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