Molecular cloning and characterization of the pseudorabies virus US1 gene

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ABSTRACT. Using polymerase chain reaction, a 1050-bp sequence of the US1 gene was amplified from the pseudorabies virus (PRV) Becker strain genome; identification of the US1 gene was confirmed by further cloning and sequencing. Bioinformatics analysis indicated that the PRV US1 gene encodes a putative polypeptide with 349 amino acids. The encoded protein, designated PICP22, had a conserved Herpes_IE68 domain, which was found to be closely related with the herpes virus immediate early regulatory protein family and is highly conserved among the counterparts encoded by Herpes_IE68 genes. Multiple nucleic acid sequence and amino acid sequence alignments suggested that the product of PRV US1 has a relatively higher homology with ICP22-like proteins of genus Varicellovirus than with those of other genera of Alphaherpesvirinae. In addition, phylogenetic analysis showed that PRV US1 has a close evolutionary relationship with members of the genus Varicellovirus, especially Equid herpes virus 1 (EHV-1), EHV-4 and EHV-9. Antigen prediction indicated that several potential B-cell epitopes are located in PICP22. Also, subcellular localization analysis demonstrated that PICP22 is predominantly located in the cytoplasm,
suggesting that it might function as a cytoplasmic-targeted protein.

**Key words:** Pseudorabies virus; US1; ICP22; Cloning; Bioinformatics; Molecular characterization

**INTRODUCTION**

Aujeszky’s disease, which is caused by pseudorabies virus (PRV), is a frequently fatal disease with a global distribution that primarily affects swine and other domestic and wild animals incidentally. PRV belongs to the genus Varicellovirus, subfamily Alphaherpesvirinae, which is a swine alphaherpesvirus closely related to the human pathogen herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) (Kramer et al., 2011). The neurotropic nature of PRV makes it a useful tracer of neuronal pathways and it is a useful model for the study of herpesvirus pathogenesis. PRV has caused great economic losses in the pig industry worldwide, while efforts to eradicate PRV have shown great progress, it remains an endemic problem in many countries (Kramer et al., 2011).

To understand the fundamental mechanisms underlying PRV spread and pathogenesis, it is particularly important to have a comprehensive understanding of the function of each gene in the course of viral replication. During the lytic cycle of PRV infection, viral genes are expressed in a cascade of 3 temporally distinct and functionally interdependent phases termed immediate-early (IE), early (E), and late (L). Although several PRV genes, including the unique IE gene IE180 (Tomioka et al., 2008), 3 E genes EP0 (Brukman and Enquist, 2006), UL54 (Li et al., 2011a,b), and UL23 (Ferrari et al., 2000), and many L genes such as capsid genes UL25 (Coller et al., 2007) and UL35 (Krautwald et al., 2008), tegument genes UL36, UL37 (Luxton et al., 2006), and UL41 (Lin et al., 2010), and envelope genes UL27 (Nixdorf et al., 2001a), UL44 (Kramer et al., 2011), US8, and UL10 (Nixdorf et al., 2001b), have been extensively studied, the function of US1 and its protein product ICP22 (PICP22) is less well understood.

In this study, the US1 gene was amplified from the PRV Becker genome using polymerase chain reaction (PCR) followed by cloning and sequencing. A comprehensive bioinformatics analysis was conducted to study the molecular characteristics of US1 and to provide molecular biological insight for future study on the function and mechanism of PICP22 during PRV infection. The analysis included a large number of bioinformatics tools such as open reading frame (ORF) Finder, Conserved Domains, DNAstar 7.0, Bioedit 7.0, SignalP-4.0, NetPhos 2.0, PSIPred, CPHmodels 3.2, and PSORT II.

**MATERIAL AND METHODS**

**Cloning and sequencing of PRV US1**

PCR amplification primers for US1 (accession No. JF797219) were designed using Oligo 6.0 and Primer 5.0; reactions were performed with TaKaRa reagents. The upstream primer 5’-TTGAAATTCATGGACGGGACGGCCTG-3’ anneals to the first 20 nucleotides of US1 and introduces an EcoRI restriction site (underlined) for cloning. The downstream primer 5’-AAGTCGAGTCACGGGGGCGGGC-3’ is complementary to the final 18 nucleotides of US1 and introduced an XhoI restriction site (underlined).
The US1 gene was PCR-amplified from the genomic DNA of PRV Becker, previously purified from vBecker2-infected PK-15 cells (Smith and Enquist, 2000; Li et al., 2011a,b). PCR profiles involved initial predenaturation for 5 min at 95°C followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 65°C for 40 s, and extension at 72°C for 1 min 15 s. The final extension step was performed at 72°C for 10 min. Purified PCR product was digested with EcoRI and XhoI and ligated into pre-digested prokaryotic expression vector pET28a(+) (Novagen) to generate pET28a(+)–US1. Presence of the appropriate insert was verified by PCR, restriction analysis, and sequencing.

Bioinformatics analysis of the nucleotide sequence of PRV US1

To determine nucleotide sequence similarity and identify the ORF, we used NCBI BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Subsequently, Clustal V in the MegAlign program of DNASTar (version 7.0, DNASTar, Inc.) was used to analyze nucleotide sequence homology of 19 ICP22-like proteins of alphaherpesvirus (listed in Table 1).

Bioinformatics analysis of the deduced amino acid sequence of PRV US1

For amino acid (aa) sequence comparison, homology search, and conserved domain analysis, the aa sequence of PICP22 was analyzed using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) and the Conserved Domains search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To compare PICP22 with ICP22-like proteins of other alphaherpesviruses (listed in Table 1), aa sequence homology and phylogenetic relationships were performed in DNASTar 7.0. To predict the signal peptide sequence, transmembrane domain, glycosylation site, phosphorylation site, and hydrophobic and hydrophilic regions, B-cell epitope, secondary structure, 3-dimensional (3-D) structure, and subcellular localization of PICP22, SignalP-4.0 Server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), Bioedit 7.0, DNAstar 7.0, PSIPred (http://bioinf.cs.ucl.ac.uk/psipred/), CPHmodels 3.2 (http://www.cbs.dtu.dk/services/CPHmodels/), and PSORT (http://psort.nibb.ac.jp/) were used.

RESULTS

PCR amplification and cloning of PRV US1

The US1 gene was PCR-amplified from the purified genome of PRV Becker. As shown in Figure 1, no specific band was amplified from the mock-infected control (Figure 1, lane 1), whereas a target fragment of 1050 bp, consistent with the expected size, was amplified from DNA purified from PRV-infected PK-15 cells (Figure 1, lane 2). The DNA fragment was cloned into prokaryotic expression vector pET28a(+) to yield pET28a(+)–US1 (Figure 1, lane 3), which was confirmed by restriction analysis (Figure 1, lanes 4 and 5), PCR amplification (Figure 1, lane 6), and DNA sequencing (Figure 2A).
Figure 1. PCR amplification and restriction enzyme analysis of the recombinant plasmid pET28a(+)–US1. Lanes 1 and 2 = PCR amplification product of the US1 gene using DNA purified from mock- and PRV-infected PK-15 cells as the template, respectively; lane 3 = the recombinant plasmid pET28a(+)–US1; lane 4 = restriction enzyme digestion product of pET28a(+)–US1 with EcoRI and SalI; lane 5 = restriction enzyme digestion product of pET28a(+)–US1 with EcoRI and XhoI; lane 6 = PCR amplification product of the US1 gene from pET28a(+)–US1. Samples were electrophoresed through a 1% agarose gel and stained with ethidium bromide. The electrophoresis migration of molecular mass marker (lane M, TaKaRa) was also shown.

Figure 2. Multiple nucleic acid sequence alignment of the US1 gene of the PRV Becker strain with other PRV strains and its homologous genes of 18 different selected species. A. Multiple nucleic acid sequence alignment of the US1 gene of the PRV Becker strain (JF797219) with Kaplan (JF797218) and Bartha strains (JF797217) using Clone Manager (version 8). B. Multiple nucleic acid sequence alignment of the US1 gene of the PRV Becker strain with its homologous genes of 18 selected species (Table 1) by using the MEGALIGN program in LASERGENE (DNAStar 7.0) with the Clustal V method, and sequence distance was calculated using weight matrix Identity. Gaps had been introduced by the alignment program to maximize the homology.
Molecular characterization of PRV US1

Bioinformatics analysis of the PRV US1 nucleotide sequence

ORF Finder analysis revealed an integrated PRV US1 ORF consisting of 1050 bp. Nucleotide sequence similarity search by NCBI BLASTN yielded 2 nucleotide sequences (accession Nos. JF797218 and JF797217) with strong similarity to PRV Becker US1 (similarity up to 92 and 89%, respectively) (Figure 2A); these accessions correspond to the US1 gene of the PRV Kaplan and Bartha strains. Multiple alignment of PRV US1 with 18 homologous reference alphaherpesviruses (Table 1) revealed remarkably high homology of 32.1 to 44.7% with the members of genus Varicellovirus, subfamily Alphaherpesvirinae, i.e., Human herpesvirus 3 (HHV-3, VZV), Cercopithecine herpesvirus 9 (CeHV-9), Bovine herpesvirus 5 (BoHV-5), Equine herpesvirus 4 (EHV-4), BoHV-1, EHV-9, and EHV-1. However, low homology was detected between PRV and other viruses of the subfamily Alphaherpesvirinae (Figure 2B).

<table>
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Table 1. Abbreviations and accession numbers of 19 US1 gene encoding proteins from different species.
Bioinformatics analysis of the PICP22 polypeptide sequence

An aa sequence similarity search in NCBI BLASTP yielded 2 aa sequences (accession Nos. AEM64036 and AEM63971) that strongly matched the target sequence of PRV Becker ICP22 (similarity up to 92 and 88%, respectively); these correspond to the ICP22 protein of the PRV Kaplan and Bartha strains, respectively (Figure 3A). Multiple sequence alignment of PICP22 with its homologs in 18 reference alphaherpesviruses (Table 1) showed relatively high homology of 30.3 to 38.8% between PICP22 and its BoHV-1, EHV-4, EHV-9, and EHV-1 counterparts. However, PICP22 shared no substantial homology with ICP22-like proteins from HHV-2 (HSV-2), HHV-1 (HSV-1), CeHV-16, CeHV-1, or CeHV-2 (16.9 to 17.8%; Figure 3B). Conserved domain analysis indicated that PICP22 contains a conserved domain of Herpes_IE68 (Figure 3C), which is an immediate early protein. Further searches revealed 13 conserved domains (data not shown), all similar to Herpes_IE68. Thus, PICP22 is closely related to the immediate early protein family and is similar to its counterparts encoded by Herpes_IE68 genes. Therefore, PICP22 might belong to the Herpes_IE68 family.

Figure 3. Conserved domain analysis and multiple aa sequence alignment of the PICP22 of PRV Becker strain with other PRV strains and its homologous proteins of 18 different selected species. A. Multiple aa sequence alignment of PICP22 of the PRV Becker strain (AEM64105) with Kaplan (AEM64036) and Bartha strains (AEM63971) using Clone Manger (version 8). B. Multiple aa sequence alignment of PICP22 of the PRV Becker strain with its homologous genes of 18 selected species (Table 1) by using the MEGALIGN program in LASERGENE (DNAStar 7.0) with the Clustal V method, and sequence distance was calculated using weight matrix PAM250. Gaps had been introduced by the alignment program to maximize the homology. C. Conserved domain analysis of PRV ICP22 by using NCBI Conserved Domains search tool.

Phylogenetic analysis of PRV and other herpesviruses was performed based on the sequences of PICP22 and the ICP22-like proteins of 18 reference alphaherpesviruses (Table 1). The proteins could be preliminarily separated into different genera, i.e., Simplexvirus, Varicellovirus, Mardivirus, and Iltovirus. This was consistent with the existing classification of subfamily Alphaherpesvirinae (Figure 4). Furthermore, PICP22 clustered with EHV-4, EHV-9, and EHV-1 first, and then clustered with other varicelloviruses, including CeHV-9, HHV-3, BoHV-1, and BoHV-5 (Figure 4). Therefore, PRV might have a closer evolutionary relationship with the members of genus Varicellovirus than other genera of Alphaherpesvirinae.

Signal polypeptide prediction indicated no cleavage site (Figure 5A) and no transmembrane domain in PICP22 (Figure 5B). N-linked glycosylation site (Asn-X-Ser/Thr) prediction demonstrated no N-glycosylation site (Figure 5C), which confirmed the absence of a signal peptide in PICP22. Interestingly, there were 22 potential phosphorylation sites in PICP22 (Figure 5D), including 15 serine, 5 threonine, and 2 tyrosine residues. Hydrophobicity analysis revealed 6 hydrophobic regions located at aa 73-80, 97-112, 120-125, 138-148, 168-182, and 198-205 (Figure 6A). The hydrophilic region was larger than the hydrophobic region (Figure 6B), suggesting that PICP22 might be a hydrophilic protein.
Figure 5. The prediction of signal peptide sequence, transmembrane domain, glycosylation site, and phosphorylation site of PICP22. Signal peptide sequence, transmembrane domain, glycosylation site and phosphorylation site of PICP22 were analyzed by SignalP-4.0 Server (A), the TMHMM program (B), the NetNGlyc 1.0 program (C), and the NetPhos 2.0 program (D), respectively.
Several potential B-cell epitopes were identified in PICP22, located in or adjacent to aa 1-8, 12-81, 109-122, 129-138, 145-198, and 202-347 (Figure 7A). Secondary structure analysis (Figure 7B) suggests that PICP22 is primarily consisted of random coils (up to 87.39%) and α-helices (12.03%); no 3-D structure model for PICP22 was found. A coiled-coil motif was predicted at 213-240 aa. Subcellular localization analysis demonstrated that PICP22 predominantly localizes to the cytoplasm (47.8%) and nucleus (26.1%). However, only a small fraction localized to the vacuoles (8.7%), mitochondria (8.7%), cytoskeleton (4.3%), and peroxisomes (4.3%). PICP22 is therefore predominantly located in the cytoplasm and functions as a cytoplasmic-targeted protein.

Figure 6. Hydrophobicity and hydrophilicity analyses of PICP22 by using BioEdit 7.0. The hydrophobicity (A) or hydrophilicity profile (B) was determined with the values of Kyte and Doolittle (1982) or Hopp and Woods (1981) by using a 13-amino acid window, respectively. The upward-pointing peaks represent the most hydrophobic regions (A) and the most hydrophilic regions (B), respectively.
DISCUSSION

PCR amplification, cloning, and sequencing verified the presence of the US1 gene in PRV Becker. Bioinformatics analysis indicated that its encoded protein PICP22 is a member of the Herpes IE68 family. HSV-1 ICP22 and VZV ORF63, homologs of PICP22, have been well documented; US1 likely acts as a real IE gene encoding for an IE protein in these cases.

Figure 7. Antigenic analysis and secondary structure prediction of PICP22. A. Antigenic analysis of PICP22 was carried out by the PROTEAN software of DNAStar based on its flexibility, surface probability and antigenic index by the determination of its primary structure. B. Secondary structure was predicted by using the PSIPred program and the letters h, e, and c indicate alpha helix, extended (beta strand) and coil, respectively.
Molecular characterization of PRV US1

However, PRV US1 is not expressed as an IE gene (Zhang and Leader, 1990). Important for HSV-1 replication, ICP22 is a multifunctional protein with different roles during infection, such as inducing the formation of discrete nuclear foci containing cellular chaperone proteins known as VICE domains (Bastian et al., 2010), ensuring proper virion morphology (Orlando et al., 2006), and modulating viral and cellular gene expression (Advani et al., 2003; Bowman et al., 2009; Kalamvoki and Roizman, 2011), which are also the main functions of VZV ORF63 (Kost et al., 1995; Jones and Arvin, 2005). VZV ORF63 is also critical for efficient establishment of latency (Ambagala and Cohen, 2007). Therefore, the important roles played by HSV-1 ICP22 and VZV ORF63 in the process of infection suggest that PICP22 may play a similar role in the course of infection. However, it is not yet known which real biological functions PICP22 has in the herpesvirus life cycle and the examination of these aspects must await further study of its functions in viral replication and interactions between herpesvirus and host.

Subcellular protein localization is closely related to its functional role. Specific aa sequences determine whether a protein will span the cell membrane and enter the cell, fuse to the intramembrane, or be exported from the cell. It appears to be a universal phenomenon that almost every protein is marked with a localization signal (Feng, 2002). Subcellular localization research of HSV-1 ICP22 suggests that in an HSV-1-infected cell, ICP22 predominantly locates in the nucleus, particularly in the small, dense nuclear bodies early in infection and in the diffuse replicative complexes after onset of DNA synthesis (Jahedi et al., 1999; Xing et al., 2011). PICP22 was predicted to be localized in the cytoplasm where it might function as a cytoplasmic-targeted protein, although it does contain a putative nuclear localization signal (NLS, 47PPKRGRY53). Although VZV ORF63 contains a functional NLS, it accumulates exclusively in the cytoplasm of latently infected sensory neurons; it shifts to nuclear and cytoplasmic localization following reactivation from latency (Walters et al., 2008). Therefore, PICP22 might have the same subcellular localization pattern as VZV ORF63, since PICP22 was more similar to VZV ORF63 than to HSV-1 ICP22. However, this inference has not been verified.

Protein phosphorylation is one of the most normal and essential types of protein modification; certain aspects of cell process modulation are regulated by protein phosphorylation. Signal transduction, proliferation, differentiation, and metabolism are all controlled by the balance of activity of protein kinases and protein phosphatases upon pivotal target proteins. Phosphorylation site prediction revealed 22 potential phosphorylation sites in PICP22, including 2 potential tyrosine phosphorylation sites. Tyrosine phosphorylation is involved in the replication of several herpesviruses (Geiss et al., 2001; Ren et al., 2001) and in shifting protein translocation from the cytoplasm to the nucleus during productive virus infection (Pomeranz and Blaho, 1999). Phosphorylation of VZV ORF63 is associated with its subcellular localization and transcriptional regulatory properties (Habran et al., 2005; Mueller et al., 2010), and phosphorylation of ICP22 is involved in HSV-1 virulence (Brandt and Kolb, 2003). Therefore, PICP22 phosphorylation may also play an important role during PRV infection, perhaps in modulating its subcellular localization or other uncharacterized functions, such as transcriptional regulation.

Nevertheless, hydrophobicity analysis indicated no strong hydrophobic region, although there were 6 hydrophobic regions principally situated in the intermediate region of the polypeptide chain. Sequence analysis showed no potential transmembrane domain, signal peptide, or N-linked glycosylation site (Asn-X-Ser/Thr), which may provide some clues regarding its expression. Secondary structure prediction (Figure 7) revealed that PICP22 pre-
dominantly contained random coils and α-helices. PICP22 also contained a coiled-coil motif, which is conserved in alphaherpesviruses (Pelletier et al., 1997; Mason and Arndt, 2004; Barbara et al., 2007) and mediates crucial protein-protein interactions, such as transcriptional control. Therefore, PICP22 might be a transcriptional factor that interacts with important host and virus proteins via the coiled-coil domain.

As more information becomes available on protein antigens, it should be possible to use this information to predict the locations of antigenic determinants prior to immunological testing. However, the elucidation of protein antigenic structures is presently a difficult, uncertain, and time-consuming task. Earlier methods were based on the assumption that the antigenic region is primarily the hydrophilic region at the surface of the protein molecule (Hopp and Woods, 1981; Welling et al., 1985). However, this assumption is limited in its accuracy. To improve accuracy, the B-cell epitopes of PICP22 were predicted using DNAStar PROTEAN programs based on flexibility, antigenic index and surface probability based on the primary structure (Figure 7A). These findings of the antigenic and structural properties of PICP22 may yield methods for developing new antibodies and immunoassays for clinical diagnosis of PRV.

In the course of evolution, viruses are generally conserved and only a few genes undergo mutation (Antunes et al., 2010). Thus, viral evolution can be discussed at the molecular level. Our analysis revealed that the nucleotide sequence similarity of US1 of PRV Becker to Kaplan and Bartha was 92 and 89% (Figures 2A and 3A); the aa sequence similarities were up to 92 and 88%, respectively, consistent with a previous report (Szpara et al., 2011). Meanwhile, it may be that this evolutionary difference produces the variable virulence of distinct PRV strains. Multiple nucleotide and aa sequence alignments of PICP22 and the ICP22-like proteins showed that PICP22 has greater homology to members of Varicellovirus, especially EHV-1 (44.7 and 38.8%, respectively). Therefore, PICP22 has relatively high homology to Varicellovirus, but not Simplexvirus, Mardivirus, or Iltovirus. Phylogenetic analysis (Figure 4) unequivocally demonstrated that PRV belongs to the genus Varicellovirus of subfamily Alphaherpesvirinae, consistent with a previous report (McGeoch et al., 2000). Moreover, PRV shares a closer evolutionary relationship with members of genus Varicellovirus than with other genera of Alphaherpesvirinae. Although the US1 gene products are conserved within the Alphaherpesvirinae subfamily, they are not conserved in other herpesvirus subfamilies, including Betaherpesvirinae and Gammaherpesvirinae, suggesting that the ICP22-like protein may execute a function that is discrete or complemented by other proteins from these subfamilies.

In conclusion, we presented the cloning and molecular characterization of the PRV US1 gene. Elucidating the molecular characterization and genetic evolution of PRV US1 will contribute to our understanding of this virus at the molecular level and will enrich the herpesvirus database. These studies will also provide insights for further research into the function and mechanism of US1 during PRV infection.

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Molecular characterization of PRV US1

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