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Molecular cloning and characterization of Pseudorabies virus *EP0* gene

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The pseudorabies virus (PRV) early protein EP0 is a homologue of the herpes simplex virus 1 (HSV-1) immediate-early protein ICP0, which is a multifunctional protein and important for HSV-1 infection. However, the exact function of EP0 is not clear. In this study, using polymerase chain reaction, a 1,104 base-pair sequence of the *EP0* gene was amplified from the PRV Becker strain genome and identification of the *EP0* gene was confirmed by further cloning and sequencing. Bioinformatics analysis indicated that the PRV *EP0* gene encoded a putative polypeptide with 367 amino acids. The encoded protein, designated as EP0 contained a conserved RING-finger superfamily domain and was found to be closely related with the herpes virus RING-finger superfamily and was highly conserved among the counterparts encoded by RING-finger genes. Multiple nucleic acid sequence and amino-acid sequence alignments suggested that PRV EP0 showed a relatively higher similarity with EP0-like proteins of genus *Varicellovirus* than with those of other genera of *Alphaherpesvirinae*. In addition, phylogenetic analysis showed that PRV EP0 had a close evolutionary relationship with members of genus *Varicellovirus*, especially bovine herpesvirus 1 (BoHV-1) and BoHV-5. Antigen prediction indicated that several potential B-cell epitopes were located in EP0. Also, subcellular localization analysis demonstrated that EP0 was predominantly localized in the nucleus, suggesting that it might function as a nuclear-targeted protein.

Keywords: Pseudorabies virus, EP0, Cloning, Bioinformatics, Molecular characterization

Aujeszky's disease caused by pseudorabies virus (PRV) is a fatal disease with a global distribution that primarily affects swine and other domestic and wild animals. PRV belongs to the genus *Varicellovirus* and subfamily *Alphaherpesvirinae* and is a swine alphaherpesvirus closely related to the human pathogens herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV)[1]. It is a useful model organism for studies of the molecular pathomechanism of herpes viruses and is also a powerful tool in neural circuit tract tracing-studies. PRV has caused great economic losses worldwide among the pig industry, although efforts to eradicate PRV have shown great progress, it remains an endemic problem in many countries[1].

To understand the fundamental mechanisms underlying PRV spread and pathogenesis, it is important to have a comprehensive understanding of the function of each gene in the course of viral replication. During the productive infection, viral gene expression of PRV can be divided into a cascade of 3 temporally distinct and functionally interdependent classes termed as immediate-early (IE), early (E) and late (L). The IE genes are expressed first, independently of de novo protein synthesis from the virus. The products of these genes are regulatory proteins that control viral gene expression through complex mechanisms. Although several PRV genes, including the unique IE gene *IE180*[2], E gene *UL54*[3,4] and L gene, such as tegument gene *US1*[5,6] have been

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Abbreviations: aa, amino acid; BEHV, bovine encephalitis herpesvirus; BoHV-1, bovine herpesvirus 1; BoHV-5, bovine herpesvirus 5; CaHV-1, canid herpesvirus 1; CeHV-1, cercopithecine herpesvirus 1; CeHV-2, cercopithecine herpesvirus 2; CeHV-9, cercopithecine herpesvirus 9; CeHV-16, cercopithecine herpesvirus 16; 3D, 3-dimensional; E, early; EAV, equine abortion virus; EHV-1, equid herpesvirus 1; EHV-4, equine herpesvirus 4; EHV-8, equid herpesvirus 8; EHV-9, equid herpesvirus 9; ERV, equine rhinopneumonitis virus; FeHV-1, felid herpesvirus 1; GHV-1, gazelle herpesvirus 1; GaHV-2, gallid herpesvirus 2; HHV-1, human herpesvirus 1; HHV-2, human herpesvirus 2; HHV-3, human herpesvirus 3; HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus 2; HVP-2, herpesvirus papio 2; IBRV, infectious bovine rhinotracheitis virus; IE, immediate-early; L, late; LHV-4, leporid herpesvirus 4; MarHV, marmoset herpesvirus; McHV-1, macacine herpesvirus 1; MDV-1, marek's disease virus type 1; ORF, open-reading-frame; PaHV-1, papiine herpesvirus 2; PCR, polymerase chain reaction; PRV, pseudorabies virus; SA8, simian agent 8; SaHV-1, saimiriine herpesvirus 1; SuHV-1, suid herpesvirus 1; SVV, simian varicella virus; VZV, varicella-zoster virus.

extensively studied, the function of *EP0* and its protein product EP0 is less well understood.

In this study, we have amplified the *EP0* gene from the PRV Becker genome using polymerase chain reaction (PCR), followed by its cloning and sequencing. A comprehensive bioinformatics analysis has been conducted using a large number of bioinformatics tools to study the molecular characteristics of *EP0* and to provide molecular biological insight for future study on the function and mechanism of EP0 during PRV infection.

Materials and Methods

Cloning and sequencing of PRV *EP0*

PCR amplification primers for *EP0* (Accession no. JF797219, contains a *Bam*HI restriction enzyme site) were designed using Oligo 6.0 and Primer 5.0 and reactions were performed with TaKaRa reagents. The upstream primer 5′-CGAAGCTTATGGACTGCCC CATCTGCCTG anneals to the first 21 nucleotides of *EP0* and introduces a *Hin*dIII restriction site (underlined) for cloning. The downstream primer 5′-GAAGATCTGAGTCGTCGTCCTGGGTGAGGTC is complementary to the final 23 nucleotides of *EP0* and introduces a *Bgl*II restriction site (underlined).

The *EP0* gene was amplified via PCR by KOD-Plus-Neo (TOYOBO), from the genomic DNA of PRV Becker, previously purified from vBecker2 infected PK-15 cells[3,4,7]. PCR profiles involved initial predenaturation for 5 min at 95°C, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 66°C for 30 s and extension at 72°C for 1 min 10 s. The final extension step was performed at 72°C for 10 min. Purified PCR product was digested with *Hin*dIII and *Bam*HI (isocaudarner of *Bgl*II) and ligated into pre-digested green fluorescent protein variant mammalian expression vector pEYFP-C1 (Clontech) to generate pEYFP-C1-EP0. Presence of the appropriate insert was verified by PCR, restriction analysis and sequencing.

Bioinformatics analysis of nucleotide sequence of PRV *EP0*

To determine nucleotide sequence similarity and identify the open-reading-frame (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 EP0-like proteins of alphaherpesvirus (Table 1).

Bioinformatics analysis of deduced amino acid sequence of PRV *EP0*

For amino acid (aa) sequence comparison, similarity search and conserved domain analysis, the aa sequences of EP0 and 24 EP0-like proteins were analyzed using BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/)[8] and the conserved domains search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[9]. To compare EP0 with EP0-like proteins of other alphaherpesviruses (Table 1), aa sequence similarity and phylogenetic relationships were performed in DNAstar 7.0.

To predict the signal peptide sequence, transmembrane domain, glycosylation site, phosphorylation site, hydrophobic and hydrophilic regions, B-cell epitope, secondary structure, three-dimensional (3D) structure and subcellular localization of EP0 or the EP0-like proteins, SignalP-4.0 Server (http://www.cbs.dtu.dk/services/SignalP/)[10,11], TMHMM (http://www.cbs.dtu.dk/services/TMHMM/)[12], NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/)[13], Bioedit 7.0[14,15], DNAstar 7.0[16,17], PSIpred (http://bioinf.cs.ucl.ac.uk/psipred/)[18], CPHmodels 3.2 (http://www.cbs.dtu.dk/services/CPHmodels/)[19] and PSORT (http://psort.nibb.ac.jp/) were used.

Results and Discussion

PCR amplification and cloning of PRV *EP0*

In an effort to study the nuclear import and export signals and subcellular transport mechanism of PRV EP0, which might be important for further investigating the biological functions of EP0 in the course of PRV infection, a recombinant expression plasmid pEYFP-C1-EP0 that drives the expression of EP0 protein fused to the carboxyl terminal of EYFP, was constructed. The *EP0* gene was PCR-amplified from the purified genome of PRV Becker. As shown in Fig. 1, no specific band was amplified from the mock-infected control (Fig. 1, lane 1), whereas a target fragment of about 1,104 bp, consistent with the expected size was amplified from DNA purified from PRV-infected PK-15 cells (Fig. 1, lane 2). Then, the DNA fragment was cloned into mammalian expression vector pEYFP-C1 to yield pEYFP-C1-EP0 (Fig. 1, lane 3), which was confirmed by restriction analysis (Fig. 1, lane 4), PCR amplification (Fig. 1, lane 5) and DNA sequencing (Fig. 2a).

Bioinformatics analysis of PRV *EP0* nucleotide sequence

In the course of evolution, viruses are generally conserved and only a few genes undergo mutation[20].

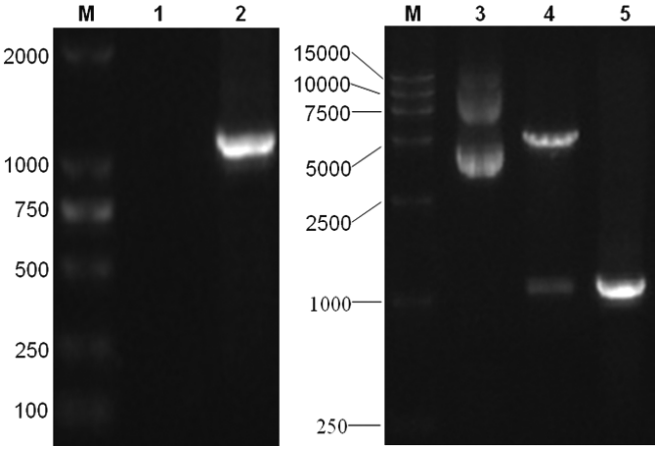

Fig. 1—PCR amplification and restriction enzyme analysis of the recombinant plasmid pEYFP-C1-EP0 [Lane 1 and 2, PCR amplification product of *EP0* gene using DNA purified from mock- and PRV-infected PK-15 cells as the template, respectively; lane 3, recombinant plasmid pEYFP-C1-EP0; lane 4, restriction enzyme digestion products (1,064 and 4,746 bp, respectively) of pEYFP-C1-EP0 with *Hin*dIII and *Bam*HI; and lane 5, PCR amplification product of *EP0* gene from pEYFP-C1-EP0. Samples were electrophoresed through a 1% agarose gel and stained with ethidium bromide. The electrophoresis migration of molecular mass marker (M, TaKaRa) is also shown]

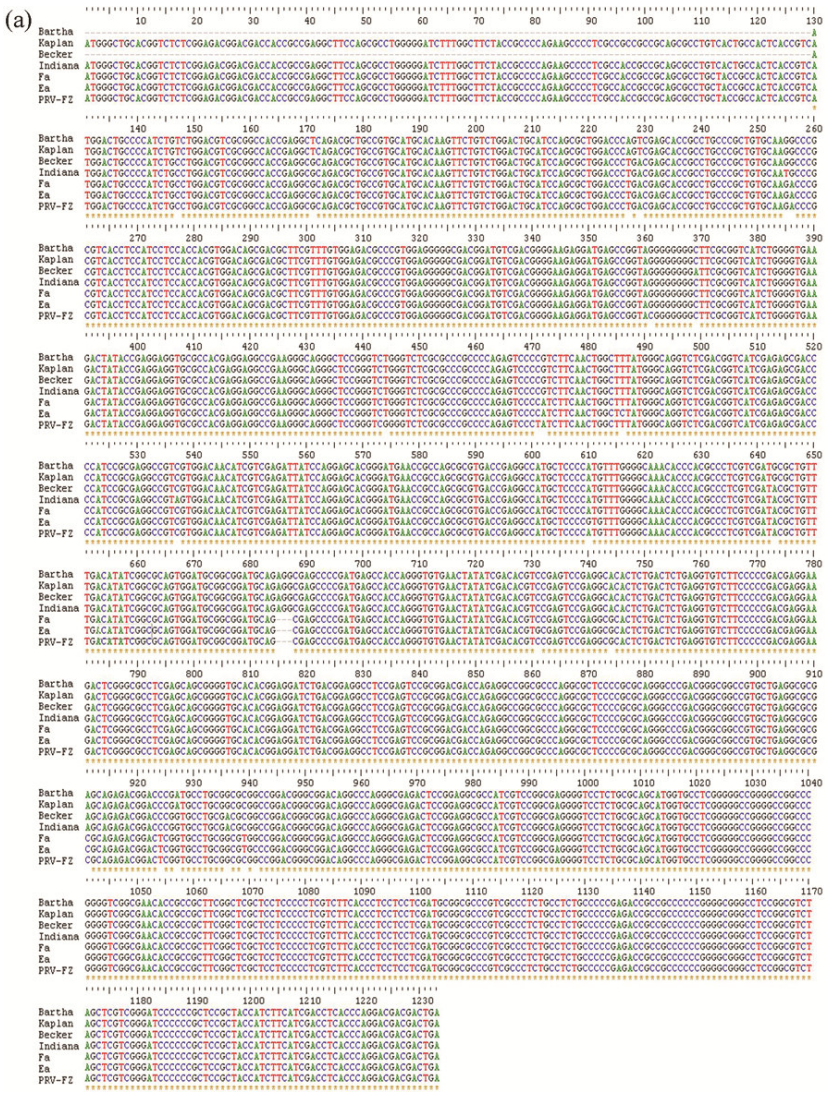

Fig. 2a—Multiple nucleic acid sequence alignment of *EP0* gene of PRV Becker strain with other PRV strains and its homologous genes of 18 different selected species [Multiple nucleic acid sequence alignment of *EP0* gene of PRV Becker strain (JF797219) with Bartha (JF797217), Ea (AF298586), Fa (EU333164), PRV-FZ (FJ477294), Kaplan (JQ809328) and Indiana-Funkhauser (M57504) strains using Clustal X (version 1.8) and Bioedit 7.0. Asterisks (*) indicate positions that have a single, fully conserved residue between the 7 EP0 proteins from different PRV strains]

(b)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		35.6	35.7	22.1	28.5	31.6	24.3	28.8	29.3	28.1	29.0	30.2	23.0	29.6	28.9	28.6	22.6	29.3	24.2	1	SuHV-1 Becker
2	41.1		72.7	18.3	26.2	27.0	21.1	27.4	23.6	22.5	24.8	25.4	21.6	31.0	25.8	30.4	20.9	29.5	22.2	2	BoHV-1
3	39.6	16.5		19.2	28.3	28.4	22.4	28.6	25.3	22.9	24.5	25.2	21.4	29.0	26.0	30.0	21.5	28.6	23.1	3	BoHV-5
4	350.0	350.0	350.0		13.2	12.8	24.3	14.5	24.9	27.2	24.4	25.2	33.4	15.1	16.4	13.1	24.5	14.2	19.5	4	CaHV-1
5	91.7	88.5	84.1	350.0		63.4	18.6	64.2	22.5	20.2	23.4	22.6	19.2	32.0	44.8	44.5	19.0	47.3	28.7	5	CeHV-1
6	87.4	84.7	82.3	350.0	21.0		20.1	73.6	23.1	20.5	21.6	22.3	17.6	30.8	39.3	45.3	19.8	46.4	25.8	6	CeHV-2
7	193.3	288.2	390.6	184.9	232.9	181.8		17.9	21.6	21.8	20.7	21.0	22.9	21.4	21.5	18.7	38.7	19.5	22.2	7	CeHV-9
8	88.3	82.7	82.0	350.0	22.8	13.3	192.8		23.3	20.6	22.5	23.4	18.5	31.7	42.6	45.3	18.9	40.2	26.8	8	CeHV-16
9	124.2	121.0	117.8	135.5	119.0	118.3	162.1	115.5		59.6	87.1	89.7	24.4	26.0	21.3	20.4	21.5	23.2	22.6	9	EHV-1
10	129.5	137.5	149.0	132.0	156.3	164.4	157.7	156.6	39.6		59.1	59.7	26.4	20.8	17.8	19.9	21.9	18.2	21.1	10	EHV-4
11	122.2	124.9	128.0	139.3	126.0	120.7	156.1	119.8	10.9	38.8		88.8	26.6	25.0	20.9	22.2	22.2	22.6	23.8	11	EHV-8
12	124.3	119.7	119.6	135.6	115.0	113.9	155.3	112.9	8.4	40.4	8.4		25.3	27.5	21.6	23.6	21.7	22.5	23.9	12	EHV-9
13	175.6	166.9	177.4	59.2	205.4	204.9	171.0	213.0	107.2	108.2	109.3	105.7		20.9	16.5	18.6	21.3	18.8	19.4	13	FeHV-1
14	93.9	94.4	78.1	350.0	37.7	34.1	180.5	33.2	114.2	134.3	119.8	102.3	144.2		29.5	30.8	19.9	32.3	25.1	14	GaHV-2
15	103.8	92.7	95.3	350.0	52.6	50.8	199.1	51.3	128.5	173.3	132.7	121.0	259.9	38.9		54.4	19.5	40.6	26.1	15	HHV-1
16	101.3	90.5	87.4	350.0	50.2	47.9	277.6	47.7	133.4	173.6	138.9	126.9	220.2	40.2	29.3		20.7	40.9	25.3	16	HHV-2
17	350.0	350.0	350.0	153.5	208.9	176.0	69.8	175.9	177.8	176.1	176.7	169.2	182.7	219.2	197.4	207.4		20.2	19.5	17	HHV-3
18	105.8	91.7	90.7	350.0	40.1	40.3	203.7	40.6	123.6	158.5	122.2	118.7	229.3	41.8	62.0	58.0	231.3		29.1	18	LHV-4
19	131.8	114.4	110.0	350.0	91.9	89.7	203.1	89.6	155.9	222.4	149.2	146.3	162.5	95.0	112.0	111.0	229.8	102.0		19	SaHV-1
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Fig. 2b—Multiple nucleic acid sequence alignment of *EP0* gene of PRV Becker strain with its homologous genes of 18 selected species (Table 1) using the MEGALIGN program in LASERGENE (DNAStar 7.0) with Clustal V Method [Sequence distance was calculated using weight matrix Identity. Gaps were introduced by the alignment program to maximize the identity]

Thus, viral evolution can be discussed at the molecular level. ORF Finder analysis revealed an integrated PRV *EP0* ORF consisting of 1,104 bp. Nucleotide sequence similarity search by NCBI BLASTN yielded 6 nucleotide sequences (Accession nos. AF298586, EU333164, FJ477294, JQ809328, M57504 and JF797217) with strong identity to PRV Becker *EP0* (similarity up to 88, 88, 88, 88, 89 and 99%, respectively) (Fig. 2a); these accessions correspond to the *EP0* gene of the PRV Ea, Fa, FZ, Kaplan, Indiana-Funkhauser and Bartha strains. Multiple alignment of PRV *EP0* with 18 homologous reference alphaherpesviruses (Table 1) revealed remarkably high similarity (30.2-35.7%) with the members of genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, i.e. Equine herpesvirus 9 (EHV-9), Cercopithecine herpesvirus 2 (CeHV-2), Bovine herpesvirus 1 (BoHV-1) and BoHV-5. However, relatively low similarity was observed between PRV and other viruses of the subfamily *Alphaherpesvirinae* (Fig. 2b).

Bioinformatics analysis of *EP0* polypeptide sequence

An aa sequence similarity search in NCBI BLASTP yielded 6 aa sequences (Accession nos. AAG17904, ABY55292, ACJ76976, AFI70837, AAA47463 and AEM64001) that strongly matched the target sequence of PRV Becker EP0 (similarity up to 87, 88, 88, 88, 89 and 99%, respectively), consistent with a previous report [21]; these sequences correspond to the EP0 protein of the PRV Ea, Fa, PRV-FZ, Kaplan, Indiana-Funkhauser and Bartha strains, respectively (Fig. 3a). Multiple sequence alignment of EP0 with its homologs from 18 reference alphaherpesviruses (Table 1) showed relatively high similarity of 22.9% to 25.1% between EP0 and its counterparts of *Varicellovirus*, i.e. EHV-8, EHV-9, BoHV-1, EHV-1, EHV-4 and BoHV-5. However, EP0 shared no substantial similarity with EP0-like proteins from CeHV-1, Saimiriine herpesvirus 1 (SaHV-1), CeHV-16, Human herpesvirus 2 (HHV-2, HSV-2), Gallid herpesvirus 2 (GaHV-2), HHV-1 (HSV-1), Leporid herpesvirus 4 (LHV-4) or CeHV-2 (16.1 to 18.8%; Fig. 3b). Therefore, EP0 had relatively high similarity to *Varicellovirus*, but not *Simplexvirus* or *Mardivirus*.

Conserved domain analysis (Fig. 3c and Fig. 6) indicated EP0 and almost all its homologues, except the *Mardivirus* member GaHV-2 (Table 2) contained a conserved domain of C3HC4 RING-finger, which is a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc and is probably involved in mediating protein-protein interactions[22,23]. Thus, EP0 was closely related to the RING-finger superfamily protein and was similar to its counterparts encoded by RING-finger genes and, therefore, might belong to the RING-finger superfamily. The RING-finger region of HSV-1 ICP0 is reported to be essential for its regulation of gene expression, stimulation of lytic infection, enhancement of reactivation from quiescence, disruption of ND10 structures, induction of proteasome-dependent degradation of cellular proteins and interaction with cyclin D3[24]. Accordingly, the important roles played by HSV-1 ICP0 in the process of infection suggested that EP0 might also play a similar role in the course of infection. Its real biological

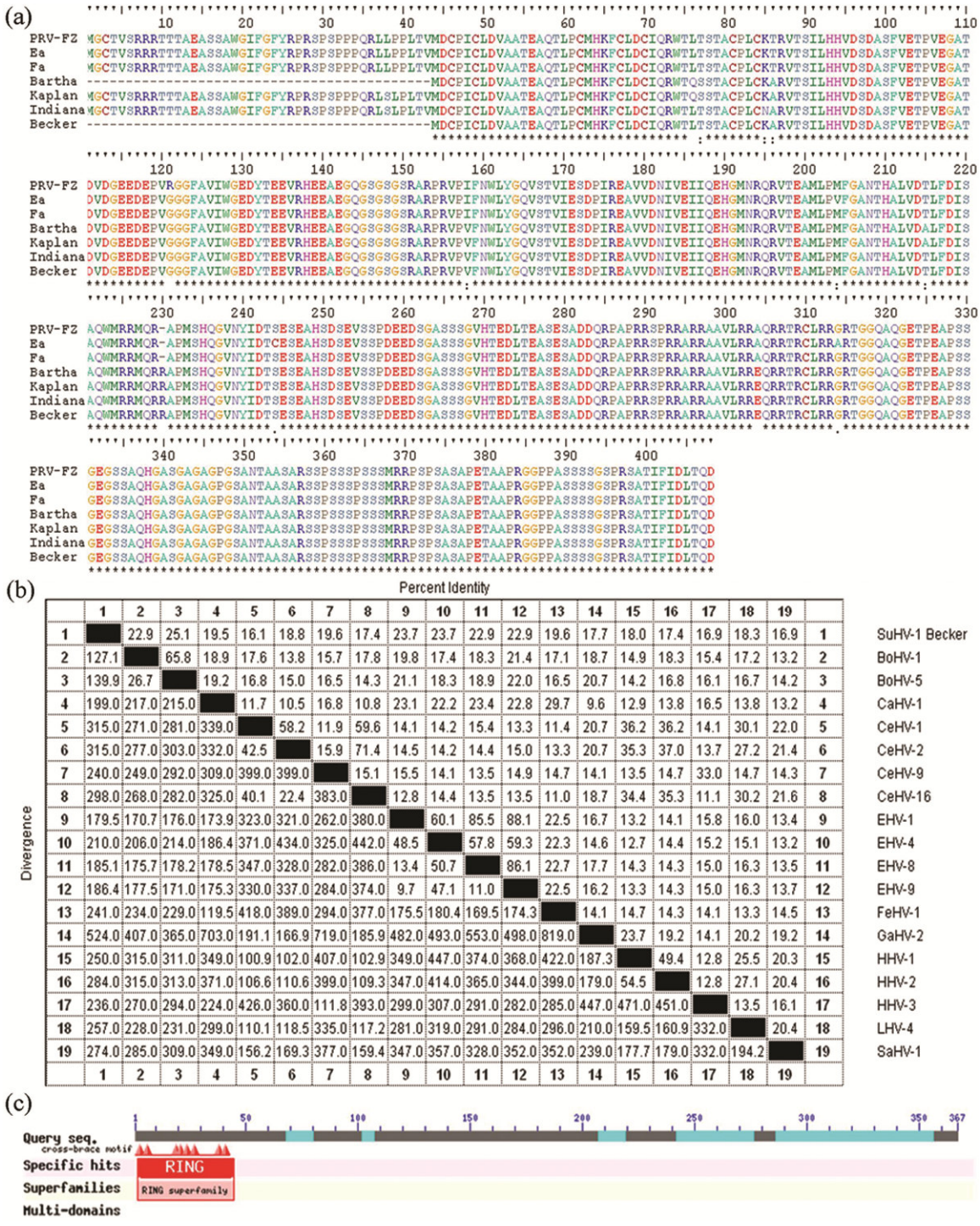

Fig. 3—Conserved domain analysis and multiple aa sequence alignment of EP0 of PRV Becker strain with other PRV strains and its homologous proteins of 18 different selected species [(a) Multiple aa sequence alignment of EP0 of PRV Becker strain (AEM64139) with Bartha (AEM64001), Ea (AAG17904), Fa (ABY55292), PRV-FZ (ACJ76976), Kaplan (AFI70837) and Indiana-Funkhauser (AAA47463) strains using Clustal X (version 1.8) and Bioedit 7.0. Asterisks (*) indicate positions that have a single, fully conserved residue between the 7 EP0 proteins from different PRV strains. Colons (:) indicate conservation of strong groups; periods (.) indicates conservation of weak groups; (b) Multiple aa sequence alignment of EP0 of PRV Becker strain with its homologous genes of 18 selected species (Table 1) using the MEGALIGN program in LASERGENE (DNAStar 7.0) with Clustal V Method and sequence distance was calculated using weight matrix PAM250. Gaps were introduced by the alignment program to maximize the identity; and (c) Conserved domain analysis of PRV EP0 by using NCBI conserved domains search tool]

Table 1—Abbreviations and accession no. of 25 *EP0*-like gene products from different species

Rank	Virus name (Abbreviation)	Genus	Strain/isolate	Description	Natural host	GeneBank accession no.	Sequence length, aa
1			Becker	EP0 gene (early protein 0), its		AEM64139	367
2			Bartha	product is ICP0, a ubiquitin E3 ligase		AEM64001	367
3	Suid		Kaplan	(functions in protein degradation and		AFI70837	410
4	herpesvirus 1	Varicellovirus	Fa	gene regulation), which contains		ABY55292	409
5	(SuHV-1)		PRV-FZ	RING-finger (Really Interesting New	Sus scrofa (Pig)	ACJ76976	409
6	Pseudorabies		Ea	Gene) domain, a specialized type of		AAG17904	409
7	virus (PRV)		Indiana-Funkha user	Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.		AAA47463	410
8	Equid herpesvirus 1 (EHV-1) Equine abortion virus (EAV)	Varicellovirus	T-529	ORF63 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome -dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Equus caballus (Horse)	BAD91104	531
9	Equine herpesvirus 4 (EHV-4) Equine rhinopneumonitis virus (ERV)	Varicellovirus	NS80567	ORF63 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Equus caballus (Horse)	NP_045280	536
10	Equid herpesvirus 8 (EHV-8) Equid herpesvirus 9 (EHV-9)	Varicellovirus	wh	ORF63 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Equus caballus (Horse)	YP_006273043	540

(*Contd.*)

Table 1—Abbreviations and accession no. of 25 *EP0*-like gene products from different species—(*Contd.*)

Rank	Virus name (Abbreviation)	Genus	Strain/isolate	Description	Natural host	GeneBank accession no.	Sequence length, aa
11	Gazelle herpesvirus 1 (GHV-1) Cercopithecine herpesvirus 9 (CeHV-9)	Varicellovirus	P19	ORF63 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Equus caballus (Horse)	YP_002333544	533
12	Simian varicella virus (SVV)	Varicellovirus	Delta	ORF61 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Erythrocebus patas (Monkey)	NP_077475	503
13	Felid herpesvirus 1 (FeHV-1)	Varicellovirus	C-27	ICP0 gene, its product is a ubiquitin E3 ligase, which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Felidae (Cat)	YP_003331582	498
14	Bovine herpesvirus 1 (BoHV-1) Infectious bovine rhinotracheitis virus (IBRV)	Varicellovirus	Composite of 5 strains	BICP0 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Bos Taurus (Cattle)	NP_045363	676
15	Bovine herpesvirus 5 (BoHV-5) Bovine encephalitis herpesvirus (BEHV)	Varicellovirus	SV507/99	BICP0 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Bos Taurus (Cattle)	YP_003662526	720

(*Contd.*)

Table 1—Abbreviations and accession no. of 25 *EP0*-like gene products from different species—(*Contd.*)

Rank	Virus name (Abbreviation)	Genus	Strain/isolate	Description	Natural host	GeneBank accession no.	Sequence length, aa
16	Canid herpesvirus 1 (CaHV-1)	Varicellovirus		CICP0 gene, its product is infected cell protein 0, which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Greyhound (Dog)	BAA95211	333
17	Human herpesvirus 3 (HHV-3) Varicella-zoster virus (VZV)	Varicellovirus	11	ORF61 gene (similar to HHV-1 RL2), its product is a ring-finger protein (functions in modulating cell state and gene expression), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Homo sapiens (Human)	ABF21707	467
18	Leporid herpesvirus 4 (LHV-4)	Simplexvirus	LHV 4012612	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Bunny (Rabbit)	AFR32439	569
19	Saimiriine herpesvirus 1 (SaHV-1) Marmoset herpesvirus (MarHV)	Simplexvirus	MV 5-4	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Saimiri (Squirrel monkeys)	YP_003933840	729
20	Human herpesvirus 1 (HHV-1) Herpes simplex virus 1 (HSV-1)	Simplexvirus	OD4	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Homo sapiens (Human)	AER38009	775

(*Contd.*)

Table 1—Abbreviations and accession no. of 25 *EP0*-like gene products from different species—(*Contd.*)

Rank	Virus name (Abbreviation)	Genus	Strain/isolate	Description	Natural host	GeneBank accession no.	Sequence length, aa
21	Human herpesvirus 2 (HHV-2) Herpes simplex virus 2 (HSV-2)	Simplexvirus	HG52	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Homo sapiens (Human)	NP_044528	824
22	Cercopithecine herpesvirus 1 (CeHV-1) Macacine herpesvirus 1 (McHV-1) Monkey B virus	Simplexvirus	E2490	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Macaca mulatta (Monkey)	NP_851918	691
23	Cercopithecine herpesvirus 2 (CeHV-2) Simian agent 8 (SA8)	Simplexvirus	B264	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Cercopithecus aethiops (Monkey)	YP_164501	709
24	Cercopithecine herpesvirus 16 (CeHV-16) Papiine herpesvirus 2 (PaHV-1) Herpesvirus papio 2 (HVP-2)	Simplexvirus	X313	RL2 gene, its product is ICP0, a ubiquitin E3 ligase (functions in proteasome-dependent degradation of several cellular proteins, disrupting ND10, gene regulation and latency), which contains RING-finger (Really Interesting New Gene) domain, a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, defined by the 'cross-brace' motif C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X2-C-X(4-48) C-X2-C.	Papio cynocephalus (Baboons)	YP_443846	713
25	Gallid herpesvirus 2 (GaHV-2) Marek's disease virus type 1 (MDV-1)	Mardivirus	Md11	RLORF1 gene, its product is ICP0	Gallus domesticus (Chicken)	AAS01621	198

Table 2—Conserved domain, signal peptide sequence, transmembrane domain, phosphorylation site, N-glycosylation site and three-dimensional structure predictions of 25 *EP0*-like gene products from different species

Virus name	RING-finger domain	Signal peptide sequence	Transmembrane domain	Phosphorylation sites	N-Glycosylation sites	3D structure
Becker strain	Yes	No	No	Ser: 36 Thr: 6 Tyr: 3	No	Yes
Bartha strain	Yes	No	No	Ser: 36 Thr: 6 Tyr: 3	No	Yes
Ea strain	Yes	No	No	Ser: 37 Thr: 9 Tyr: 3	No	Yes
Fa strain	Yes	No	No	Ser: 39 Thr: 9 Tyr: 3	No	Yes
PRV-FZ strain	Yes	No	No	Ser: 39 Thr: 9 Tyr: 3	No	Yes
Kaplan strain	Yes	No	No	Ser: 40 Thr: 9 Tyr: 3	No	Yes
Indiana-Funkhauser strain	Yes	No	No	Ser: 39 Thr: 9 Tyr: 3	No	Yes
BoHV-5	Yes	No	No	Ser: 26 Thr: 11 Tyr: 3	No	Yes
BoHV-1	Yes	No	No	Ser: 28 Thr: 10 Tyr: 3	No	Yes
HHV-3	Yes	No	No	Ser: 32 Thr: 16 Tyr: 3	No	Yes
CeHV-9	Yes	No	No	Ser: 34 Thr: 9 Tyr: 5	Yes	Yes
FeHV-1	Yes	No	No	Ser: 31 Thr: 19 Tyr: 2	Yes	Yes
CaHV-1	Yes	No	No	Ser: 22 Thr: 2 Tyr: 4	Yes	Yes
EHV-4	Yes	No	No	Ser: 36 Thr: 12 Tyr: 2	Yes	Yes
EHV-1	Yes	No	No	Ser: 33 Thr: 8 Tyr: 3	Yes	Yes
EHV-8	Yes	No	No	Ser: 30 Thr: 10 Tyr: 3	Yes	Yes
EHV-9	Yes	No	No	Ser: 33 Thr: 9 Tyr: 3	Yes	Yes
LHV-4	Yes	No	No	Ser: 19 Thr: 13 Tyr: 1	No	Yes
SaHV-1	Yes	No	No	Ser: 54 Thr: 15 Tyr: 3	Yes	Yes
CeHV-2	Yes	No	No	Ser: 21 Thr: 18 Tyr: 3	Yes	Yes
CeHV-16	Yes	No	No	Ser: 18 Thr: 16 Tyr: 2	Yes	Yes
CeHV-1	Yes	No	No	Ser: 21 Thr: 14 Tyr: 4	Yes	No
HHV-1	Yes	No	No	Ser: 49 Thr: 16 Tyr: 5	Yes	Yes
HHV-2	Yes	No	No	Ser: 71 Thr: 16 Tyr: 3	Yes	Yes
GaHV-2	No	No	No	Ser: 9 Thr: 5 Tyr: 0	No	No

functions of EP0 in the herpesvirus life cycle is not yet known and further studies are warranted on its functions in viral replication and interactions between herpesvirus and host.

Phylogenetic analysis of PRV and other herpesviruses was performed based on the sequences of EP0 and the EP0-like proteins of 18 reference alphaherpesviruses (Table 1). The proteins could be preliminarily separated into different genera, i.e. *Simplexvirus*, *Varicellovirus* and *Mardivirus*, which was consistent with the existing classification of subfamily *Alphaherpesvirinae* (Fig. 4). Furthermore, EP0 clustered with BoHV-1 and BoHV-5 first and then with other varicelloviruses, including HHV-3, CeHV-9, FeHV-1, and CaHV-1 (Fig. 4). Therefore, phylogenetic analysis unequivocally demonstrated that PRV shared a closer evolutionary relationship with the members of genus *Varicellovirus* than other genera of *Alphaherpesvirinae*, consistent with a previous report[25]. Although the *EP0*-like gene products are conserved within the *Alphaherpesvirinae* subfamily[26], they are not conserved in other herpesvirus subfamilies, including *Betaherpesvirinae* and *Gammaherpesvirinae*, suggesting that the EP0-like protein might function that is discrete or complemented by other proteins from these subfamilies.

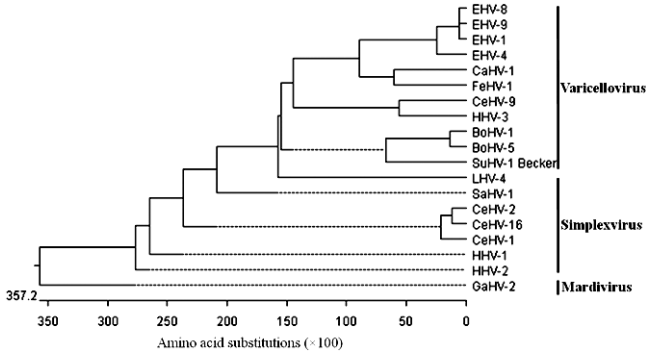

Fig. 4—Evolutionary relationships of putative PRV EP0 protein with its 18 reference alphaherpesviruses from different species (Table 1) [Phylogenetic tree of these proteins was generated by using the MEGALIGN program in LASERGENE (DNAStar 7.0) with Clustal V Method and sequence distance was calculated using weight matrix PAM250. Gaps had been introduced by the alignment program to maximize the identity]

Signal polypeptide or transmembrane domain prediction indicated no cleavage site (Fig. 5a) or transmembrane domain in EP0 (Fig. 5b) and the homologues of EP0 (Table 2). Furthermore, N-linked glycosylation site (Asn-X-Ser/Thr) prediction demonstrated no potential N-glycosylation site (Fig. 5c) in EP0 and its BoHV-1, BoHV-5, HHV-3, LHV-4 and GaHV-2 counterparts (Table 2), which might be related with their evolutionary relationships and provide some clues regarding their expression. Nevertheless, hydrophobicity analysis revealed 6 hydrophobic regions located at aa 1-47, 50-55, 78-85, 117-143, 157-176, and 353-358 (Fig. 7a). The hydrophilic region was larger than the hydrophobic region (Fig. 7b), suggesting that EP0 might be a hydrophilic protein.

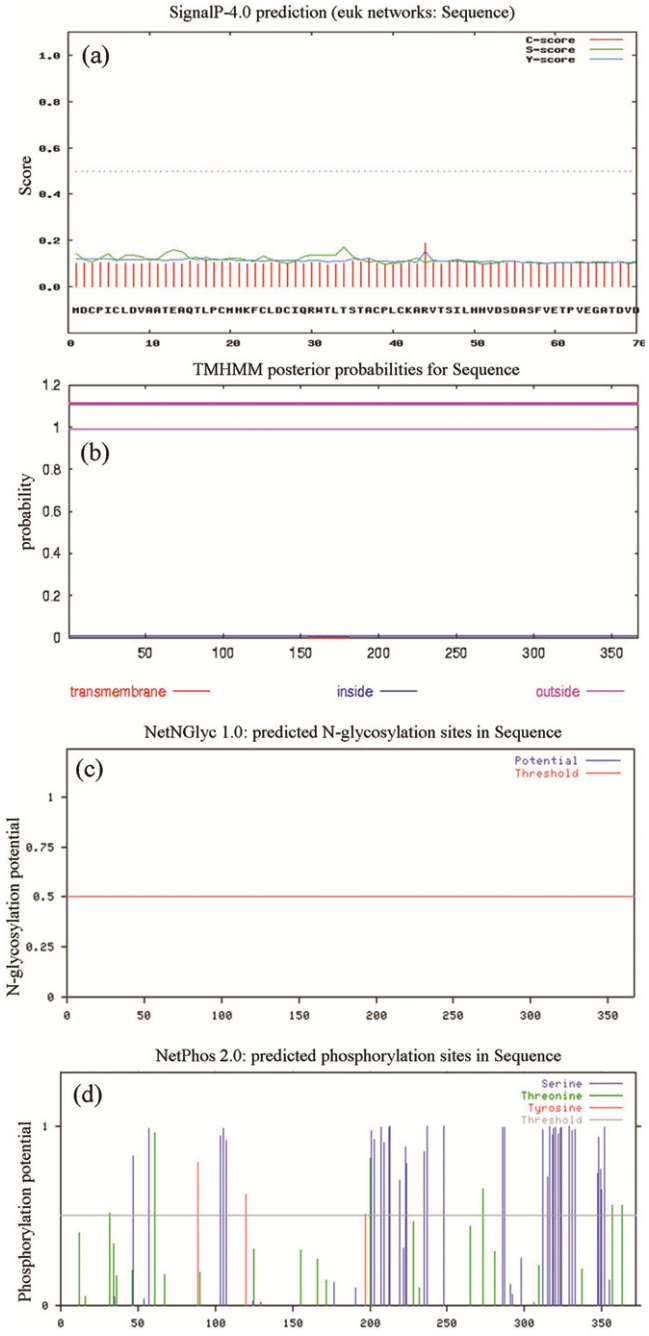

Fig. 5—Prediction of signal peptide sequence, transmembrane domain, glycosylation site and phosphorylation site of PRV EP0 [Signal peptide sequence, transmembrane domain, glycosylation site and phosphorylation site of EP0 were analyzed by SignalP-4.0 Server (a), TMHMM program (b), NetNGlyc 1.0 program (c) and NetPhos 2.0 program (d), respectively]

Protein phosphorylation is one of the most normal and essential types of protein modification and certain aspects of cell process modulation are regulated by protein phosphorylation. Like signal transduction, proliferation, differentiation and metabolism are all controlled by the balance of activity of protein kinases and protein phosphatases upon pivotal target proteins[27]. Phosphorylation site prediction revealed 45 potential phosphorylation sites in EP0 (Fig. 5d and Fig. 6), including 36 Ser, 6 Thr, and 3 Tyr potential phosphorylation sites. Tyrosine phosphorylation is reported to be involved in the replication of several herpesviruses[28,29] and shifting protein translocation from the cytoplasm to the nucleus during productive virus infection[30]. Phosphorylation of HSV-1 ICP0 is associated with its regulatory function[31], and VZV ORF63 is involved in its subcellular localization and transcriptional regulatory properties[32,33]. Therefore, EP0 phosphorylation might also play an important role during PRV infection, perhaps in modulating its subcellular localization or other uncharacterized functions, such as transcriptional regulation[34,35]. Moreover, the results indicated that almost all the homologues of EP0, except GaHV-2 ICP0 had potential Ser, Thr and Tyr phosphorylation sites (Table 2 and Fig. 6), suggesting the EP0-like proteins were relatively conserved to be phosphorylated throughout alphaherpesvirus. However, some of the orthologous sites in these proteins (especially in the conserved domain) were not phosphorylated modified (not occupied by the same modifiable aa), suggesting genome-specific modification or noise might play a role in the protein modification and the prediction was not relevant without experimental validation.

As more information becomes available on protein antigens, it is 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

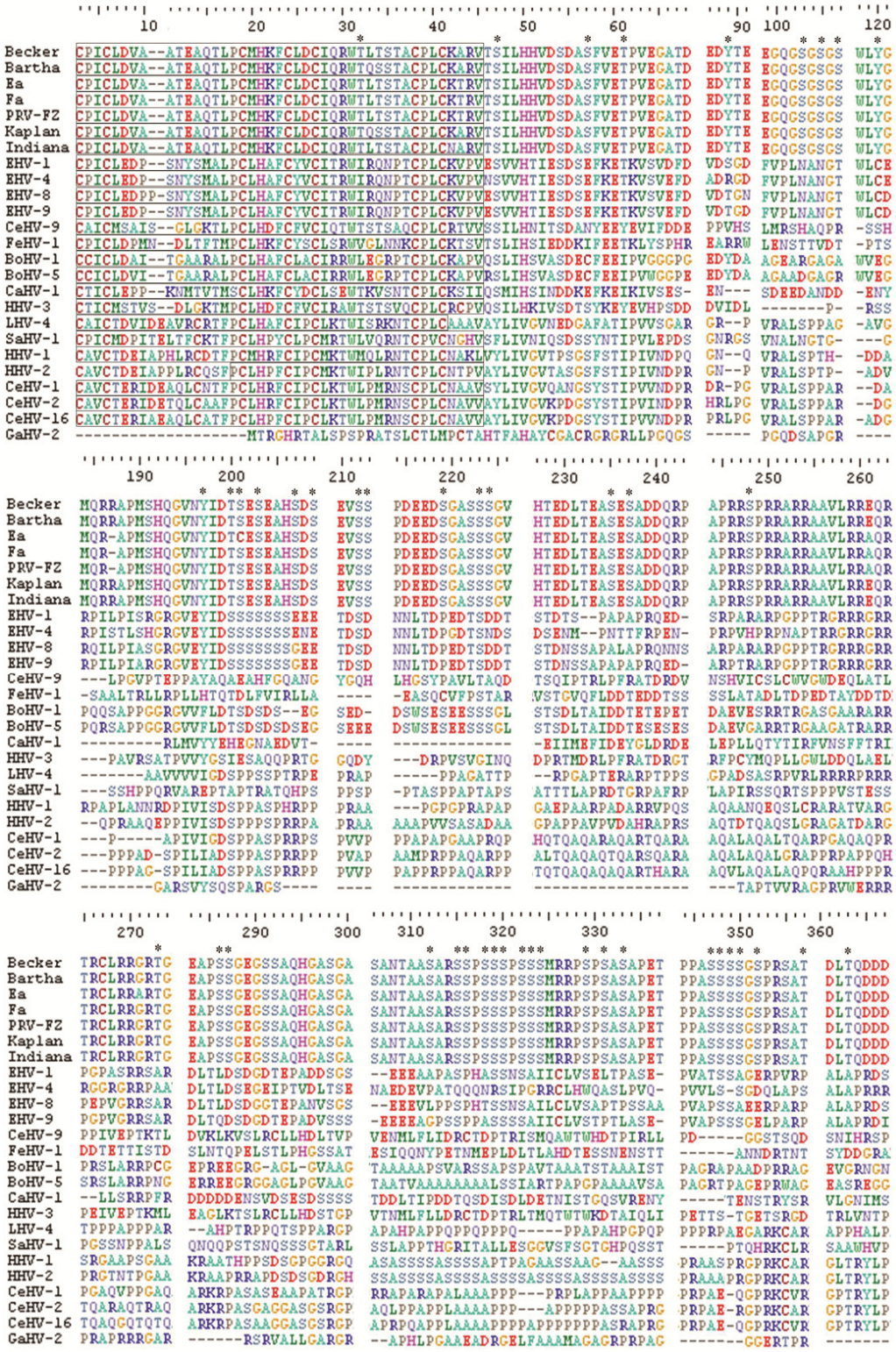

Fig. 6—Conserved domains and detailed multiple aa sequence alignment of EP0 of PRV Becker strain with its homologous proteins of 24 different selected species [Ruler shows the aa sequence localization of EP0 of PRV Becker strain and sequence alignment was conducted by using Clustal X. Asterisks (*) indicate the predicted phosphorylation sites of EP0 of PRV Becker strain and rimmed aas indicate the predicted conserved domains of the EP0-like proteins from different species]

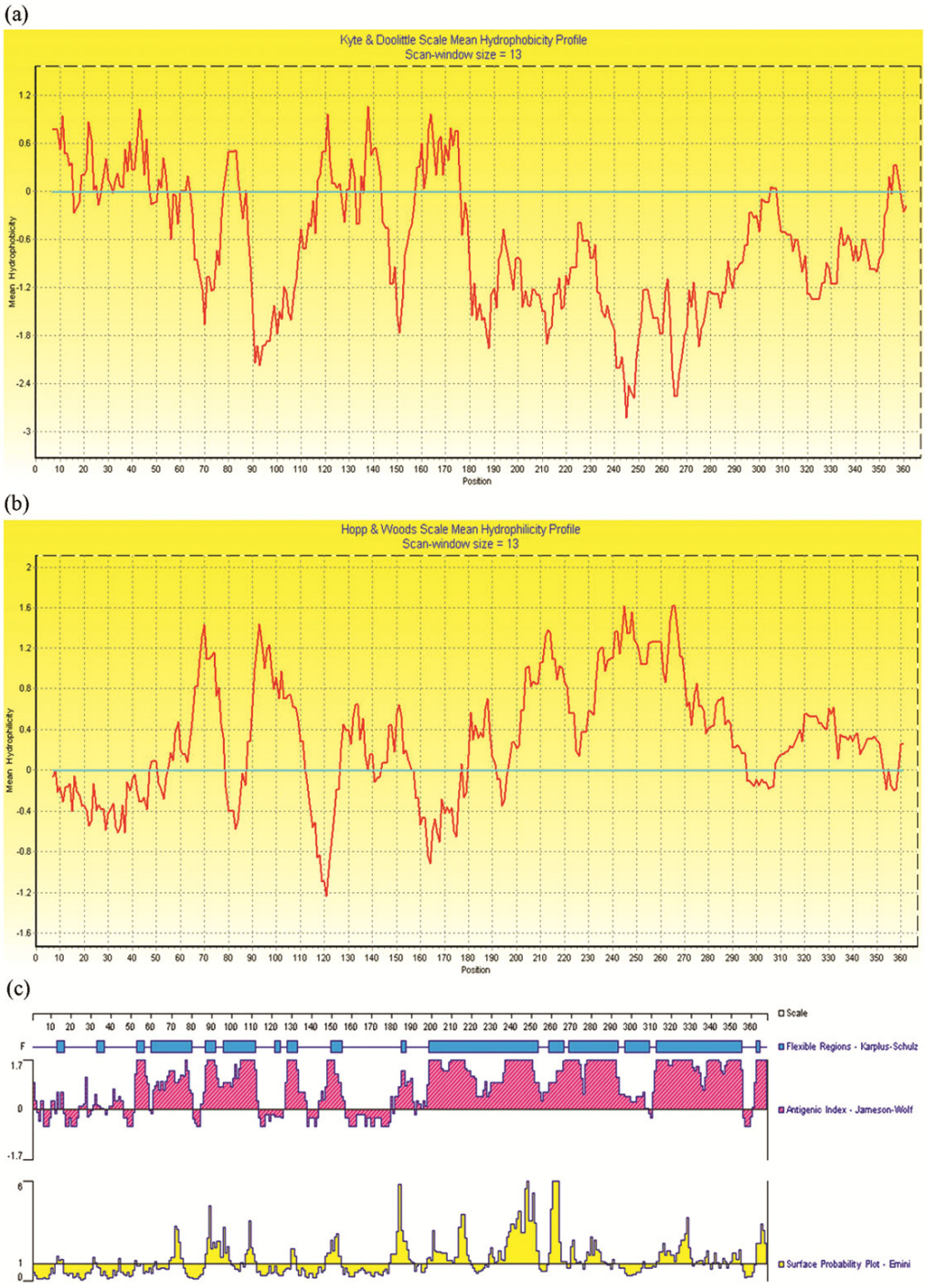

Fig. 7—Hydrophobicity, hydrophilicity and antigenic analyses of PRV EP0 [The hydrophobicity (a) or hydrophilicity profile (b) of EP0 was determined with the values of Kyte and Doolittle (Kyte and Doolittle, 1982) or Hopp and Woods (Hopp and Woods, 1981) using a 13-amino-acid window, respectively. The upwardpointing peaks represent the most hydrophobic regions (a) and the most hydrophilic regions (b), respectively. (c) Antigenic analysis of EP0 was carried out by PROTEAN software of DNAStar based on its flexibility[37], surface probability[38] and antigenic index[39] by the determination of its primary structure]

uncertain and time-consuming task. Earlier methods have been based on the assumption that the antigenic region is primary the hydrophilic region at the surface of the protein molecule[14,36]. However, this assumption is limited in its accuracy. To improve accuracy, the B-cell epitopes of EP0 have bear predicted using DNAStar PROTEAN programs based on flexibility[37], antigenic index[38] and surface probability[39] based on

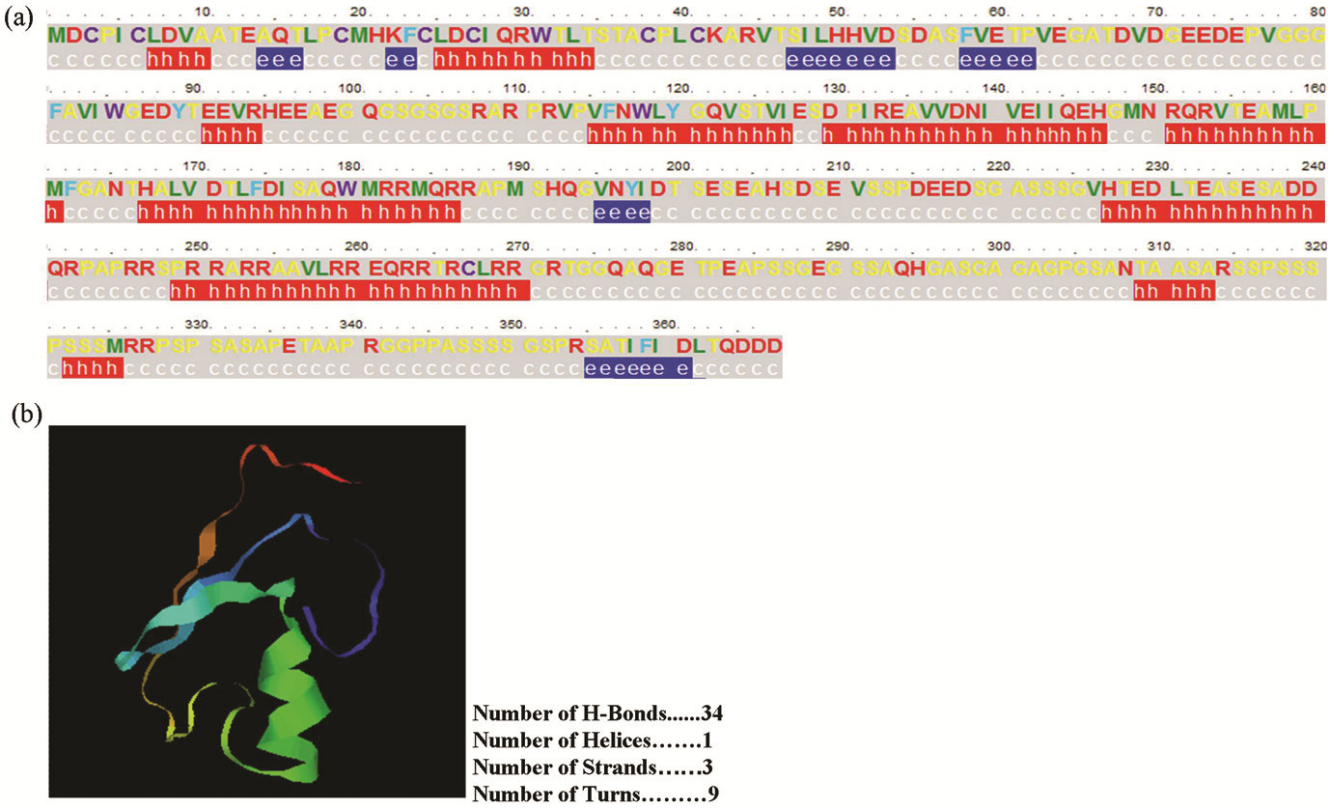

Fig. 8—Secondary structure and three-diamension (3D) structure prediction of PRV EP0 [(a) Secondary structure of EP0. The secondary structure was predicted by using PSIpred program and the letters h, e and c indicate alpha Helix, extended (beta strand) and Coil, respectively; and (b) 3D structure of EP0. The 3D structure was predicted by employing the protein modeling server database CPHmodels 3.2 Server. The number of H-Bond, Helice, Strand and Turn included in this model were 34, 1, 3 and 9, respectively]

the primary structure (Fig. 6c). In our study, several potential B-cell epitopes were identified in EP0, located in or adjacent to aa 13-18, 70-77, 88-93, 97-101, 108-113, 131-133, 151-156, 186-188, 200-220, 229-254, 259-267, 270-274, 278-289, 314-339, 341-344, 347-356 and 364-365 (Fig. 7c). These findings of antigenic and structural properties of EP0 might provide methods for developing new antibodies and immunoassays for clinical diagnosis of PRV.

Secondary structure analysis (Fig. 8a) suggested EP0 primarily consisted of random coils (up to 58.20%) and α-helices (34.15%), whereas the β-strand accounted for the lowest component (7.65%). Therefore, the principal component of α-helix of EP0, which contains many highly conserved residues[40,41], might be engaged in its self-association or the interactions with other constituents of the tegument[42], nucleocapsid[40,42] and the host proteins[41], whereas the β-turn might play a fundamental role for its stabilization. Three-dimension (3D) structure prediction showed that a known 3D structure model having homology with EP0 (predominantly composed by β-turn and β-strand) (Fig. 8b) and its homologues, except its CeHV-1 and GaHV-2 counterparts (Table 2), was found, which could be explained by the weakest similarity between EP0 and its homologues of CeHV-1 and GaHV-2.

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[43]. Subcellular localization research of HSV-1 ICP0 suggests that in an HSV-1-infected cell, ICP0 predominantly locates in the nucleoli[44]. However, our subcellular localization analysis demonstrated that EP0 was predominantly localized in the nucleus and only a small fraction of EP0 was localized in the cytoskeleton and mitochondria and, therefore, it functions as a nuclear-targeted protein, although no typical nuclear localization signal can be found in the EP0 protein sequence.

In conclusion, we presented the cloning and molecular characterization of the PRV *EP0* gene. Elucidating the molecular characterization and genetic evolution of PRV *EP0* will contribute to our understanding of this virus at the molecular level and will enrich the herpesvirus database. These works will also provide insights for further research into the function and mechanism of *EP0* during PRV infection.

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References

1 Kramer T, Greco T M, Enquist L W & Cristea I M (2011) *J Virol* 85, 6427-6441
2 Tomioka Y, Miyazaki T, Taharaguchi S, Yoshino S, Morimatsu M, Uede T, Ono E & Watanabe M (2008) *Eur J Neurosci* 27, 2115-2132
3 Li M L, Wang S, Cai M S & Zheng C F (2011) *J Virol* 85, 10239-10251
4 Li M L, Wang S, Cai M S, Guo H & Zheng C F (2011) *Virology* 417, 385-393
5 Li M L, Zhao Z Y, Chen J H, Wang B Y, Li Z, Li J & Cai M S (2012) *Virol Sin* 27, 303-315
6 Li M L, Chen J H, Zhao Z Y, Zhang K J, Li Z, Li J, Mai J Y, Zhu X M & Cai M S (2013) *Genet Mol Res* 12, 85-98
7 Smith G A & Enquist L W (2000) *Proc Natl Acad Sci (USA)* 97, 4873-4878
8 Altschul S F, Madden T L, Schaffer A A, Zhang J, Zhang Z, Miller W & Lipman D J (1997) *Nucleic Acids Res* 25, 3389-3402
9 Marchler-Bauer A, Anderson J B, Cherukuri P F, DeWeese-Scott C, Geer L Y, Gwadz M, He S, Hurwitz D I, Jackson J D, Ke Z, Lanczycki C J, Liebert C A, Liu C, Lu F, Marchler G H, Mullokandov M, Shoemaker B A, Simonyan V, Song J S, Thiessen P A, Yamashita R A, Yin J J, Zhang D & Bryant S H (2005) *Nucleic Acids Res* 33, D192-196
10 Nielsen H, Engelbrecht J, Brunak S & von Heijne G (1997) *Protein Eng* 10, 1-6
11 Bendtsen J D, Nielsen H, von Heijne G & Brunak S (2004) *J Mol Biol* 340, 783-795
12 Moller S, Croning M D & Apweiler R (2001) *Bioinformatics* 17, 646-653
13 Blom N, Gammeltoft S & Brunak S (1999) *J Mol Biol* 294, 1351-1362
14 Hopp T P & Woods K R (1981) *Proc Natl Acad Sci (USA)* 78, 3824-3828
15 Kyte J & Doolittle R F (1982) *J Mol Biol* 157, 105-132
16 Burland T G (2000) *Methods Mol Biol* 132, 71-91
17 Van Regenmortel M H (1992) *Mol Biol Rep* 16, 133-138
18 Jones D T (1999) *J Mol Biol* 292, 195-202
19 Nielsen M, Lundegaard C, Lund O & Petersen T N (2010) *Nucleic Acids Res* 38, W576-581
20 Antunes R S, Gomes V N, Prioli S M, Prioli R A, Julio H F, Jr., Prioli L M, Agostinho C S & Prioli A J (2010) *Genet Mol Res* 9, 674-684
21 Szpara M L, Tafuri Y R, Parsons L, Shamim S R, Verstrepen K J, Legendre M & Enquist L W (2011) *PLoS Pathog* 7, e1002282
22 Freemont P S (1993) *Ann N Y Acad Sci* 684, 174-192
23 Cheung A K (1991) *J Virol* 65, 5260-5271
24 Hagglund R & Roizman B (2004) *J Virol* 78, 2169-2178
25 McGeoch D J, Dolan A & Ralph A C (2000) *J Virol* 74, 10401-10406
26 Everett R D, Boutell C, McNair C, Grant L & Orr A (2010) *J Virol* 84, 3476-3487
27 Kyriakis J M (2014) *J Biol Chem*
28 Ren X, Harms J S & Splitter G A (2001) *J Virol* 75, 9010-9017
29 Geiss B J, Tavis J E, Metzger L M, Leib D A & Morrison L A (2001) *J Virol* 75, 10721-10729
30 Pomeranz L E & Blaho J A (1999) *J Virol* 73, 6769-6781
31 Davido D J, von Zagorski W F, Lane W S & Schaffer P A (2005) *J Virol* 79, 1232-1243
32 Mueller N H, Walters M S, Marcus R A, Graf L L, Prenni J, Gilden D, Silverstein S J & Cohrs R J (2010) *J Gen Virol* 91, 1133-1137
33 Habran L, Bontems S, Di Valentin E, Sadzot-Delvaux C & Piette J (2005) *J Biol Chem* 280, 29135-29143
34 Brukman A & Enquist L W (2006) *J Virol* 80, 10871-10873
35 Watanabe S, Ono E, Shimizu Y & Kida H (1995) *J Gen Virol* 76 (Pt 11), 2881-2885
36 Welling G W, Weijer W J, van der Zee R & Welling-Wester S (1985) *FEBS Lett* 188, 215-218
37 Karplus P A & Schulz G E (1985) *Naturwissenschaften* 72, 212-213
38 Emini E A, Hughes J V, Perlow D S & Boger J (1985) *J Virol* 55, 836-839
39 Jameson B A & Wolf H (1988) *Comput Appl Biosci* 4, 181-186
40 Benach J, Wang L, Chen Y, Ho C K, Lee S, Seetharaman J, Xiao R, Acton T B, Montelione G T, Deng H, Sun R & Tong L (2007) *J Biol Chem* 282, 31534-31541
41 Li-Smerin Y, Hackos D H & Swartz K J (2000) *J Gen Physiol* 115, 33-50
42 Granzow H, Klupp B G & Mettenleiter T C (2004) *J Virol* 78, 1314-1323
43 Feng Z P (2002) *In Silico Biol* 2, 291-303
44 Morency E, Coute Y, Thomas J, Texier P & Lomonte P (2005) *Arch Virol* 150, 2387-2395