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Molecular analysis of *US10*, *S3*, and *US2* in duck enteritis virus

Yan Zhao · Jun Wei Wang · Fengyuan Liu · Bo Ma

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Abstract A 4554-bp fragment was amplified from the DEV C-KCE vaccine strain by single oligonucleotide nested polymerase chain reaction with partially known sequences for the DEV *US1* and *US10* genes. Three open reading frames containing the genes encoding *US10*, *S3*, and *US2* were predicted using the Editseq program (DNAStar). The *S3* and *US2* genes have the same transcription orientation but are oriented head-to-head with respect to *US10*. The promoters and polyadenylation signals were predicted. Two poly A sequences were predicted in *S3*, but none were predicted in *US2*. These results provide partial sequence of US region for the physical map of the DEV genome. Phylogenetic analysis suggests that the DEV C-KCE strain is more closely related to Mardivirus in the *alphaherpesvirus* subfamily of the Herpesviridae.

Keywords SON-PCR · Duck enteritis virus · *US10* · *S3* · *US2*

Introduction

Duck viral enteritis (DVE) is an acute contagious disease that is highly lethal in all ages of birds from the order Anseriformes (ducks, geese, and swans) [1]. Duck enteritis virus (DEV), a member of the family Herpesviridae, is the causative agent for DVE. DVE was first known as an acute hemorrhagic disease found in domestic ducks in Holland as early as 1923 [2]. DVE was first reported in China in 1957 [3]. Several studies indicate that DVE is difficult to monitor and control, because DEV establishes an asymptomatic carrier state in both farmed and wild waterfowl and it is only detectable during the intermittent shedding period of the virus [4].

The genome of DVE is a linear, double-stranded DNA molecule of approximately 180 kb, consisting of unique long (UL) and unique short (US) regions with a structure similar to that of other alphaherpesviruses**.** The G + C content of the genome is 64.3%, which is higher than any other reported avian herpesvirus in the subfamily *Alphaherpesvirinae* [5]. DEV was classified as an unassigned virus in the family Herpesviridae according to the Eighth International Committee on Taxonomy of Viruses (ICTV) [6], although it was previously grouped in the subfamily *Alphaherpesvirinae* [7, 8]. The majority of DEV sequences are limited in the UL region; however, only partial sequences of US1 and *US10* have been published and other sequences in the US region were not clear. In order to clarify the genomic organization, we have amplified the unknown sequences in the US region of DEV.

Here, we report the cloning of the full-length *US10*, *S3*, and *US2* genes by combining single oligonucleotide nested polymerase chain reaction (SON-PCR) [9] with common PCR. We have characterized the acquired sequences and performed phylogenetic analyses of the evolutionary relationship of DEV with reference strains of *Alphaherpesvirinae*. These results further supported the phylogenetic classification of DEV. Our findings help in illustrating the structure and function of these genes in DEV.

Y. Zhao · J. W. Wang (✉) · F. Liu · B. Ma
Northeast Agricultural University, Harbin 150030, China
e-mail: jwwang@neau.edu.cn

Materials and methods

Virus propagation and preparation of viral genomic DNA

DEV C-KCE was obtained from the station of veterinary drugs censorship (Beijing, China). The virus was propagated in chicken embryo fibroblasts (CEF) in Dulbecco's Minimal Essential Medium. Viral particles were harvested when the cytopathic effect reached 80%. After three frozen-thawed cycles cell lysate was then centrifuged to remove cell debris and stored at −70°C until use.

Viral DNA preparation was described previously [10]. The presence of DEV was confirmed by using PCR, The primers, D1 (+) 5′-GTAGACGAAGGCGGGTATG-3′ and D2 (−) 5′-CGTATTGGTTTCTGAGTTGG-3′, were designed according to the partial sequence available for UL30 (AF064639).

Primer design and PCR amplification of *US10*, *S3*, and *US2*

The procedure for amplifying the DEV genomic fragment is shown in Fig. 1. Several specific primers were designed according to the DEV *US1* and *US10* partial sequence, and synthesized as reported previously [11]. All the primers are listed in Table 1.

PCR was carried out in a reaction mixture containing 2.5 μl 10 × reaction buffer, 2.0 μl dNTPs (2.5 mM for each of the four dNTPs), 0.5 μl of each primer (10 pmol each), 2.0 μl DNA template, 0.3 μl ExTaq DNA polymerase (5 U/μl), and water up to 25 μl (all the reagents were purchased from TaKaRa). PCR conditions used are shown in Table 2.

Table 1 Oligonucleotide primers used for PCR amplification

Name	Sequence
110up	5′ TTTGTGCTTCGCCGTGGTT 3′
110dn	5′ CCTGGACGCTTGATAAATCCTTAC 3′
sp1	5′ TCTGTTTCCGACCTGGCTCTC 3′
sp2	5′ TATTCCATCCAGTTGCTCCCG 3′

Cloning and sequencing of PCR products

PCR products were analyzed on 1% agarose gels and purified using a DNA Gel Extraction Kit (Bioteke, Peking). They were then cloned into the pMDl8-T vector (TaKaRa) according to the manufacturer's instructions. Each fragment was sequenced by Sangon.

Analysis of DNA sequence

The full-length assembled sequence was analyzed using the Editseq program (DNAStar) to search for open reading frames (ORFs), and then submitted to GenBank for BLAST search analysis. The predicted amino acid sequences of these ORFs were compared with those from

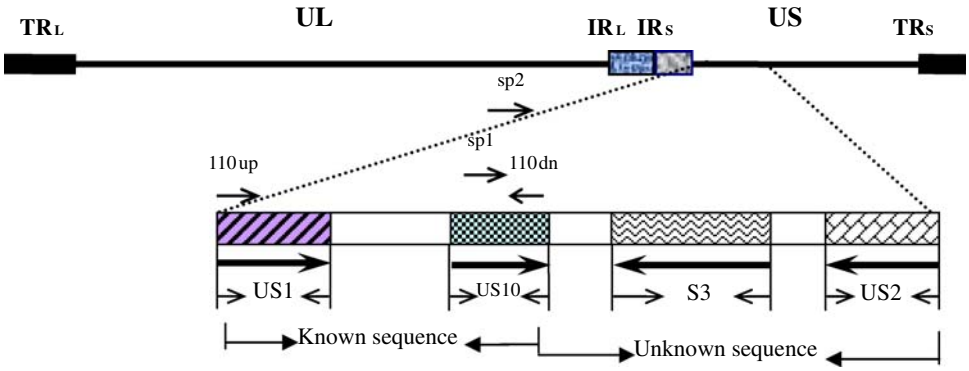

Fig. 1 A schematic illustrating the arrangement of *US1–US2* genes in DEV genome and the PCR amplification strategy. The DEV genomic structure is presented here in schematic form, and the relative positions of *US1*, *US10*, *S3*, and *US2* are displayed between dashed lines. The putative ORFs are shaded, while the non-encoding regions are shown in blank, and the putative mRNA of each gene is shown with a boldfaced arrow. *S3* and *US2* have the same transcription orientation but are in a head-to-head transcription orientation with *US10*. The strategy for PCR amplification of the genes from *US1* to *US2* is presented here. Primers 110up and 110dn were used to amplify the known sequences of *US1* and *US10*, on the basis of which sp1 and sp2 were designed. During the high temperature cycle of SON-PCR, the sp1 can bind to its specific position in *US10*; however, during low temperature cycles, sp1 can bind non-specifically downstream of *US2* to form a product containing sp1 on both ends. In order to prove that the product was in the right position, internal identification was carried out using the product as a template, and sp1 and 110dn as well as sp2 and 110dn as internal detection primers. Primer positions are shown with black arrows

Table 2 PCR cycling conditions

Reaction	Cycle No.	Thermal condition
Primary reaction	1	95°C, 5 min
	5	94°C, 30 s, 57.5°C, 30 s, 72°C, 2 min
	1	94°C, 30 s, 30°C, 3 min, ramp to 72°C by 2°C s^{-1}, 72°C, 2 min
	30	94°C, 20 s, 57°C, 30 s, 72°C, 2 min
	1	72°C, 10 min
Internal	1	95°C, 2 min
Identification	30	94°C, 20 s, 56.5°C, 30 s, 72°C, 30 s
	1	72°C, 10 min

other alphaherpesviruses using the DNAStar program (version 7.1, DNAStar, Inc.). The promoters of these genes were analyzed by the program of Berkeley Drosophila Genome project's Neural Network Promoter Prediction, which is a eukaryotic (human) core promoter search engine (http://www.fruitfly.org/seq_tools/promoter.html) [12]. The core promoters were examined for the presence of TATA box consensus sites using the TRANSFACFind search engine (http://motif.genome.jp/). POLYADQ, analyzed by a eukaryotic (human) polyadenylation (poly A) signal search engine (Cold Spring Harbor Laboratory, [http://rulai.cshl.org/tools/polyadq/polyadq_form.html]). The potential *N*-glycosylation sites transmembrane region, and signal peptide were analyzed using the program of NetNGlyc 1.0 Server, TMHMM, and SignalP 3.0 Server from the search engine (http://www.cbs.dtu.dk/services/). The DNA sequence identity to the Kozak consensus sequence (GCCGCCRCCATGG, R = A/G) [13] around the initiator ATG of each protein was also measured. Phylogenetic analysis (maximum likelihood method) was performed by using the MEGA4.1, while multiple alignment was performed by Clustal X.

Results

ORF determination and molecular characteristics of three genes

A 4554-bp sequence was amplified from DEV C-KCE genome by SON-PCR. Three complete ORFs were predicted, containing the genes *US10*, *S3*, and *US2* homolog of MDV-1. The ORF sizes were 507, 888, and 720-bp, respectively. The sequence obtained in this study is available from GenBank under the accession number (EF619046). The gene arrangement of the three ORFs in DEV was similar to MDV-1. The relative positions of the three genes in the DEV genome are shown in Fig. 1.

These predicted functional regions are shown in Table 3. The canonical polyadenylation signal AATAAA was found have an overlap of 5 nucleotides with *US10*, while two potential polyadenylation signals were predicted downstream of *S3*, but none was found for *US2*. The three genes were found to have a Kozak ribosome-binding sequence of 5, 6, and 7, respectively, at their translation start sequence.

The US10 protein is encoded by 168 amino acids (aa) and possesses a putative zinc finger motif, 93-C-X3-C-X3-H-X3-C-105, where C = cysteine, H = histidine, X = any amino acid. Comparing the putative US10 protein with other alphaherpesvirus strains revealed a highly conserved stretch of 13 amino acid residues, which lies in the putative zinc finger domain.

The *S3* ORF was predicted to encode 295 aa. Only avian herpesviruses have been shown to contain this gene. This gene is conserved, with sequence homology of as high as 41.84%, but its functions are unknown.

The US2 protein is composed of 239 aa. It possesses two potential *N*-linked glycosylation sites at amino acid residues 85 and 127. Only VZV was absent from the multiple alignment; the result predicted two conserved regions mainly centered on the *N*-terminal region. The first region,

Table 3 Core promoters searched in the neural network and polyadenylation signals predicted by POLYADQ

Gene	Promoter location[a]	Promote Score	TATA sequence	TATA location[a]	TSS location	Kozak (of 13)[b]	Poly(A) sequence	Poly(A) location	Poly(A) score
US10	1176–1225	0.99	ATATATG	1185–1191	1216	5	AATAAA	1908–1913	0.218646
S3	3174–3125r[a]	1.00	TTTAAAA	3166–3160r	3134r	6	ATTAAA	2150–2155r	0.023327
							AATAAA	2094–2099r	0.138697
US2	4301–4252r	0.80	TCTAAAA	4292–4286r	4261r	7	NP	NP	NP

[a] r indicates reverse direction

[b] Identity of the ATG to the Kozak consensus, GCCGCCRCCATGG

The initial search was performed at a high stringency (the cutoff score of 0.85 out of 1.00)

Potential polyadenylation signal sequences were predicted using the Po1yADQ program

Cut-off parameters were initially set at zero to return the location of all AATAAA and ATTAAA consensus signals, along with an associated score between 0 and 1

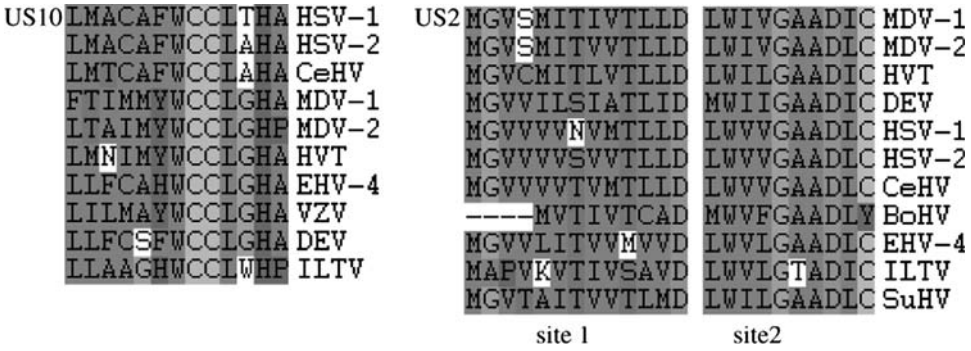

Fig. 2 Conserved sites in the deduced amino acid sequences of *US10* and *US2* among reference strains. Highly conserved sites are shown with dark shading. *US10* contained a highly conserved region with seven continuous amino acids; in *US2*, two conserved regions were predicted, and were mainly located in 5′ half

a 14 hydrophobic residue domain was conserved in all *Alphaherpesvirinae* (Fig. 2).

Phylogenetic analysis

The phylogenetic tree was performed by comparing the three putative protein of DEV with that of 11 reference strains of closely related herpesviruses (Fig. 3), DEV is closely related to members of *Alphaherpesvirinae*, consistent with that reported previously [14, 15]. Furthermore, the phylogenetic distance showed that DEV is closer to Mardivirus of the *Alphaherpesvirinae*. The three genes and deduced amino acid sequence of DEV shared high homology with Mardivirus of *Alphaherpesvirinae* (Table 4). These results will provide evidence for the taxonomic classification of DEV.

Discussion

Phylogenetic trees based on the three putative proteins show that DEV C-KCE is evolutionarily most closely related to the Avian herpesviruses. Our findings are quite similar to those who proposed that this virus should be classified as an alphaherpesvirus [14, 15]. All the three phylogenetic analyses we have performed indicate that DEV forms a single cluster, but is more closely related to Avian herpesviruses in the alphaherpesvirus subfamily of the *Herpesviridae*.

US10 homologs of EHV-1 and HSV-1 are known to possess a sequence of 13 amino acids (C-X3-C-X3-H-X3-C), which is a perfect match to the consensus zinc finger motif [16], which was also present in DEV 93-C-X3-C-X3-H-X3-C-105. The *US10* gene encodes a protein in HVT which is non-essential for virus replication in vitro and in vivo and has been used as a site for the insertion and expression of foreign sequences [17]. There is very little absolute sequence conservation in the *N*-terminal 70% of the polypeptide. In contrast, the *C*-terminal region of US10 is highly conserved among all of the primate viruses.

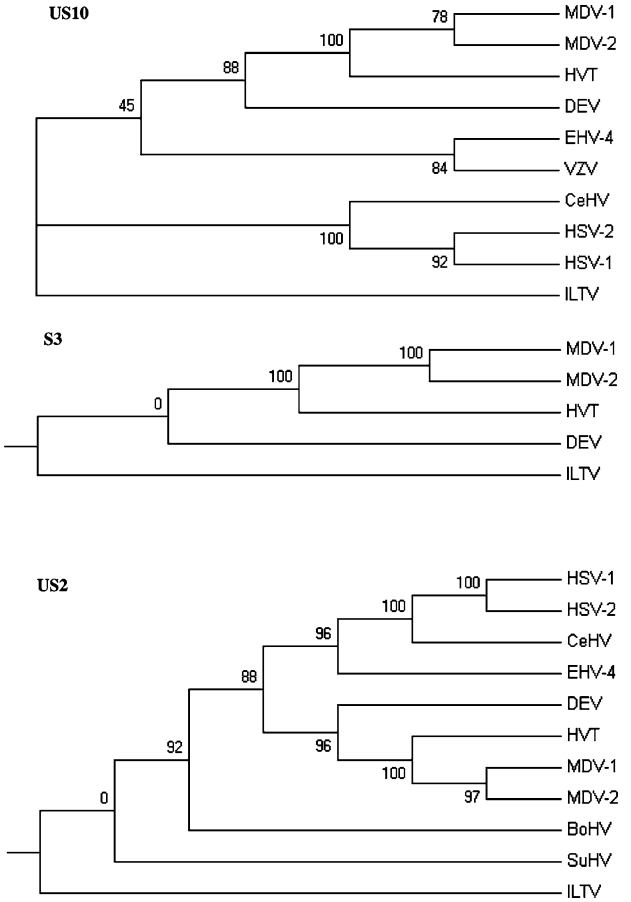

Fig. 3 Phylogenetic analysis of *US10*, *S3*, and *US2* deduced protein in DEV and 11 reference strains of the subfamily *Alphaherpesvirinae*. Phylogenetic trees for these three proteins in these alphaherpesviruses were generated using the MEGA4.1. The reference strains of *Alphaherpesvirinae* are listed below: [duck enteritis virus (DEV, EF619046). Simplexvirus: including herpes simplex virus 1 (HSV-1, X14112), herpes simplex virus 2 (HSV-2, NC_001798), Cercopithecine herpesvirus 1 (CeHV, NC_004812). Varicellovirus: including varicellazoster virus (VZV, X04370), bovine herpesvirus 1 (BoHV, AJ004801), equine herpesvirus 4 (EHV-4, NC_001844), Suid herpesvirus 1 (SuHV, BK001744). Mardivirus: including Marek's disease virus 1 (MDV-1, AF243438), Marek's disease virus 2 (MDV-2, NC_002577), turkey herpesvirus (HVT, NC_002641). Infectious laryngotracheitis virus (ILTV, NC_006623) belonged to Iltovirus. BoHV and SuHV were not included in the multiple alignment of US10 sequences; VZV was not included in the multiple alignment for *US2*; only MDV-1, MDV-2, HVT, and ILTV were included in the multiple alignment for *S3*

Table 4 Percentage of identity among herpesviruses *US10*, *S3*, and *US2* at the level of nucleotide and deduced amino acid sequences

Viruses	1	2	3	4	5	6	7	8	9	10	11
US10											
DEV	***	31.0	30.2	28.8	23.9	23.3	22.1	27.4	23.1	18.3	
MDV-1	18.6	***	54.8	39.5	19.3	20.1	19.8	18.7	25.2	19.5	
MDV-2	16.0	56.2	***	39.0	19.7	19.3	19.1	24.2	25.4	20.0	
HVT	15.2	34.6	32.0	***	20.3	20.6	21.4	26.0	25.0	23.5	
HSV-1	17.3	14.5	14.9	15.8	***	67.7	46.9	22.1	24.1	22.3	
HSV-2	18.6	13.4	11.7	14.7	59.0	***	44.4	24.5	26.5	21.3	
CeHV-1	17.3	16.3	12.7	16.2	32.2	32.9	***	25.0	23.2	24.1	
EHV-4	20.8	19.9	18.3	21.0	12.2	15.1	15.4	***	31.5	23.4	
VZV	16.5	16.0	13.6	14.0	14.8	12.8	16.0	24.7	***	22.5	
ILTV	15.6	12.3	12.7	14.7	11.0	11.5	12.8	14.7	12.3	***	
S3											
DEV	***	35.2	36.1	33.9	20.2						
MDV-1	32.5	***	56.2	46.1	19.3						
MDV-2	33.8	55.7	***	39.2	18.6						
HVT	30.2	41.7	39.8	***	18.9						
ILTV	18.3	13.0	15.8	11.8	***						
US2											
DEV	***	38.9	37.5	37.9	26.5	24.9	24.7	27.1	25.5	25.8	25.2
MDV-1	41.2	***	67.4	56.0	23.9	25.3	24.2	27.7	21.6	22.0	24.6
MDV-2	39.0	72.0	***	54.9	25.0	26.5	26.7	28.2	25.5	25.0	24.5
HVT	36.8	65.3	59.4	***	25.4	26.6	23.1	29.3	24.4	23.7	22.2
HSV-1	30.0	29.1	30.1	24.8	***	71.3	51.4	25.5	31.4	27.8	23.6
HSV-2	28.2	28.8	31.0	25.6	77.8	***	50.7	27.7	31.4	24.6	24.2
CeHV-1	23.2	28.5	29.9	24.3	53.8	51.5	***	25.0	33.2	31.5	21.6
EHV-4	24.1	30.5	29.9	26.4	27.3	29.0	28.0	***	26.7	23.1	26.1
BoHV-1	21.1	21.4	17.8	20.4	24.3	25.0	24.7	24.3	***	29.6	24.3
SuHV-1	18.3	20.6	21.8	15.9	21.8	18.8	18.2	19.7	17.8	***	21.4
ILTV	20.1	18.8	16.9	19.2	18.8	19.5	20.4	24.3	16.4	18.2	***

The right upper part represents nucleotide identity, the left lower part is the amino acid identity

Among the reference strains of *Alphaherpesvirinae*, only Avian herpesviruses have the *S3* gene, which has no orthologue among HSVs or any other mammalian herpesvirus. The function of HVT SORF3 or its MDV homolog, SORF3 [18], is not currently known.

The conservation of the US2 ORF among diverse herpesviral genomes is consistent with the proposal that this gene appeared early in the herpesvirus lineage. The gene encoding the US2 protein had no obvious *N*-terminal signal sequence or transmembrane domain, and only two *N*-linked glycosylation sites. It is conserved among alphaherpesviruses family members with the notable exception of the varicella-zoster virus; despite its wide conservation of sequence, no alphaherpesvirus requires the US2 protein for replication in cultured cells [19]. Even in animals, US2-null viruses show only modest attenuation. US2 contributes to virus penetration and efficient cell-to-cell spreading. Furthermore, it plays a role in sustained virus replication in vivo [20]. All *US2* homologs share a highly conserved stretch of 14 hydrophobic amino acids at the extreme amino terminus. It is suggested that the *US2* homologs may encode either a secreted or an *N*-terminally anchored transmembrane (glyco) protein [21, 22] and perhaps a common structure or function will be found in *US2*.

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