Identification and DNA Sequence Analysis of the Marek's Disease Virus Serotype 2 Genes Homologous to the Herpes Simplex Virus Type 1 UL20 and UL21

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ABSTRACT. We determined 3,135 bp of the nucleotide sequence located in an 8.5 kb *Eco*RI-E fragment in the unique long (UL) genome region of Marek's disease virus serotype 2 (MDV2), and identified UL20 and UL21 homologous genes of herpes simplex virus type 1 (HSV-1). The UL20 and UL21 homologous genes of MDV2 are arranged colinearly with the prototype sequence of HSV-1. In addition, an open reading frame (MDV2 ORF 273), which has been identified within the UL21 homologous gene of MDV2, has no apparent relation to any other known herpesvirus genes. Northern blot analysis and reverse transcriptase polymerase chain reaction confirmed the existance of RNA transcripts related to the UL20 and ORF 273 genes in MDV2-infected cells, except no transcript related to the UL21 gene being detected. The putative protein product of the MDV2 UL20 gene had a relatively low homology but that of the MDV2 UL21 gene had a moderate homology among herpesviruses. Further, the possible functions and features of the predicted proteins encoded within the sequenced region are discussed.—KEY WORDS: Marek's disease virus serotype 2, nucleotide sequence, ORF 273, UL20, UL21.

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Marek's disease virus (MDV) serotype 1 (MDV1) causes a naturally occurring malignant T cell lymphoma in chickens, known as Marek's disease (MD). The disease has attracted attention because it can be prevented by vaccination with antigenically related MDV, including attenuated MDV1, nonpathogenic MDV serotype 2 (MDV2) and herpesvirus of turkeys (HVT), which is also termed MDV serotype 3. A bivalent vaccine consisting of MDV2 and HVT shows better protection against MD caused by a very virulent MDV1 than a monovalent vaccine, MDV2 or HVT alone [22]. Because of similar biological properties, especially its lymphotropism, three serotypes of MDV have been classified as subfamily *Gammaherpesvirinae* [22]. However, all three MDV serotypes resemble alphaherpesviruses in their genomic structure and gene arrangement [5, 11, 20]. They consist of two covertly linked components, L and S, each consisting of unique sequences (UL and US) flanked by inverted repeats [8, 23, 27]. However, the restriction endonuclease digestion patterns vary among MDV1, MDV2, and HVT [10, 21, 26]. In MDV2 genome, genes encoding for glycoproteins gB [30], gC [15], gD [13], gE [12], gH [24], and gI [13], thymidine kinase and UL24 [25], and phosphoprotein pp38 [19] homologues have been identified and analyzed.

The mechanism of virus maturation and egress to the extracellular space of alphaherpesviruses have not been fully understood. Recently, it is reported that UL20 protein of herpes simplex virus type 1 (HSV-1) associates with viral egress as intrinsic membrane protein [2], and UL21 protein of HSV-1 plays an important role in viral replication in cultured cells [1, 4].

In this article, we report complete nucleotide sequences of genes encoding for the UL20 and UL21 in the MDV2 UL genome region and compare with other herpesvirus counterparts including HSV-1, varicella-zoster virus (VZV), bovine herpesvirus type 1 (BHV-1) and pseudorabies virus (PRV) of the subfamily *Alphaherpesvirinae*, and Epstein-Barr virus (EBV) of the subfamily *Gammaherpesvirinae*. Further, we examine transcripts of each gene, and describe some of the properties of the putative proteins encoded by the MDV2 UL20 and UL21 genes.

MATERIALS AND METHODS

Cloning, DNA sequencing and computer analysis: A DNA library of MDV2 strain HPRS24 constructed previously [20] was used. The 120 bp of 3' terminus of the MDV2 UL21 gene was previously reported [24]. To obtain the entire MDV2 UL21 gene and further upstream sequence of the MDV2 UL21 gene, a 3.1 kb *Cla*I-*Pst*I subfragment from the *Eco*RI-E fragment (Fig. 1) was cloned into pBluescript SK+ vector (Stratagene, La Jolla, CA).

DNA sequences were determined on both strands using the Dye primer cycle sequencing kit (Applied Biosystem, Foster City, CA) by an autosequencer system (Applied Biosystem, Foster City, CA). DNA and amino acid sequences were analyzed with the computer program GENETYX-MAC (version 9.0). Homology searches and multiple sequence alignments from the GenBank and EMBL Data Libraries were performed by using the UWGCG programs BESTFIT and PILEUP, respectively.

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Preparation of total RNA from MDV2-infected cells: Primary chicken embryo fibroblasts (CEFs) were infected with MDV2 strain HPRS24 or mock infected at 3×10^6 cells per a 60-mm-diameter dish as described previously [12–14]. To isolate total RNA, monolayer cells of virus- or mock-infected CEF were harvested at 96 hr postinfection and treated with 0.05% ethylenediamine tetraacetic acid in phosphate-buffered saline. Total RNAs were extracted from these lysates by ISOGEN (Nippon Gene, Tokyo, Japan) according to the recommendation of the supplier.

Preperation of DNA probes and northern blot analysis: In order to identify the transcriptional products of the sequenced region, four DNA probes were prepared for the northern blot analysis. Probe I was prepared from the *Eco*RI-E fragment by digestion with *Eco*RI and *Cla*I restriction enzymes (approximately 0.8 kb). Probe II was amplified with the sequenced subfragment using primers 5'-CACAGCTACTGTAGCGTAGG-3' (nucleotide position from 504 to 523) and 5'-TTCTGGTGTACGCGATGGAC-3' (nucleotide position from 838 to 819) as described elsewhere [13]. Probe III was also prepared from the sequenced subfragment by digestion with *Xba*I and *pfl*MI enzymes (approximately 0.7 kb) (Fig. 1). Probe IV was amplified with the sequenced subfragment using primers 5'-GGTTGTGGAAATAGTTGCGGG-3' (nucleotide position from 2,462 to 2,482) and 5'-CAAAGTGTTCCAT GTAGCCGC-3' (nucleotide position from 2,787 to 2,767) as described elsewhere [13]

Total RNA (10 μg per lane) was separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane sheet (Biodyne, Pall BioSupport, NY). DNA probes were radiolabeled with [α-^{32}P]dCTP using a nick translation kit (Boehringer Manheim GmbH, Germany), and hybridized to RNA under stringent conditions as described previously [9].

Reverse transcriptase (RT) PCR analysis: Total RNA was treated with DNase I (Boehringer Mannheim) at 25°C for 30 min and heated to 94°C for 5 min to denature DNase I. Each of the DNase I-treated samples were further treated with 10 units of RT (Gibco BRL, Grand Island, NY) at 45°C for 1 hr with only the first primer (Fig. 2) and heated to 96°C for 5 min. Each of the RT-treated RNA sample was thermocycled at 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min for 33 cycles with the GeneAmp PCR reagent kit and AmpliTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan) with both first and second primers.

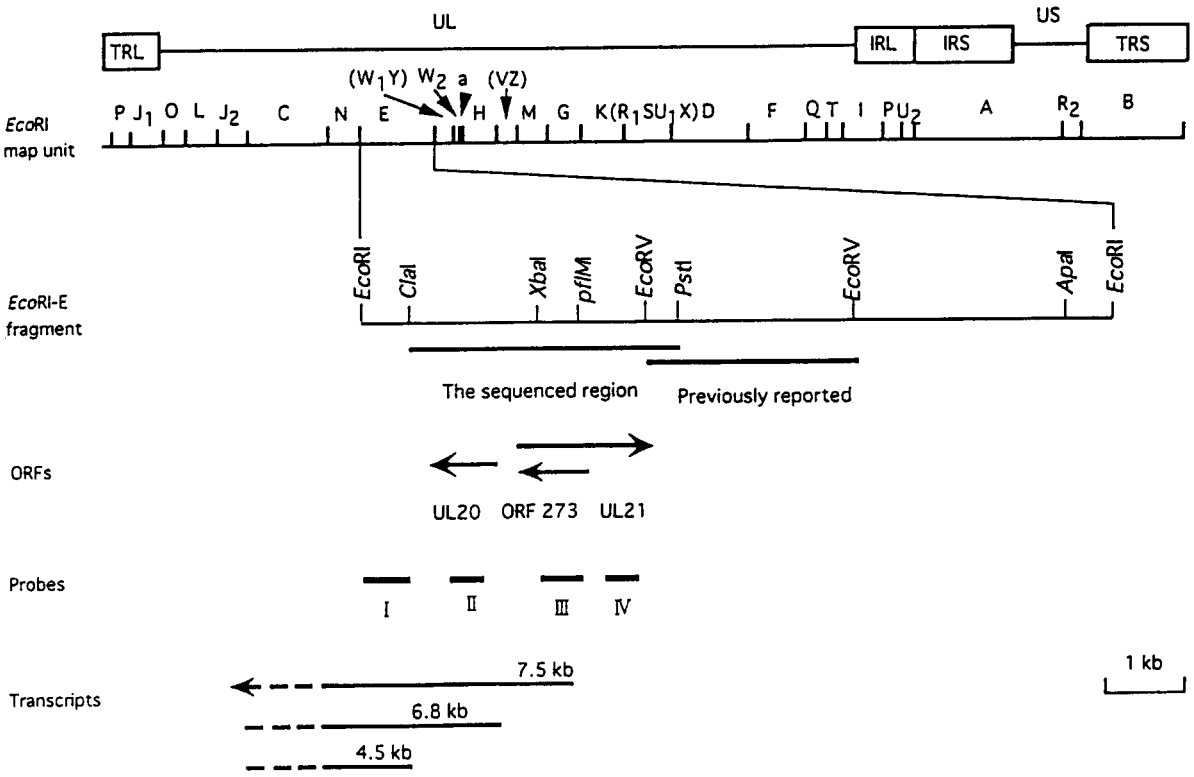

Fig. 1. Diagram of the MDV2 genome. The genome is organized into the unique long (UL) and short (US), internal repeat long (IRL) and short (IRS), and terminal repeat long (TRL) and short (TRS) regions. A 3.1 kb subfragment between *Cla*I and *Pst*I site from an 8.5 kb *Eco*RI-E fragment was cloned, and the DNA sequence of UL20, ORF 273 and UL21 homologous genes of MDV2 was determined. The locations and directions of the UL20, ORF 273 and UL21 genes are shown under the sequenced region. The locations of four DNA fragments used as probes in the northern blot analyses, and three transcripts detected by the probes are indicated under three identified genes. The approximate sizes of the three probes are as follows: I, 0.8 kb; II, 0.3 kb; III, 0.4 kb; IV, 0.3 kb.

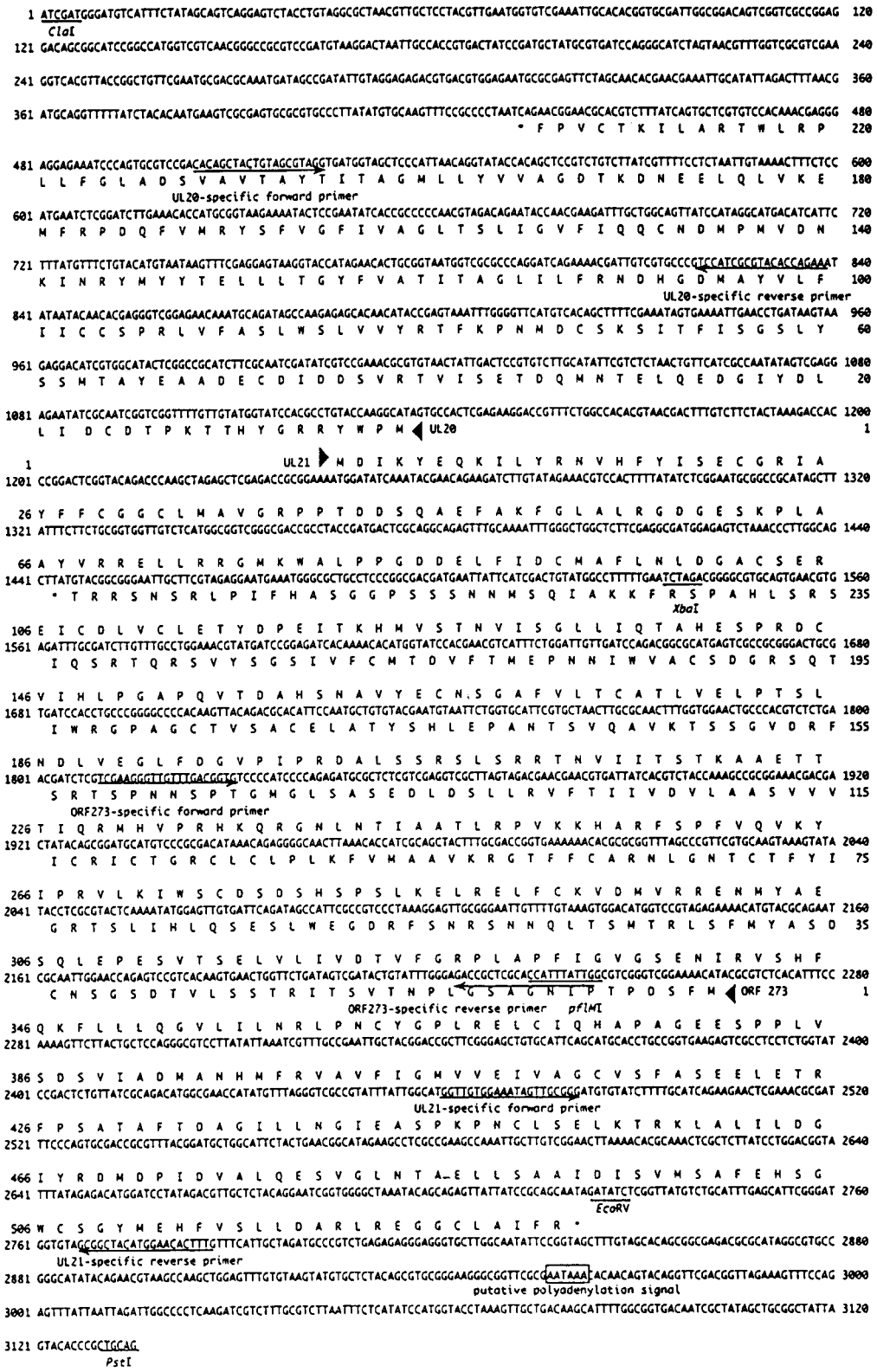

Fig. 2. Nucleotide and predicted amino acid sequences of the 3,135 bp of sequenced region of MVD2. The predicted amino acid sequence encoded by the forward strand (UL21) is shown above the corresponding nucleotide sequences in a single-letter code, while the reverse strand are shown below (UL20 and ORF 273), and stop codons are represented by asterisks. Restriction enzyme sites are underlined and putative polyadenylation signal is shown by boxes.

The reacted mixtures were electrophoresed on a 2% agarose gel with ethidium bromide, and detected by UV trans-illuminator.

RESULTS AND DISCUSSION

Nucleotide sequence and gene arrangement: We determined the nucleotide sequence of the *Cla*I-*Pst*I subfragment located in the *Eco*RI-E fragment of the MDV2 UL region (Fig. 1). This sequenced region comprises 3,135 nucleotides with an overall G+C content of 49.2%. The complete DNA sequence is shown as the rightward 5' to 3' strand only (Fig. 2). Details have been deposited in the DDBJ Data Library with an accession number of AB018252. Within the family *Herpesviridae*, this region has been reported in EBV [3], HSV-1 [18], VZV [6], BHV-1 [31] and PRV [16], and we took advantage of these sequenced data from the GenBank and EMBL Data Libraries to identify the corresponding MDV2 genes.

An open reading frame (ORF) encoding a protein homologous to the HSV-1 UL20 gene was found in the leftward transcriptional orientation of the sequenced region (Fig. 1). With a translational start codon at position 1,136 and a stop codon at position 432, it comprised 702 nucleotides (Table 1). The MDV2 UL20 homologous gene was able to encode for the protein product of 234 amino acids with a molecular weight of 26.6 kDa (Table 1). It is conserved in all alphaherpesviruses examined (HSV-1, VZV, BHV-1 and PRV), but not in gammaherpesvirus (EBV).

The second ORF encoding a protein homologous to the HSV-1 UL21 gene was found in the 5' end of the MDV2 UL20 gene in a head-to-head orientation (Fig. 1). With a translational start codon at position 1,245 and a stop codon at position 2,843, it comprised 1,596 nucleotides (Table 1). The MDV2 UL21 homologous gene was able to encode for the protein product of 532 amino acids with a molecular weight of 58.8 kDa (Table 1). It is conserved in all herpesviruses examined (EBV, HSV-1, VZV, BHV-1 and PRV).

The third ORF, which have no apparent relation to any herpesviral sequences at present, was found with the same transcriptional orientation upstream of the MDV2 UL20 homologous gene, and completely overlapped to the MDV2 UL21 homologous gene (Fig. 1). With a translational start codon at position 2,263 and a stop codon at position 1,442, it comprised 819 nucleotides (Table 1). The ORF 273 gene was able to encode for the protein product of 273 amino acids with a molecular weight of 29.6 kDa (Table 1). The similar gene arrangement of the MDV2 ORF 273 is not found in most of the herpesviruses such as BHV-1 [31], canine herpesvirus [29], EBV [3], PRV [16] and VZV [6].

Transcriptional mapping of the UL20, UL21 and ORF 273 genes of MDV2: In order to determine whether the transcripts of the identified genes truly exist, northern blot analysis was performed. As shown in Fig. 3A, three RNA transcripts (7.5 kb, 6.8 kb and 4.5 kb) were determined. A 7.5 kb transcript was commonly hybridized to the gene-specific DNA probes I, II and III, respectively (Fig. 3A). A 6.8 kb transcript was hybridized by probes I and II, but not probe III (Fig. 3A). A 4.5 kb transcript was hybridized only by probe I. Although a putative polyadenylation signal sequence (AATAAA) was observed in downstream of the MDV2 UL21 gene, and we tried to detect transcripts related to the MDV2 UL21 gene by using probe IV, no respective transcript was hybridized at least 20 μg per lane total cellular RNA-blotted membranes (data not shown). These results indicate that the RNA transcripts, from which the MDV2 UL20 and ORF273 genes are likely to be translated, may exist in the MDV2 infected cells.

Table 1. Properties of the identified MDV2 genes

Gene	Start	Stop	Primary translation products: Numbers of residues	Molecular weight (Da)	Isoelectric point (pKa)
UL20*	1,136	432	234	26,638	4.5
UL21	1,245	2,843	532	58,845	5.5
ORF273*	2,263	1,442	273	29,590	9.0

* Located in the reverse DNA strand.

Furthermore, the 7.5 kb RNA transcript was implied by reverse transcriptase (RT) PCR (RT-PCR) to be the strand encoding the UL20 and ORF273 genes (Fig. 3B). Specific amplified products, whose length were approximately equal to the length between the two primers shown in Fig. 2, were found in RT-PCR with RT treatments by using a forward primer in the UL20 region and reverse primers in UL20 and ORF273 regions, whereas no product was found in RT-PCR with RT treatments by using forward primers in ORF273 and UL21 regions and a reverse primer of UL 21 region (Fig. 3B). These results represent that the 7.5 kb RNA is transcribed from the leftward transcriptional orientation of the sequenced region.

From these results, it is likely that the 7.5 kb transcript was considered to be a large read-through transcript including both UL20 and ORF 273 genes and possibly other genes of downstream on this strand in the MDV2 UL region. On the other hand, the orientation of the 4.5 kb and 6.8 kb transcripts could not be confirmed at least in this study. Moreover, it is not known at present whether its translational products are expressed indeed in the virus-infected cells.

Properties of encoded proteins of the MDV2 UL20 and UL21 genes: Compared from the three putative primary translation products of the MDV2 genes, UL20 protein had notably low isoelectric point (pKa=4.5) (Table 1). As shown in Fig. 4, hydropathicity analysis indicated that the MDV2 UL20 protein had three notably hydrophobic domains, which might be possible transmembrane sequences as observed other herpesviral counterparts [28]. From the homology analysis of the deduced protein with the corresponding protein products of the HSV-1 UL20 gene [18] and its homologues of VZV [6], BHV-1 [31] and PRV [16], the MDV2 UL20 protein exhibits relatively low homology to

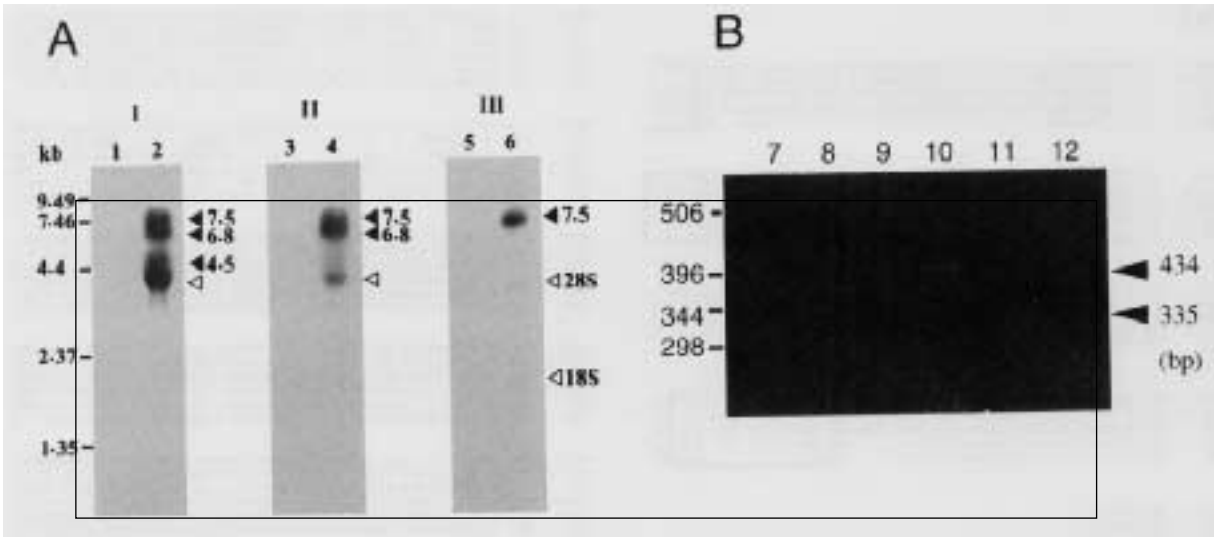

Fig. 3. Transcripts from the region whose sequence was determined. (A) Northern blot analysis in the sequenced region of the MDV2 UL region. Lanes 1, 3 and 5 show the hybridization results between virus-specific probes and total RNA from mock-infected cells for control. Lanes 2, 4 and 6 show the hybridization results between virus-specific probes and total RNA from MDV2-infected cells. Molecular sizes (kb) of the RNA markers (Gibco BRL, Grand Island, NY) are indicated at the leftmost. Open arrowheads denote locations of 28S and 18S rRNAs. (B) Reverse transcriptase (RT) PCR analysis. Lane 7 and 8 show the RT-treatment results by UL20-specific forward and reverse primers. Lane 9 and 10 show the RT-treatment results by ORF273-specific forward and reverse primers. Lane 11 and 12 show the RT-treatment results by UL21-specific forward and reverse primers. Molecular sizes (kb) of the DNA markers (Gibco BRL, Grand Island, NY) are indicated at the leftmost.

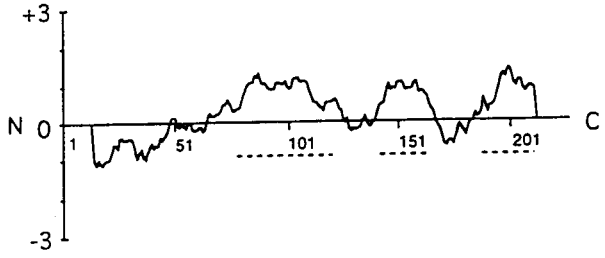

Fig. 4. Hydropathicity plot of the putative protein of the MDV2 UL20 gene. N and C represent the amino and carboxyl termini of the predicted proteins. Hydrophobic regions are shown above and hydrophilic regions below the X-axes. The algorithm of Kyte and Doolittle [19] was used, with a sliding window set to 10 amino acid residues. The dashed lines indicate notably hydrophobic region that also considered likely to be membrane-spanning.

Table 2. Identity of the predicted amino acid sequences between MDV2 and other herpesviruses*

Gene	Identity (%)				
	EBV	HSV-1	VZV	BHV-1	PRV
UL20	DC	25	28	26	30
UL21	42	38	29	36	39

* The entire UL20 and UL21 amino acid sequences were used. Values were obtained using the UWGCG program BESTFIT and expressed as percentage identity. DC, does not contain this homologue.

those of the other four alphaherpesviruses (between 30% and 25%) (Table 2). However, three hydrophobic domain sequences are relatively conservative among all alphaherpesviruses, particularly in the third hydrophobic domain (Fig. 5A). From these results, the property of the MDV2 UL20 protein appears to be similar among them as membrane spanning. The HSV-1 UL20 protein is necessary for viral replication in cultured cells [2]. Therefore, clarification of the role of this essential membrane protein may be important to understand the MDV replication.

On the other hand, the MDV2 UL21 gene is conserved in all herpesviruses examined (EBV, HSV-1, VZV, BHV-1 and PRV). It encodes the protein moderately homologous to the predicted UL21 products of EBV [3], HSV-1 [18], VZV [6], BHV-1 [31] and PRV [16] (between 42% and 29%), paticuraly in the amino-terminal half (Fig. 5B). It was reported that the HSV-1 UL21 protein was a virion component, probably tegument, but dispensable for growth in cultured cells [1], while the PRV UL21 homologue is essential element [7]. Moreover, unlike the PRV UL21 homologue, the HSV-1 UL21 does not facilitate DNA cleavage and packaging in cultured cells [1]. The apparent discrepancy may reflect the possibilities that the respective genes have different functions each other in some herpesviruses.

Although the three homologous genes of MDV2 were firstly identified among MDV genomes, further analysis for the role of these genes are required.

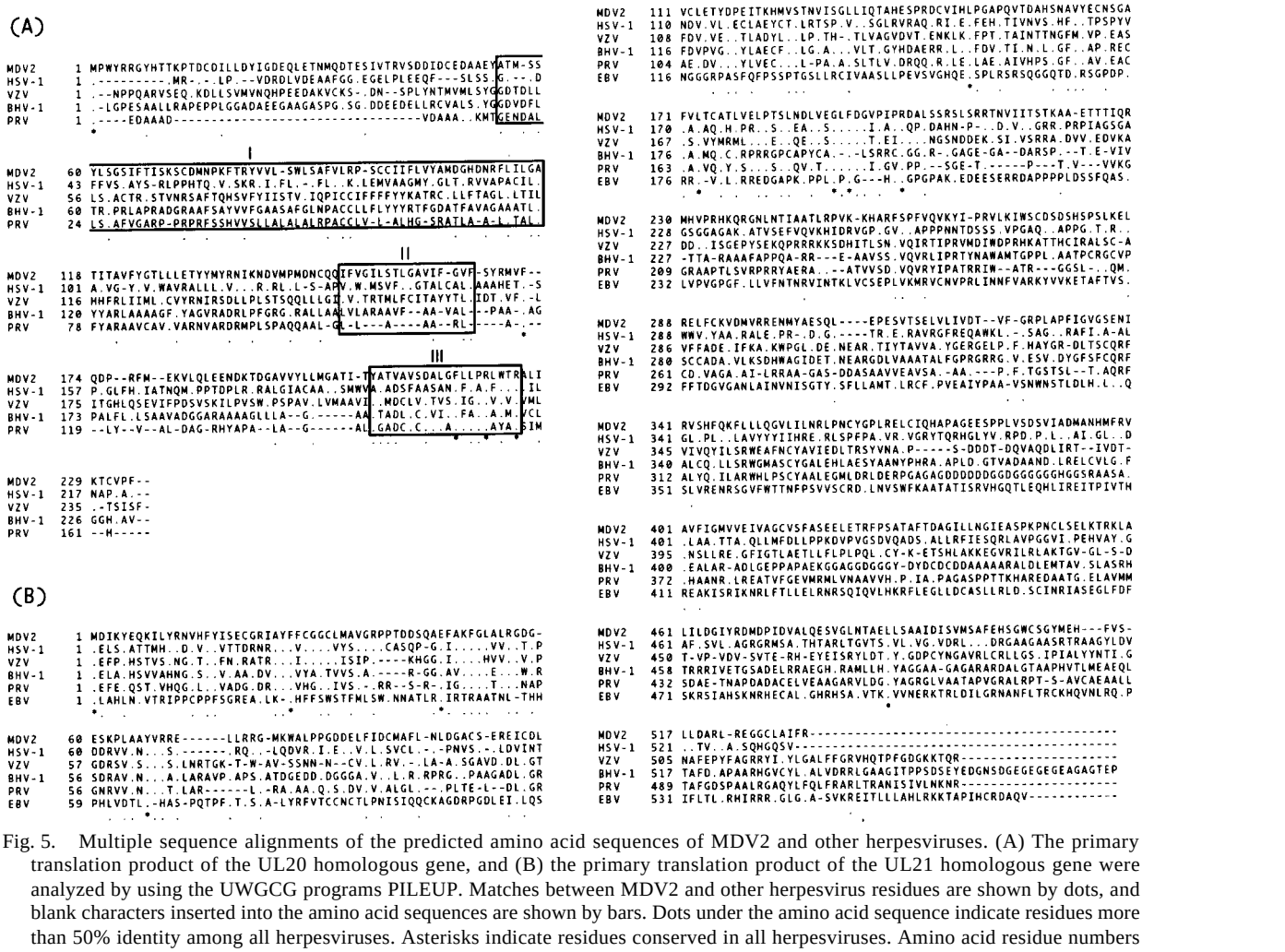

Fig. 5. Multiple sequence alignments of the predicted amino acid sequences of MDV2 and other herpesviruses. (A) The primary translation product of the UL20 homologous gene, and (B) the primary translation product of the UL21 homologous gene were analyzed by using the UWGCG programs PILEUP. Matches between MDV2 and other herpesvirus residues are shown by dots, and blank characters inserted into the amino acid sequences are shown by bars. Dots under the amino acid sequence indicate residues more than 50% identity among all herpesviruses. Asterisks indicate residues conserved in all herpesviruses. Amino acid residue numbers (not including blank characters) are shown on left side of the alignment. Three boxed regions (I, II, III) represent hydrophobic domains in the UL20 homologous protein.

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