Identification and DNA Sequence Analysis of the Marek's Disease Virus Serotype 2 Gene Homologous to the Herpes Simplex Virus Type 1 Glycoprotein H

Yukako SHIMOJIMA, Hyung-Kwan JANG, Mitsuru ONO[1)], Chieko KAI, and Takeshi MIKAMI*

Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113 and [1)]Biological Research Laboratories, Sankyo Co., Ltd., 1–2–58 Hiromachi, Sinagawa-ku, Tokyo 140, Japan

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ABSTRACT. Marek's disease virus (MDV) serotype 2 (MDV2) gene homologous to the glycoprotein H (gH) gene of herpes simplex virus type 1 was identified and sequenced. The predicted region encoding for the MDV2 gH gene was 2436 nucleotide and the primary translation product was 812 amino acids with a molecular weight of 89.4 kDa. The protein encoded by MDV2 gH gene has a number of features characteristic of a membrane-associated glycoprotein. First, there are 9 potential N-linked glycosylation sites and 11 cysteine residues, and 6 of the sites and 8 of the residues were conserved among all of the three MDV serotypes. Second, this protein had N-terminal and C-terminal hydrophobic regions, which were a signal sequence and a transmembrane-anchor domain, respectively. From the northern blot analysis, it was suggested that a transcript encoding MDV2 gH and a poly-cistronic transcript encoding MDV2 thymidine kinase, gH, and possibly other genes of downstream on this strand existed. Alignment of the amino acid sequences of the gH homologues among the three MDV serotypes showed 57.5% (MDV1 and MDV2), 56.2% (MDV1 and HVT), and 50.1% (MDV2 and HVT) identities.
— KEY WORDS: glycoprotein H, Marek's disease virus serotype 2.

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Marek's disease virus (MDV) serotype 1 (MDV1) causes a naturally occurring contagious 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) known as a 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 [34]. Because of similar biological properties, especially its lymphotropism, three serotypes of MDV have until recently been classified as gammaherpesviruses [31, 32]. However all three MDV serotypes resemble alphaherpesviruses in their genomic structure and gene arrangement [2, 14, 28]. The restriction endonuclease digestion patterns vary among MDV1, MDV2, and HVT [12, 33, 37]. It has also been reported that the different three serotypes of MDV could produce serotype-specific monoclonal antibodies [24]. The surface glycoproteins of alphaherpesviruses play prominent roles in the infectious process, and are predominant targets for both humoral and cellular immunity in the host. Therefore, studies on the MDV2 genome structure and its gene products may be important for vaccine improvement and understanding of the difference in characteristics among the MDV three serotypes. So our laboratory has already reported the full nucleotide sequences of genes encoding for glycoproteins C (gC), D (gD), E (gE), and I (gI) in the genome of MDV2 strain HPRS24 [15–17, 20]. Further, glycoprotein B (gB) has also been reported in MDV2 strain SB-1 [40].

Glycoprotein H (gH) is conserved in herpesviruses belonging to all of the three subfamilies of alpha-, beta- and gamma-herpesviruses such as herpes simplex virus type 1 (HSV-1) [8, 25], varicella zoster virus (VZV) [19], human cytomegalovirus [3], Epstein-Barr virus [1], and herpesvirus saimiri [9]. In alphaherpesviruses, gH appears to be essential for viral replication [4] and implicated in virus entry and cell-to-cell spread [8, 19, 29]. Further, antibodies raised against gH homologues neutralize the infectivity of HSV-1, VZV and so on [7, 18]. However, HSV-1 gH alone synthesized in a mammalian cell expression system or recombinant vaccinia virus-infected cells was slightly smaller than authentic HSV-1 gH and antigenically distinct from it, and not transported to the cell surface [6, 10]. Because HSV-1 gH required glycoprotein L (gL) for proper posttranslational processing and transport to the cell surface [13]. Similarly, gH and gL associations have also been described in VZV [5], pseudorabies virus (PRV) [22], and equine herpesvirus type 1 (EHV-1) [38].

In MDV1 and HVT genomes, the potential open reading frames (ORFs) which are homologous to the HSV-1 gH gene were reported [35]. The predicted primary polypeptide products of the MDV1 gH and HVT gH ORFs were 813 and 808 amino acids, respectively. Using polyclonal antiserum against MDV1 gL, gL coprecipitated with a molecular weight of 115 kDa which was predicted to be MDV1 gH from the size of the primary translational product. Further, MDV1 gL expressed alone was not properly processed to a mature form [41]. However, there is no evidence of the complex formation of gH and gL in MDV. Further it is unknown that the gH-gL complex is required

* CORRESPONDENCE TO: Prof. MIKAMI, T., Department of Veterinary Microbiology, Graduate School of Agriculture and Agricultural Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.

for normal process and transportation to the cell surface. The purpose of the present study was to identify the gene encoding the gH homologue of the MDV2 strain HPRS24 (MDV2 gH) which might be involved in vaccinal immunity and viral replication, and to characterize its transcript(s). We also compared the nucleotide sequences of the gH homologous genes among the three serotypes of MDV.

MATERIALS AND METHODS

Cloned viral DNA: A DNA library of MDV2 strain HPRS24 constructed previously [28] was used. The 5' terminal part of MDV2 gH ORF was previously reported [36]. To obtain the rest part of MDV2 gH ORF, a 2.5 kb *Eco*RV subfragment from the *Eco*RI-E fragment (Fig. 1C) was cloned, and then various subfragments digested with restriction endonucleases were subcloned into pBluescript SK+ vector (Stratagene, La Jolla, CA).

DNA sequencing and computer analysis of data: DNA sequences were determined on both strands using an autosequencer system (Applied Biosystems, Foster City, CA). DNA and amino acid sequences were analyzed with the computer program GENETYX-MAC (version 8.0). Homology searches and multiple sequence alignments from the GenBank and EMBL Data Libraries were performed using the UWGCG programs BESTFIT and PILEUP, respectively.

Preparation of total RNA from MDV2-infected cells: Primary chicken embryo fibroblasts (CEF) were infected with MDV2 strain HPRS24 or mock infected at 3×10^6 cells per a 60-mm-diameter dish and maintained as previously described conditions [15–17]. To isolate total RNA, monolayers of virus- or mock-infected CEF were harvested at 96 hr postinfection (PI) and treated with 0.05% ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Total RNAs were extracted from these lysates by ISOGEN (Nippon Gene, Tokyo, Japan) according to the recommendation of the supplier.

Northern blot analysis: Three subclones from the *Eco*RI-E genomic DNA were used as probes, designated in the order of their mapping as I, II, and III (Fig. 1D). Total RNA (10 μg per lane) was separated on a 1.2% agarose formaldehyde-gel and transferred to a nylon membrane filter (Biodyne, Pall BioSupport, NY). DNA probes were radiolabeled with [α-^{32}P]dCTP by Nick Translation Kit (Boehringer Mannheim GmbH, Germany), and hybridized to RNA under stringent conditions as described previously [11].

RESULTS

Identification and sequencing of the MDV2 gH gene: The gH homologous genes are conserved downstream of the thymidine kinase (TK) homologous genes in various herpesviruses [8, 21, 25] including MDV1 and HVT [35]. As shown in Fig. 1, the complete nucleotide sequences of MDV2 UL24 and TK homologues, and the partial nucleotide sequences of the N-terminal region of the potential MDV2

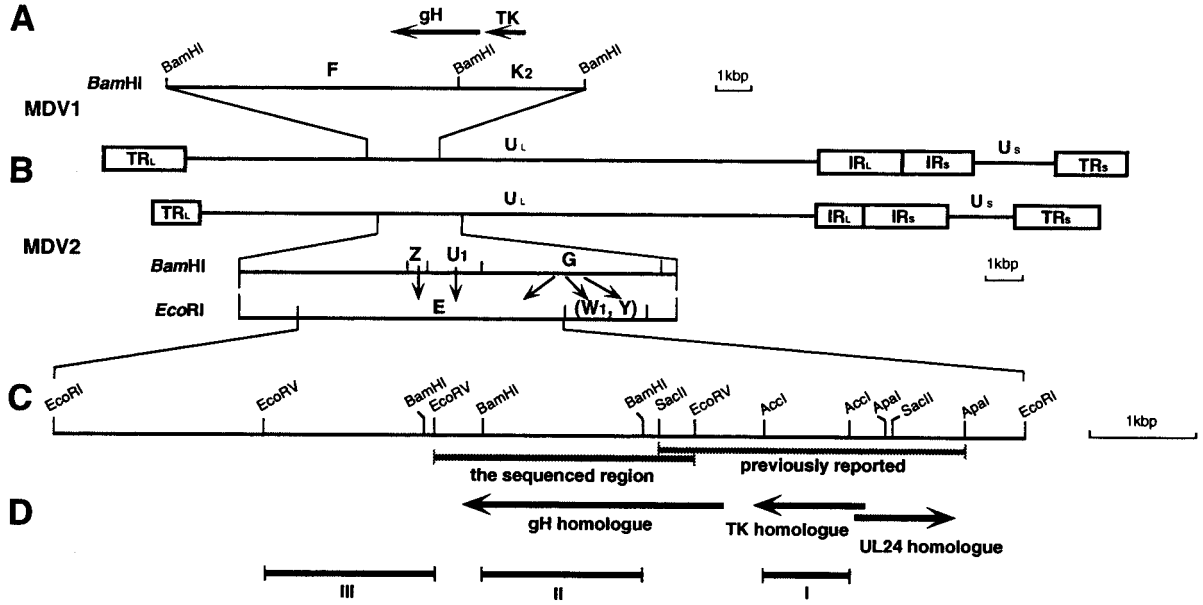

Fig. 1. Diagrams of the MDV1 and MDV2 genomes. These genomes are organized into the unique long (U_L) and short (U_S), internal repeat long (IR_L) and short (IR_S), and terminal repeat long (TR_L) and short (TR_S) regions. (A) The MDV1 TK and gH homologous regions are determined by Scott *et al.* [35]. (B) A *Bam*HI-G fragment showed colinearity with two *Bam*HI-K2 and -F fragments in MDV1, and with three *Eco*RI-W1, -Y, and -E fragments. *Bam*HI-U1 and -Z fragments showed colinearity with *Bam*HI-F fragment in MDV1, and with *Eco*RI-E fragment in MDV2 [28]. (C) A 2.4 kb *Eco*RV subfragment from a 9.1 kb *Eco*RI-E fragment were cloned, and the nucleotide sequence of a total 2451 bp containing the partial gH homologous genes was determined. (D) The locations and directions of the MDV2 UL24 , TK [36], and gH ORFs are indicated by bold arrows. The subclones used as probes in the northern blot analysis are shown as bold lines. The approximate sizes of the subclones are as follows: I, 0.8 kb; II, 1.5 kb; III, 1.5 kb.

gH, have been submitted to the DDBJ Data Library Accession No. D85421 [36]. To investigate the remaining C-terminal region of the MDV2 gH based on the DNA sequences, we cloned a 2.5 kb *Eco*RV subfragment from a *Eco*RI-E fragment of MDV2 (Fig. 1C), and determined the nucleotide sequence of 2451 bp (Fig. 2). As a result, an ORF starting at position −275 and ending at position 2161 was identified. The predicted primary translation product of this ORF was 812 amino acids long, which is similar in length when compared with gH homologues of HSV-1 (838 amino acids) [8, 25], VZV (841 amino acids) [19], bovine herpesvirus type 1 (BHV-1; 842 amino acids) [26], equine herpesvirus type 4 (EHV-4; 855 amino acids) [27], EHV-1 (848 amino acids) [30], MDV1 (813 amino acids), and HVT (808 amino acids) [35].

Analysis of the MDV2 gH nucleotide sequence: As observed in various MDV genes [15–17, 20, 40] including MDV1 and HVT gH genes [35], translation start codon of the ORF corresponds quite well to functional initiation site according to a report by Kozak [23], and several potential transcriptional regulatory sites lie adjacent to the ORF (Fig. 2). A TATA box lies at position −361 to −358, and a CAAT box lies at position −487 to −484 upstream of the start codon of MDV2 gH. A potential poly (A) signal sequence, AATAAA, is located at position 2167 to 2172, and the GT cluster, TGTTGTGT, that is required for efficient formation of mRNA 3' termini, is located at position 2205 to 2212. The nucleotide composition of the MDV2 gH gene is 25.7% A, 24.5% T, 25.4% C, and 24.4% G.

Northern blot analysis: In order to confirm the transcription of the gH gene, northern blot analyses were performed. The TK-specific probe (probe I) hybridized to transcripts of 7.8, 5.0, 3.5, and 1.1 kb. The gH-specific probe (probe II) hybridized to transcripts of 2.4 and 4.2 kb, along with 7.8, 5.0, and 3.5 kb transcripts. These results using the TK-specific probe (probe I) and the gH-specific probe (probe II) were thoroughly consistent with our previous report [36], and suggested that the 2.4 kb transcript detected by the probe II encodes the gH, which was a reasonable length when compared with the length from the TATA box to the poly (A) signal of gH ORF, approximately 2.5 kb. The 2.4 kb transcript was not hybridized by the probe III localized downstream the MDV2 gH ORF. Furthermore, the probe III hybridized to a transcript of 7.8 kb, which was hybridized by the probe I and II (data not shown). This result suggested that the 7.8 kb transcript detected by all of the probes I, II, and III, encodes the TK, gH, and possibly other genes of downstream encoded on this strand as observed in other alphaherpesviruses [21, 39].

Characteristics of the predicted amino acid sequence of MDV2 gH: As presented in Fig. 2, the predicted amino acid sequence of the gH homologue was deduced from the nucleotide sequence. This predicted polypeptide has a molecular weight of 89.4 kDa, compared to 90.8 kDa of MDV1 and 91.1 kDa of HVT [35]. The protein encoded by MDV2 gH gene has a number of features characteristic of a membrane-associated glycoprotein. There are nine potential N-linked glycosylation sites and eleven cysteine residues. By hydropathicity analysis of the predicted MDV2 gH, this protein had the N-terminal hydrophobic signal sequence for the transport of membrane-associated proteins between residues 1 and 15, and the C-terminal hydrophobic region, of which predicted function was transmembrane-anchor domain, between residues 761 and 787.

Alignment of the predicted amino acid sequences of MDV2 gH with MDV1 and HVT gHs: Alignment of the predicted amino acid sequences of the gH homologues among the three MDV serotypes was shown in Fig. 3. Homologies between MDV1 and MDV2, MDV1 and HVT, and MDV2 and HVT were 57.5%, 56.2%, and 50.1%, respectively. Further comparison of the amino acid composition of the MDV2 gH with other alphaherpesvirus gH homologues showed homology of 26.9% with PRV [21], 26.1% with VZV [19], 25.5% with BHV-1 [26], 24.1% with EHV-1 [30], 24.0% with EHV-4 [27], and 23.5% with HSV-1 [8, 25]. Out of eleven cysteine residues in MDV2 gH, eight were conserved among all of the three MDV serotypes. Similarly, six of the nine potential N-linked glycosylation sites in MDV2 gH were also conserved among all of the three MDV serotypes. Judging from the location of conserved N-glycosylation sites and cysteine residues, the C-terminal part has higher homology in gH homologues of all MDV serotypes. These results suggest that the structures of gH homologues may be similar among MDV three serotypes.

DISCUSSION

In the present study, we determined 2451 bp of the nucleotide sequence located in the 9.1 kb *Eco*RI-E fragment, and presented additional 672 bp upstream of the nucleotide sequence (Fig. 2). This sequence contains an ORF capable of encoding a polypeptide of 812 amino acids with homology to HSV-1 gH, which is downstream of the MDV2 TK homologous gene. The gene order is identical to those of MDV1, HVT, and other alphaherpesviruses. In the restriction endonuclease map of MDV2 strain HPRS24, the exact order of *Bam*HI-U1 and -Z fragments, which were flanked with *Bam*HI-G fragment in their internal region, had not been determined [28]. When we sequenced the partial *Bam*HI-U1 and -Z fragments and compared their nucleotide sequences with that of the 2451 bp *Eco*RV subfragment (data not shown), the sizes of *Bam*HI-U1 and -Z fragments were about 1.5 and 0.5 kb, respectively [28], and a *Bam*HI fragment next to *Bam*HI-G fragment was 1477 bp (Fig. 2). From these results, it was suggested that the *Bam*HI-Z fragment was located in the more terminal region than the *Bam*HI-U1 fragment in the unique long (U_L) region (Fig. 1B).

By northern blot analysis, besides the possible transcript of MDV2 gH, a large readthrough transcript predicted to encode both of the MDV2 TK and gH was detected. The significance of bi-cistronic transcripts encoded by TK and

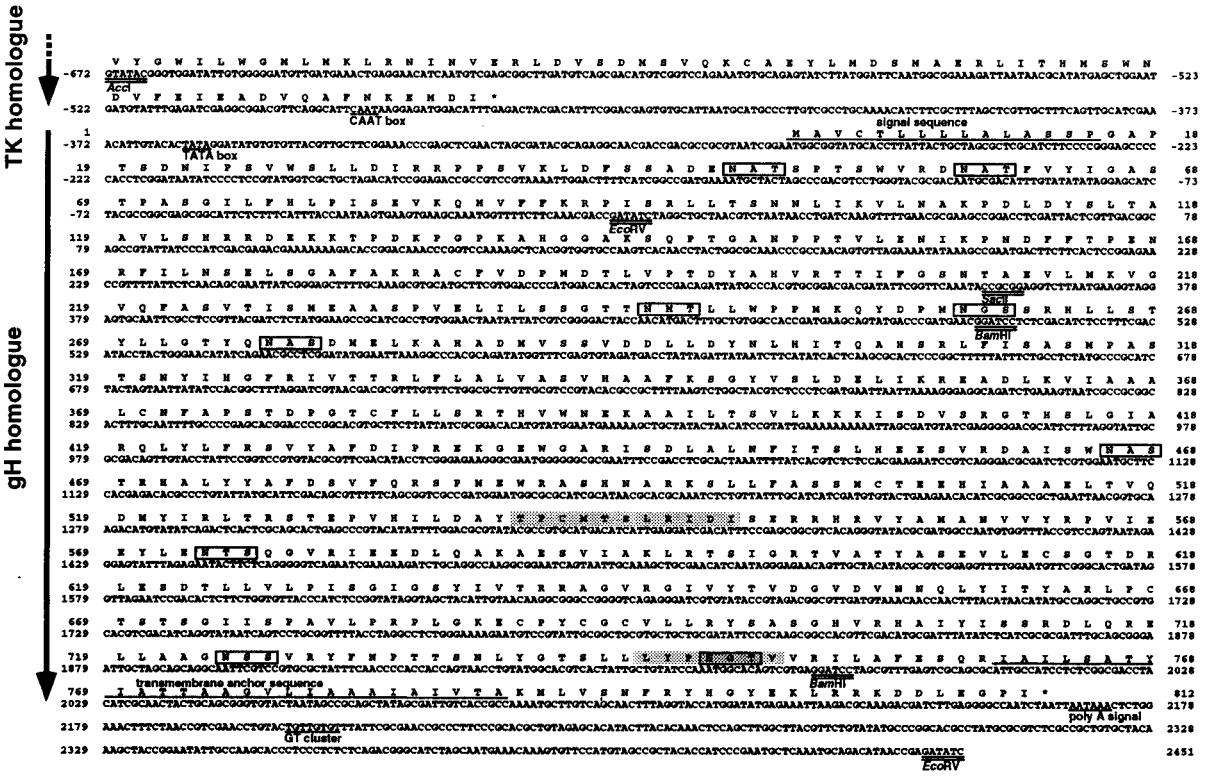

Fig. 2. Nucleotide and predicted amino acid sequences of a portion of the gH ORF (-275 to 2161). The predicted amino acid sequences are shown above the corresponding nucleotide sequences in a single-letter code and the stop codon is represented by asterisk. The ORF orientation is shown in the left of the sequence panel with a bold arrow. Restriction enzyme sites are double-underlined. Potential transcription regulatory elements, such as TATA box, CAAT box, poly A signal, and GT cluster are indicated by dashed lines. The conserved motif regions in gH homologues [21] are shaded and hydrophobic signal and transmembrane anchor sequences are underlined. Potential N-linked glycosylation sites are shown by boxes.

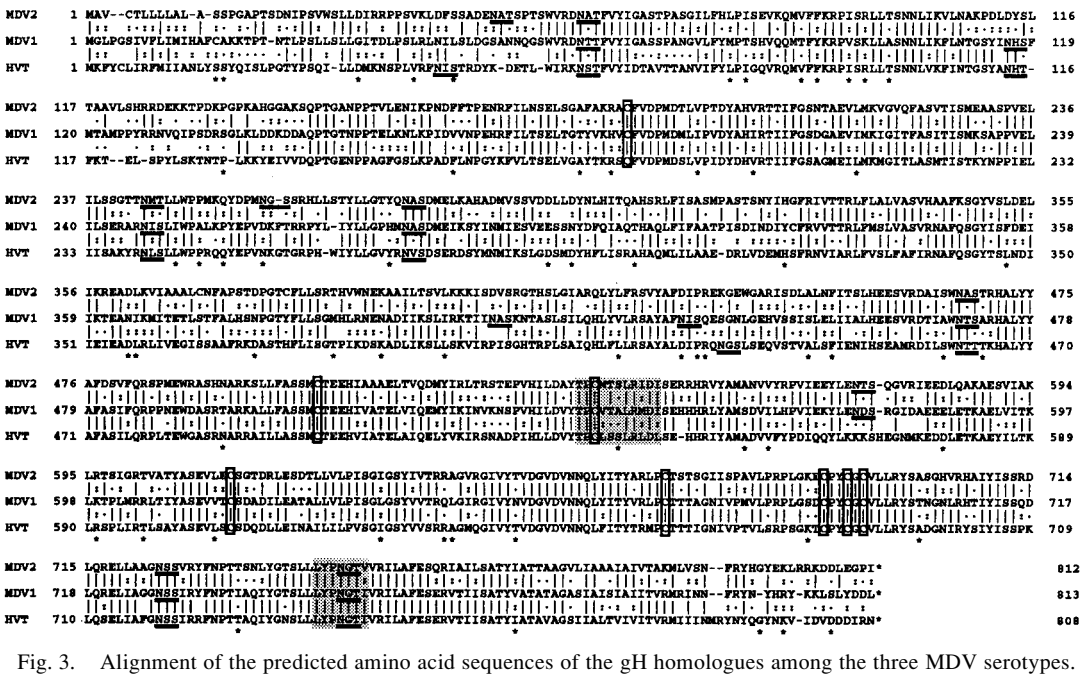

Fig. 3. Alignment of the predicted amino acid sequences of the gH homologues among the three MDV serotypes. The conserved motif regions in gH homologues [21] are shaded. Potential N-linked glycosylation sites and conserved cysteine residues among the three MDV serotypes are underlined and boxed, respectively. Dot in the sequence, gaps; vertical line, identities between amino acids; two vertical dots, high similarity between amino acids; single dots, low similarity. The asterisks indicate identical amino acids between MDV2 and HVT but not MDV1, or translation stop sites.

Table 1. Homology of amino acids deduced from glycoprotein genes among the three MDV serotypes*

serotype \ glycoprotein	gB	gC	gD	gE	gH	gI
MDV1 and MDV2	83	73	55	47	57	49
MDV1 and HVT	82	71	46	44	56	40
MDV2 and HVT	76	65	41	39	50	36

* Values were obtained using the UWGCG program BESTFIT, and are expressed as percentage identity. References: gB [40], gC [20], gD [16], gE [17], gI [15].

gH has not been clear in herpesviruses and need to be clarified. Further studies on analyses for the genes downstream of the gH encoding region and their transcriptional products should be also performed.

Alignment of the deduced amino acid sequence of MDV2 gH with other serotype MDV gH homologues showed many conservative substitutions throughout the entire sequence. The greatest similarity was apparent near the C termini, where seven cysteine residues are conserved (Fig. 3). Three cysteine residues (residues 505, 540, and 618) show the perfect alignment in gH peptides of all MDV serotypes and other alphaherpesviruses including PRV, VZV, BHV-1, EHV-1, EHV-4, and HSV-1. The positions of the N-linked glycosylation sites among the three MDV gH proteins are conserved well, and the last ones are also conserved in other alphaherpesviruses (data not shown). In addition, two highly conserved regions in herpesviruses [21] were similar in the MDV2 gH. The first region, located between MDV2 gH residues 538 and 548, includes a conserved cysteine residue. The second region, located between MDV2 gH residues 744 and 750, near the transmembrane anchor domain, includes the perfectly conserved N-linked glycosylation site (Figs. 2 and 3). These results suggest that gH homologues of alphaherpesviruses have a common function and relate evolutionary.

Homology of amino acids deduced from glycoprotein genes, gB, gC, gD, gE, gI, and gH, among three MDV serotypes demonstrated that homology between MDV1 and MDV2 was higher than that between MDV1 and HVT or that between MDV2 and HVT (Table 1). This result suggests that MDV1 and MDV2 are more closely related to each other than HVT. Further, overall identities of the glycoproteins in the U_L region, gB [40], gC [20], and gH, among the three MDV serotypes are more than those in the unique short (U_S) region, gD, gE, and gI [15–17], though gH has a little less identity than the other glycoproteins in the U_L region. These results suggest that the U_S region may have genes specific to the serotype, and gH seems to be comparatively specific to the serotype.

In future, based on our identification of MDV2 gH, expression of the gH homologues and analysis of the products among three MDV serotypes are needed to investigate if gH of the three MDV serotypes could be a candidate antigen of polyvalent vaccines in chickens.

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