Marek's Disease Virus Serotype 2 Glycoprotein I Gene: Nucleotide Sequence and Expression by a Recombinant Baculovirus

Hyung-Kwan JANG, Mitsuru ONO, Tae-Jong KIM[1)], Jin-Shun CAI, Yoshinori TSUSHIMA, Masahiro NIIKURA[2)], and Takeshi MIKAMI*

Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, Japan, [1)]Department of Veterinary Microbiology, Faculty of Animal Husbandry, Kun-Kuk University, 93–1 Mojin-dong, Kwangjin-gu, Seoul 143–701, Korea, and [2)]Avian Disease and Oncology Laboratory, USDA ARS, 3606 East Mount Hope Road, East Lansing, Michigan 48823, U.S.A.

(Received 4 June 1996/Accepted 5 July 1996)

ABSTRACT. In the Marek's disease virus (MDV) serotype 2 (MDV2) genome, a gene equivalent to the glycoprotein I (gI) of other alphaherpesviruses was identified and sequenced. The primary translation product comprises 355 amino acids with a M_r of 38.4 kDa. The predicted amino acid sequence possesses several characteristics typical of membrane glycoproteins, including a N-terminal hydrophobic signal sequence, C-terminal transmembrane and cytoplasmic domains, and extra-cellular region containing three potential *N*-linked glycosylation sites. Compared to other MDV serotypes, MDV2 gI showed 49% identity with MDV1 gI, and 36% identity with HVT gI at the amino acid level. In transcriptional analyses, a 3.5 kb mRNA which starts between 56 and 147 bps upstream of the potential translational initiation codon of gI was identified as the gI-specific transcript. By a recombinant baculovirus, this potential gI encoding region was expressed as two specific products 45 and 43 kDa. Both products were susceptible to tunicamycin treatment, indicating that they were glycoprotein. Further, the expressed gI reacted with all chicken-antisera raised to each of the three serotypes of MDV (strains GA, SB-1, and FC126), suggesting that gI is expressed by all three serotypes of MDV in infected cells and conserves common antigenic epitope(s) beyond serotypes. — KEY WORDS: cross-reactive epitope, glycoprotein I, Marek's disease virus serotype 2, recombinant baculovirus.

J. Vet. Med. Sci. 58(11): 1057–1066, 1996

Marek's disease virus (MDV) is a cell-associated herpesvirus which cause Marek's disease (MD), a naturally occurring T-cell lymphoma in chickens. The disease, caused by pathogenic MDV serotype 1 (MDV1), has been prevented by vaccination with attenuated MDV1 or herpesvirus of turkeys (HVT) which is antigenically related to MDV1 and is also termed MDV serotype 3. The efficacy of MD vaccine can be improved by co-vaccination with HVT and MDV serotype 2 (MDV2), which is a naturally avirulent strain [6, 46]. All three serotypes of MDV genome structures are similar to those of other alphaherpesviruses [7, 11, 17, 38] and have numerous genes that are homologous to and colinear with those of herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) [5]. The MDV2 genome differs in restriction endonuclease digest patterns from those of either MDV1 or HVT [16, 44, 47]. However, molecular analysis of MDV2 has lagged behind other MDV serotypes and alphaherpesviruses.

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. Among HSV-1 glycoproteins, glycoprotein I (gI) elicits protective immunity against a lethal intraperitoneal and ocular challenge with HSV-1 in mice [12, 13] as well as several other glycoproteins. Further, antibodies to a recombinant gI of HSV-1 expressed by a baculovirus system significantly prevent the establishment of latency and neutralize the infectivity of HSV-1 in the presence of complement. Based on these findings, it was proposed that gI may be of value as part of a subunit vaccine for HSV-1 [13]. HSV-1 gI forms a complex with glycoprotein E (gE) that can bind the Fc portion of immunoglobulin G (IgG) [21, 22]. VZV glycoprotein gpIV, homolog of HSV-1 gI, also forms a heterodimer with gpI (gE) which is likely to be involved in the Fc receptor activity induced on the surface of VZV-infected cells [28, 51]. The significance of this receptor is obscure, although involvement in a mechanism of immune evasion of the virus by blocking effector functions requiring Fc recognition is speculated [1]. The gI and gE complex of HSV-1 and the homologous complex of pseudorabies virus (PRV) are dispensable for virion entry but are required for efficient cell-to-cell transmission and cell fusion [9, 54, 55].

In MDV1 and HVT genomes, the potential open reading frames (ORFs) which are homologous to the HSV-1 gI gene were reported [4, 53]. The gI homolog of MDV1 (MDV1 gI) was identified as a 45 kDa glycoprotein with an unglycosylated 35 kDa precursor polypeptide in MDV1-infected duck embryo fibroblasts (DEF) by immunoprecipitation [3]. MDV1 gI-specific antisera coprecipitated MDV1 gE with gI [3], suggesting that gI and gE of MDV1 can form a complex as observed in other

* CORRESPONDENCE TO: MIKAMI, T., Department of Microbiology, Faculty of Agriculture, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

alphaherpesviruses [14, 21, 55]. However, the role(s) of gI and gE homologs of MDV in the course of infection and in the protective immunity against MD has not yet been studied.

Since MDV2 is naturally occurring apathogenic or nononcogenic strain of MDV in *Gallus* birds [26, 27], comparative studies on the virus genome and its function of MDV2 with those of MDV1 might be a crucial subject for understanding viral oncogenicity and natural immunity. The purpose of the present studies is to identify the gene encoding gI homolog of MDV2 strain HPRS 24 (MDV2 gI) which might involve in protective immunity, and to characterize its mRNA. We also expressed MDV2 gI by a baculovirus expression system, and investigated the antigenic property of MDV2 gI in relation to other serotypes of MDV.

MATERIALS AND METHODS

Cells and viruses: *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV (rAcNPV) were grown in *Spodoptera frugiperda* clone 9 (Sf9) cells in TC-100 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 0.3% tryptose phosphate broth (TPB), and antibiotics. Primary chicken embryo fibroblasts (CEF) were grown in an equal amount of Medium 199 and Ham's F-10 nutrient mixture (Gibco BRL, Grand Island, NY) containing 5% calf serum, 10% TPB, and antibiotics. MDV2 (strain HPRS 24) was passaged in CEF at least 30 times prior to use in this study.

Plasmids and cloning: Plasmid DNA manipulations were performed essentially as described by Sambrook *et al.* [45]. A 9.1 kb *Bam*HI-F fragment of MDV2 genome cloned in pUC19 (Fig. 1A) was reported [38]. To obtain the complete sequence of MDV2 gI ORF, a 2.6 kb *Sca*I-*Hind*III subfragment (Fig. 1B) was cloned into pBluescript SK+ and SK- vectors (Stratagene, La Jolla, CA), and then deleted by exonuclease III to construct deletion clones. The plasmid transfer vector for AcNPV, pAcYM1, was described by Matsuura *et al.* [30].

DNA sequencing and computer analysis of data: DNA sequence was determined on both strands using an autosequencer system (Applied Biosystems, Foster City, CA). DNA and amino acid sequences were analyzed with UWGCG (Versio 7.0) and run through a SUN SPARC serve 690MP. Homology searches from the GenBank and EMBL databases were performed using the UWGCG program FASTA [41].

Virus infection and isolation of total RNA: CEF were infected with MDV2 strain HPRS 24 or mock infected at 3×10^6 cells per a 60-mm-diameter dish. To prepare total RNA, monolayers of virus- or mock-infected CEF were harvested at 96 hr postinfection (PI) and treated with 0.05% ethylenediaminetetraacetic 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 *Bam*HI-F 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 fractionated on 1.2% agarose gels containing 2.2 *M* formaldehyde as described by Sambrook *et al.* [45]. RNA was transferred to nylon membrane filters (Biodyne, Pall BioSupport, NY) by the method of Southern [49]. DNA probes were radiolabeled with [α-^{32}P]dCTP by the nick translation method as described by Rigby *et al.* [43] using nick translation kit (Boehringer Mannheim GmbH, Germany), and hybridized to RNA on the filters under stringent conditions as previously described [15].

5'-end amplification of cDNAs: To identify transcriptional initiation site(s) of the upstream of the MDV2 gI homolog gene, rapid amplification of cDNA end (RACE) method [10] was performed. Total RNA (5 μg) was treated with RNase-free DNase I (Boehringer Mannheim GmbH, Germany) at 25°C for 30 min and heated to 94°C for 5 min to inactivate DNase I. To synthesize first strand cDNA, each of the DNase I-treated samples was incubated with 10 units of reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 45°C for 2 hr in the presence of antisense primer-1, 5'-GATCCGTCGATCGCCATAAC-3', and RNase inhibitor (Toyobo Co., Ltd., Osaka, Japan). After first strand cDNA was synthesized, cDNA molecules were added homopolymeric tails of dATP at the 3'-ends by terminal deoxynucleotide transferase (Gibco BRL, Gaithersburg, MD). The samples were then thermocycled using $(dT)_{17}$-adapter (5'-GACTCGAGTCGACATCGATTTTTTTTTTTT TTTTT-3') and adapter (5'-ACTCGAGCGACATCGA-3') primers, and another antisense primer-2, 5'-GGACGCCGCACTCCAATGTG-3' under the conditions of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 2 min) for 30 cycles with the GeneAmp PCR reagent kit and AmpliTaq DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). The RACE-amplified cDNAs were cloned by a TA cloning system (Invitrogen, San Diego, CA) and sequenced.

Construction of recombinant AcNPV: The *Sna*BI-*Sma*I fragment was excised from the cloned 2.6 kb *Sca*I-*Hind*III fragment, and inserted into blunt-ended pAcYM1 cloning site (Fig. 5). The recombinant transfer vector was designated pAcMDV2gI. The orientation of the inserted fragment was confirmed by nucleotide sequencing of the 5'-junction region (data not shown). pAcMDV2gI and BaculoGold-linearized baculovirus DNA (Pharmingen, San Diego, CA) were cotransfected into Sf9 cells, and the recombinant AcNPVs (designated rAcMDV2gI) were obtained.

Preparation of antisera: Antisera against MDV2 (HPRS 24) and HVT (FC126) were prepared by repeated inoculations of each of the virus-infected CEF (1000 PFU per chicken) into specific-pathogen-free chickens. Chicken polyclonal antisera against MDV1 (GA) and MDV2 (SB-1) were kindly provided by Dr. K. Imai (National Institute of Animal Health, Japan).

Immunofluorescence analysis (IFA): Sf9 cells infected with rAcMDV2gI or wild-type AcNPV (wAcNPV) at a multiplicity of 10 PFU per cell were harvested 48 hr PI as previously described [34], with the following modifications. In order to examine total fluorescence, cells were smeared on slides, fixed with acetone, and incubated with one of the three serotypes of anti-MDV polyclonal antibodies for 30 min at 37°C. To examine cell surface immunofluorescence, unfixed cells were suspended in ice-cold PBS containing 3% FCS and 0.1% sodium azide, and then incubated with anti-MDV2 HPRS 24 polyclonal antibody for 30 min at 4°C. The cells were washed with PBS and further incubated with fluorescein isothiocyanate-conjugated (FITC) rabbit anti-chicken IgG (Cappel, Durham, NC) for 30 min at 37°C or 4°C, respectively. The cells were washed again with PBS and examined for fluorescence.

Immunoprecipitation analysis: Sf9 cells (1×10^6 per 35-mm-diameter dish) were infected with rAcMDV2gI or wAcNPV at a multiplicity of 5 PFU per cell. At 24 hr PI, the cells were radiolabeled for 12 hr with 50 μCi of [^{35}S]methionine (1175.0 Ci/mmol; New England Nuclear, Boston, MA) per dish in Grace's methionine-free insect cell medium (Gibco BRL, Grand Island, NY) containing 1/10th the normal concentration of methionine and 5% FCS. At 36 hr PI, the cells were lysed in ice-cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 1 *mM* PMSF, 0.15 *M* NaCl, and 0.02% sodium azide in 50 *mM* Tris-HCl, pH 8.0) with aprotinin (0.2 U/m*l*), and immunoprecipitated with chicken polyclonal antibody and rabbit anti-chicken IgG (Cappel, Durham, NC) as previously described [18]. The immune complexes were precipitated with 2 to 4 mg of Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden), and analysed by SDS-PAGE as previously described [33, 34]. For tunicamycin (TM) (Sigma Chemical Co.) treatment, rAcMDV2gI- and wAcNPV-infected cells were cultured in the presence of 10 μg of TM per m*l* as previously described [34].

RESULTS

DNA sequence analysis of the MDV2 gI gene: The location of the MDV2 gD and gI genes on the virus genome, pertinent restriction endonuclease sites, and the sequencing strategy are illustrated in Fig. 1. A pUC-based plasmid library containing the *Bam*HI fragments of the MDV2 (strain HPRS 24) genomic DNA was previously established in our laboratory [38]. The nucleotide sequences of the MDV2 gD gene and a part of the HSV-1 protein kinase (PK) and gI homologous genes have been submitted to the DDBJ, EMBL and GenBank Databanks with the Accession No. D83055.

Based on the submitted DNA sequence, we cloned an about 2.6 kb *Sca*I-*Hind*III subfragment from the *Bam*HI-F fragment (Fig. 1B), and then determined the nucleotide sequence of a total of 1713 bp containing the putative MDV2 gI homologous gene (Fig. 2). An ORF of 1065 bp (position 193 to 1257) encoding 355 amino acids was identified, which is reasonable length compared to gI homologs of MDV1 strain GA (355 amino acids; [4]) and HVT strain FC126 (366 amino acids; [53]). Two possible initiation codons were located at positions 193 and 214.

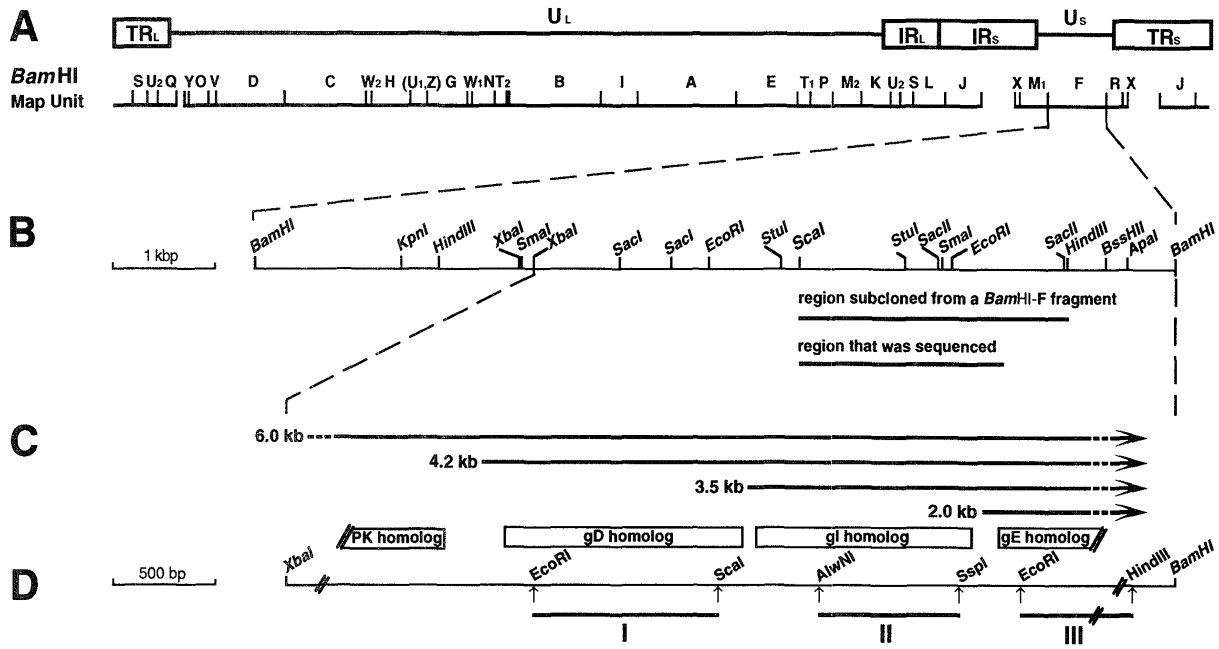

Fig. 1. Diagram of the MDV2 genome. (A) The genome is organized into the unique long (U_L) and short (U_S), internal repeat long (IR_L) and short (IR_S), and terminal repeat long (TR_L) and short (TR_S) regions. (B) A 2.6 kb fragment between *Sca*I and *Hind*III sites from a 9.1 kb *Bam*HI-F fragment was cloned, and the DNA sequence of a total 1713 bp containing the gI homologous gene was determined. (C) The locations and directions of the gD, gI, and gE mRNAs as well as the 6.0 kb mRNA are indicated by bold arrows. The ORF positions are shown as boxes. (D) The subclones used as probes in the northern blot analyses are shown as bold lines. The approximate sizes of the subclones are as follows: I, 0.9 kb; II, 0.7 kb; III, 1.1 kb.

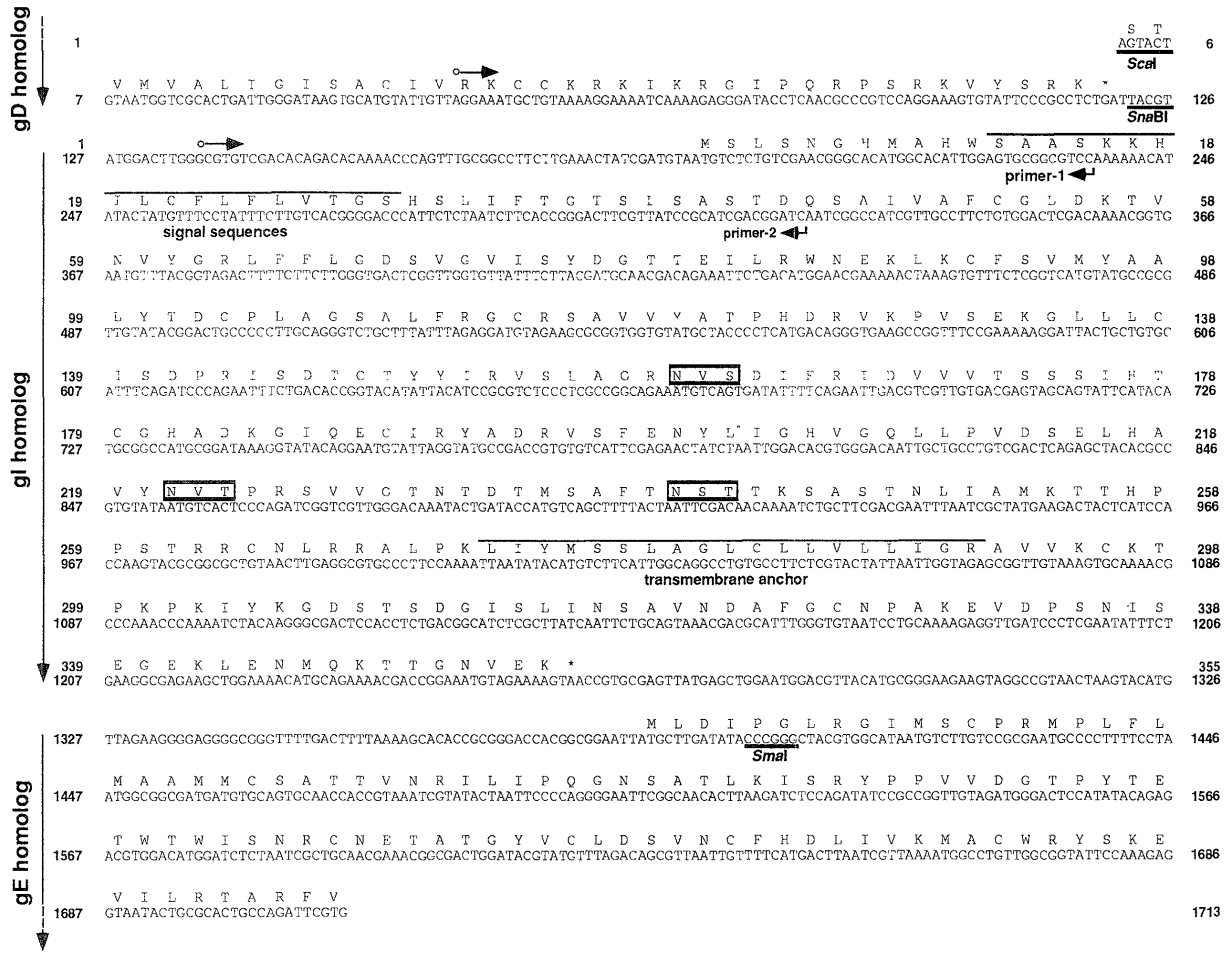

Fig. 2. Nucleotide sequence and predicted amino acid sequence of a portion of gI (nucleotides 194 to 1258) homolog. The predicted amino acid sequence is given in the single-letter code above the DNA sequence and the stop codons are represented by asterisks. The ORF orientations are shown with bold arrows on the left of the sequence panel. Proposed gI mRNA transcriptional start positions (○→) which were determined using two primers (↵) by the RACE method as described in Materials and Methods are marked. Restriction enzyme sites are boldly underlined. Potential *N*-glycosylation sites are boxed, and the predicted N-terminal signal peptide and C-terminal transmembrane sequences are indicated above the deduced amino acid sequence.

Although a methionine codon exists directly before the hydrophobic region at position 214 in Fig. 2, the scanning model for translation [25] favors more usage of the 5'-proximal initiation codon at position 193 (Fig. 2). No major *cis*-acting transcriptional regulatory sites (TATA-like elements) were evident in the region 5' to this ATG initiation codon. Also, no canonical polyadenylation consensus signal was found downstream of the TAA termination codon. The nucleotide composition of the MDV2 gI ORF was 26.5% A, 27.0% T, 22.7% C, and 23.8% G.

Analysis of the predicted amino acid sequence of MDV2 gI: The predicted amino acid sequence of MDV2 gI was deduced from the nucleotide sequence, as shown in Fig. 2. Analysis of the amino acid sequence revealed a number of features common to membrane-associated glycoproteins. The MDV2 gI contains three potential sites (NXT/S; where X is any amino acid except proline and cysteine) for *N*-linked glycosylation, one of which was located in a highly conserved region in three MDV gIs (Fig. 3). A hydorphobic segment (residues 273 to 291) was predicted to be a transmembrane anchor element. Although no obvious signal peptide could be predicted from the computer analysis, the MDV2 gI ORF displays a markedly hydrophobic region (residues 12 to 29) at the N terminal that can function as a signal peptide.

Comparison of the amino acid composition of the MDV2 gI with other alphaherpesvirus gIs revealed broad homologies with HSV-1 gI [32], VZV gpIV [8], PRV gp63 [42], and their counterparts in equine herpesvirus type 1 (EHV-1; [2]), feline herpesvirus type 1 (FHV-1; [48]), bovine herpesvirus type 1 (BHV-1; [29]), HVT [53], and MDV1 [4] (Table 1). Multiple alignments indicated that several amino acid residues, mainly located in the first half of the protein, were conserved in the gIs of all three MDV serotypes (Fig. 3) and other alphaherpesviruses (data not shown). Further features of these homologies are discussed below (see discussion).

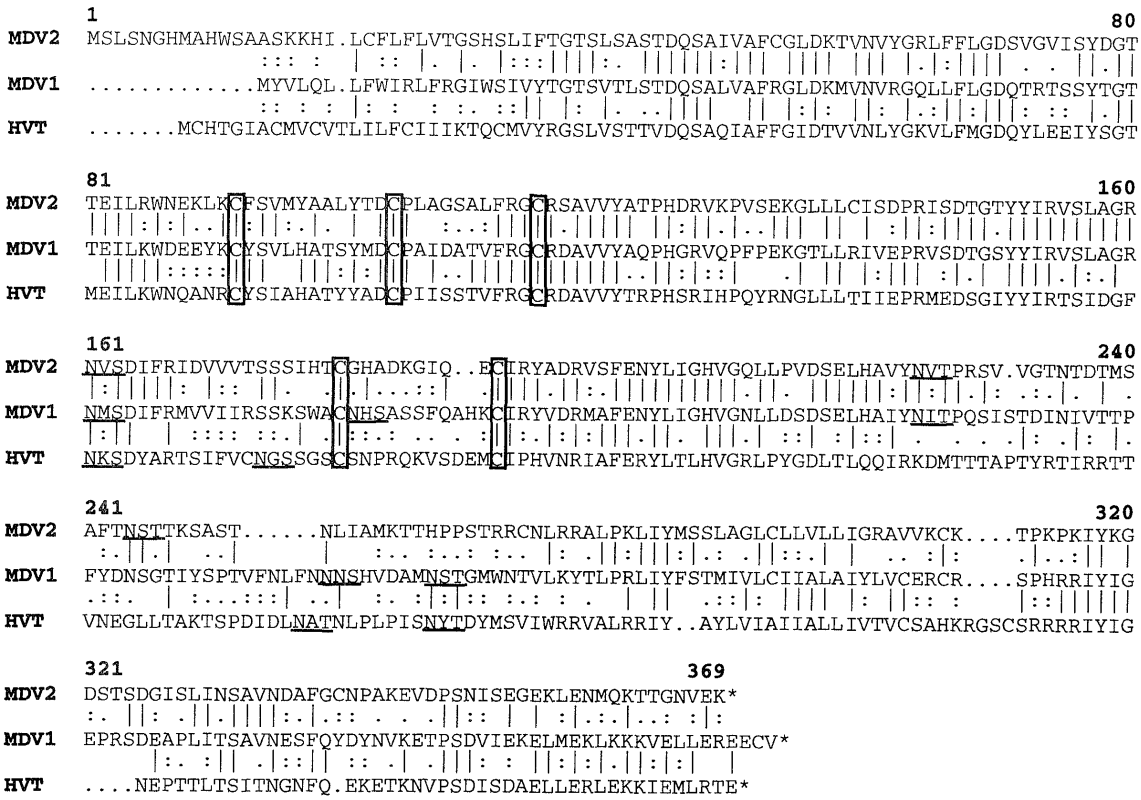

Fig. 3. Alignment of the predicted amino acid sequence of MDV2 gI homolog to those of the MDV1 [4] and HVT [53]. Alignment was carried out using the computer program FASTA (see Materials and Methods). Dots in sequence, gaps; vertical line, identities between amino acids; two vertical dots, high similarity between amino acids; single dots, low similarity between amino acids; boxes, conserved cysteine residues; underline, *N*-glycosylation sites; asterisk, translational termination sites.

Table 1. Homology among the predicted amino acid sequences of 9 alphaherpesvirus gI glycoproteins*

	MDV1	HVT	EHV-1	PRV	FUV-1	BHV-1	VZV	HSV-1
MDV2	49	36	27	26	25	24	24	22
MDV1	–	39	27	27	24	24	25	23
HVT	–	–	26	28	25	26	26	21
EHV-1	–	–	–	28	39	32	29	25
PRV	–	–	–	–	29	28	30	26
FHV-1	–	–	–	–	–	36	25	28
BHV-1	–	–	–	–	–	–	31	28
VZV	–	–	–	–	–	–	–	28
HSV-1	–	–	–	–	–	–	–	–

* Values were obtained using the UWGCG progrom FASTA [39], and are expressed as percentage identity. The entire gI amino acid sequences were used. References: MDV1 [4], HVT [51], EHV-1 [2], FHV-1 [46], BHV-1 [27], PRV [40], VZV [8], HSV-1 [30].

Transcritional analysis of the MDV2 gI gene: In order to analyze the transcription of the gI gene, northern blot analyses were performed. Probe I, which covers -1100nt to -188nt to translational initiation of gI, specifically hybridized to two abundant RNAs of approximately 6.0 and 4.2 kb (Fig. 4-I). Probe II (+322 to +1011) hybridized to three abundant RNAs of 6.0, 4.2, and 3.5 kb (Fig. 4-II). Probe III, which localizes downstream of gI encoding region and a partial gE homolog of MDV2, hybridized to a highly abundant transcript of 2.0 kb, along with 6.0, 4.2, and 3.5 kb transcripts (Fig. 4-III). However, when probed with *a ApaI-BamHI* fragment (shown in restriction map of Fig. 1B), which maps near to the Us/short terminal repeat (TRs) junction, no transcript was detected (data not shown). Two common transcripts of 6.0 and 4.2 kb were considered to be a large read-through transcripts including both gD and gI

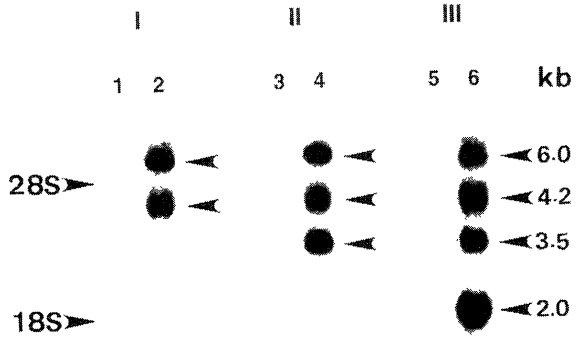

Fig. 4. Northern blot analyses of MDV2 U_S transcripts. Panels I through III represent hybridization with probes I, II, and III, as shown in Fig. 1D. The ribosomal RNAs 28S and 18S were used as size standards. Molecular sizes of the transcripts are shown in kilobases.

ORFs and possibly other genes of downstream encoded on this strand in the MDV2 U_S region as shown in Fig. 1C [20]. The 3.5 kb transcript(s) detected by both probes II and III probably overlap and may be 3' coterminal with an abundant 2.0 kb mRNA. These results indicated that gI is transcribed most likely from 3.5 kb transcripts and possibly from other 6.0 and 4.2 kb transcripts.

5'-end sequence of cDNAs amplified from the MDV2 gI mRNA: To examine the gI-encoding mRNA transcriptional initiation site(s), 5'-terminal of mRNA was cloned by using the RACE method [10] and sequenced. We examined 27 clones in total. Among 15 clones which seemed to represent 5'-terminal of 3.5 kb mRNA, six clones were initiated at "A" residue at the -147 position to translational initiation codon, while nine clones were initiated at "G" residue at -56 position. The remaining 12 clones contained a part of the potential promoter region of MDV2 gI and seemed to be amplified by the 6.0 and/or 4.2 kb transcript templates (data not shown). These results suggest that the 3.5 kb mRNA was initiated either or both -56 or -147 positions from the potential translation initiation site of MDV2 gI.

Baculovirus expression and characterization of gI: We examined whether the recombinant gI expressed by rAcMDV2gI is recognized using anti-MDV2 (strain HPRS 24) chicken serum by IFA. Specific fluorescence was detected on rAcMDV2gI-infected cells regardless of fixation of the cells (Fig. 6), while no specific fluorescence was observed in rAcMDV2gI- or mock-infected cells using normal chicken serum (data not shown). This result suggests that the recombinant gI is recognized by anti-HPRS 24 chicken serum and located in the inside or outside of the infected cells.

To analyze the size of the rAcMDV2gI, lysates from the rAcMDV2gI- or mock-infected Sf9 cells were examined by immunoprecipitation analysis using anti-HPRS 24 chicken serum. The apparent M_r of the recombinant protein showed 45 to 43 kDa (Fig. 7A). The different M_r might be due to the different degree of glycosylation in Sf9 cells. When

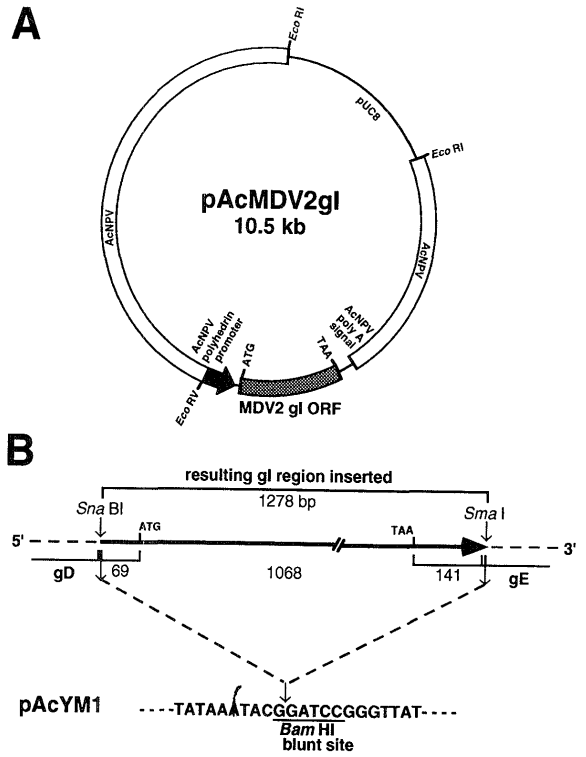

Fig. 5. Construction of the transfer vector containing the MDV2 gI gene. (A) Structure of the transfer vector plasmid pAcMDV2gI used to produce the recombinant baculovirus with the gI gene of MDV2 strain HPRS 24. (B) The thick solid black arrow indicates the 1278 bp isolated gI fragment that was inserted into the baculovirus vector. The gD and gE designations show the location of the adjacent ends of these flanking genes that have been removed. Nucleotide numbers in the regions are indicated by brackets.

rAcMDV2gI-infected cells were treated with TM, an apparently unique band of 35 kDa was detected. The reduced size of the 45 kDa is relatively close to that of unglycosylated MDV2 gI calculated as a M_r of 38.4 kDa from the amino acid sequence, suggesting both the 45 and 43 kDa polypeptides contained *N*-linked sugars (Fig. 7B). Another diffuse minor band below the 35 kDa is considered to be non-specific protein of the MDV2 gI-expressing cells.

The antigenic property of the rAcMDV2gI was further examined by immunoprecipitation analysis using three chicken antisera raised to MDV1 (strain GA), MDV2 (strain SB-1), or HVT (strain FC126), respectively (Fig. 8). All antisera precipitated proteins of M_r 45 and 43 kDa (lanes 2, 4, and 6), although the 43 kDa band was significantly fainter than the 45 kDa band. By IFA, the specific fluorescence was detected by all antisera (data not shown). These results indicate one or more antigenic epitopes on gI are conserved on all gIs of three serotypes of MDV.

DISCUSSION

Studies on MDV2 genome may be important for

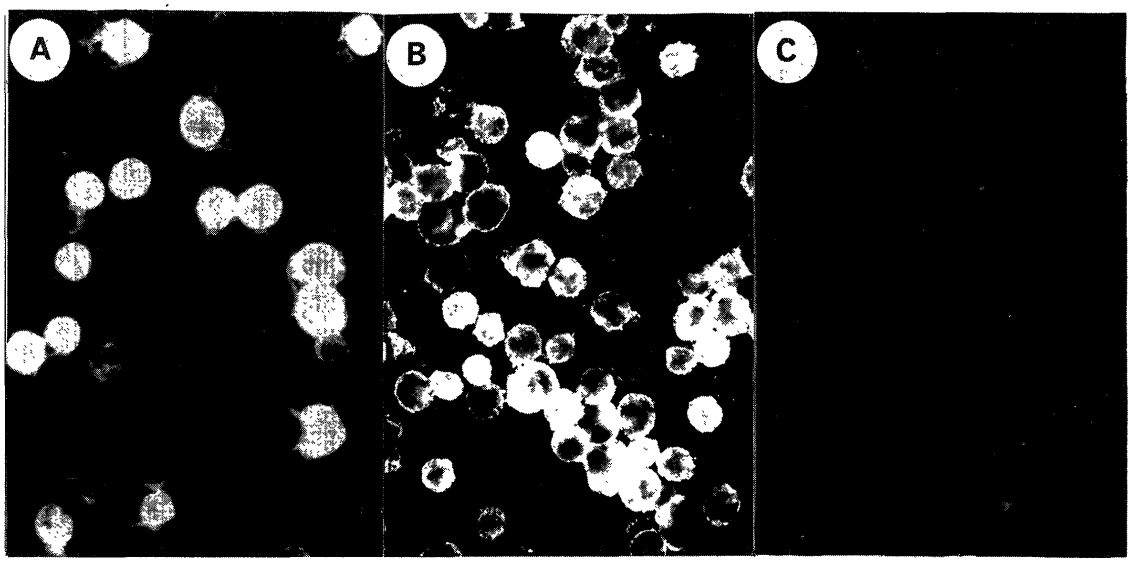

Fig. 6. Immunofluorescence analyses of rAcMDV2gI-infected cells. Acetone-fixed or unfixed infected Sf9 cells were incubated with anti-MDV2 (HPRS 24) chicken serum followed by FITC rabbit anti-chicken IgG and examined by fluorescence microscopy as described previously [34]. (A) rAcMDV2gI-infected cells, acetone-fixed; (B) rAcMDV2gI-infected cells, unfixed; (C) wAcNPV-infected ells, acetone-fixed.

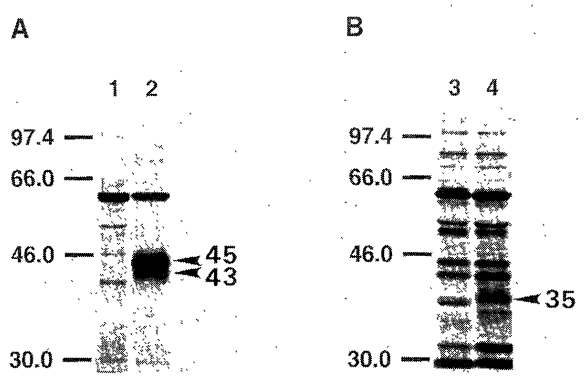

Fig. 7. Characterization of the recombinant MDV2 gI (rAcMDV2gI) by immunoprecipitation. (A) Sf9 cells were infected with wAcNPV (lane 1) or rAcMDV2gI (lane 2) and incubated for 36 hr without TM. (B) Sf9 cells infected with wAcNPV (lane 3) or rAcMDV2gI (lane 4) and incubated for 36 hr with TM (0–36 hr PI). The positions of M_r markers (Electrophoresis Calibration Kit for low M_r proteins; Pharmacia) are indicated on the left of each panel.

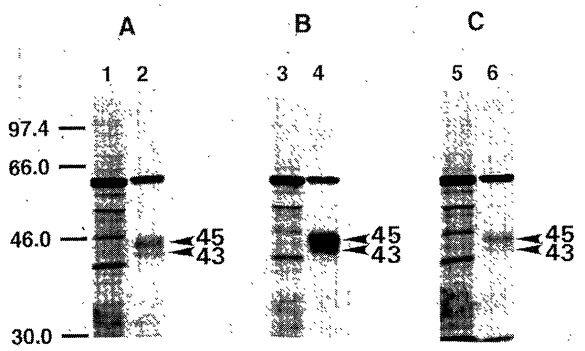

Fig. 8. Antigenicity of the MDV2 gI expressed by rAcMDV2gI. Lysates of Sf9 cells infected with wAcNPV (lanes 1, 3, and 5) or rAcMDV2gI (lanes 2, 4, and 6) were immunoprecipitated with antiserum of (A) MDV1 strain GA, (B) MDV2 strain SB-1, and (C) HVT strain FC126, as described under Materials and Methods, respectively.

understanding the biologically nononcogenic nature of the virus. Our goal in the present and previous studies [19, 20, 24, 34–40] has been focused to dissect precisely on the genomic structure and function of different viral proteins among MDVs. In this communication, we described the nucleotide sequence of a 1.7 kb segment on the MDV2 *Bam*HI-F fragment. Analysis of the DNA sequence revealed two complete ORFs and two partial ORFs, all of which orientated in the same direction. Three of them appear to encode membrane-associated glycoproteins based on the biochemical characteristics deduced from the amino acid sequence and their apparent homology with glycoproteins of other alphaherpesviruses. We propose to designate these three putative MDV2 glycoproteins gD, gI and gE, on the basis of their respective homologies with the HSV-1 gD, gI and gE glycoproteins.

Alignment of the deduced amino acid sequence of MDV2 gI with other serotype MDV gI homologs showed many conservative substitutions throughout the entire sequence. The greatest similarity was apparent near the N termini, where five cysteine residues are conserved (Fig. 3). Three cysteine residues show the perfect alignment in gI peptides of all MDV serotypes and other alphaherpesviruses. The first and second cysteine residues are 12 amino acid residues apart. At the same time, the second and third cysteine residues are 11 residues apart as observed on HSV-1, VZV, PRV, and EHV-1 [2, 31, 42]. In addition, the fourth and

fifth cysteine residues are somewhat out of alignment with those of other alphaherpesviruses (data not shown). The positions of the *N*-linked glycosylation sites among the three MDV gI proteins are not conserved well, although the asparagines at positions 160 and 221 of MDV2, and 147 and 210 of MDV1 [4] fall in very close alignment with each other, as do the sites at positions 160 of MDV2 and 152 of HVT [53]. The significantly conservative region between MDV2 gI and the other MDV gIs indicates that these sequences may be important for a similar tridimensional structure and some common biological function of these glycoproteins.

Transcriptional analyses revealed that the MDV2 gI gene is transcribed as a part of polycistronic mRNA family (3.5 kb) in a manner analogous to MDV1 gI transcription in which MDV1 gI mRNA (3.5 kb) contains both gI and gE encoding sequences [50]. Moreover, when the downstream of MDV2 gI, which supposed to include the potential gE ORF, was used as a probe, another abundant mRNA of about 2.0 kb were found, in addition to the 3.5 kb mRNA. In MDV1, the 2.0 kb gE-specific mRNA was also detected, along with 3.5 kb mRNA by northern blot analysis [50]. Although the MDV2 gE gene is not completely analyzed, there is no AATAAA or ATTAAA polyadenylation signal in the downstream of the potential MDV2 gI homologous gene (Fig. 2). Further, we confirmed that a *Apa*I-*Bam*HI subclone, which is near to the U_S/TR_S junction, was probed, no transcript was detected. Therefore, two transcripts of 3.5 and 2.0 kb detected in the present study, might be similar to those of MDV1.

A recombinant baculovirus expressing MDV2 gI was constructed and used to characterize this glycoprotein. In MDV genomes, the primary non-processed gI polypeptides deduced from the nucleotide sequences have a M_r of 38.3 kDa of MDV1 [4], and 40.4 kDa of HVT [53]. In MDV1-infected DEF, this protein is glycosylated to the mature form of gI with an apparent M_r of 45 kDa, and inhibited synthesis of unglycosylated precursor polypeptide of a M_r 35 kDa in the presence of TM [3]. Our recombinant MDV2 gI showed two major and minor bands on SDS-PAGE with a M_r of 45 kDa and 43 kDa, respectively. Further, TM treatment reduced the apparent size of the expressed gI to 35 kDa, which is relatively close to that of unglycosylated MDV2 gI calculated as a M_r of 38.4 kDa from the amino acid sequence. Therefore, this size seemed to be reasonable, because the molecular weight of the recombinant protein is similar to those of MDV1. Three glycoproteins, A antigen (gp57–65), B antigen (gp100, gp60, gp49), and gD homolog, of MDV1 have been expressed by recombinant baculoviruses and the recombinant proteins showed slightly smaller sizes than those proteins expressed in MDV1-infected CEF [34–36]. In HSV-1, the recombinant gI expressed by the baculovirus was also slightly smaller than the authentic proteins [13].

Overall identities of the Us glycoproteins among the three MDV serotypes are less than those of gene products in the U_L region [24, 52]. These results could suggest that the U_S region has genes specific to the serotype because higher DNA homology between MDV1 and HVT exists in the U_L region, but not in the U_S region [17]. The recombinant gI was recognized in IFA and immunoprecipitation analysis by the antisera from chickens inoculated with each of the three serotypes of MDV (strains GA, SB-1, and FC126). The observation indicates that one or more antigenic epitopes are conserved among the three serotypes as gB and gC [34, 35]. Further, this results also suggest that gI is actually expressed by all three serotypes of MDV in infected cells.

The gB homolog (B antigen) of MDV is the only viral protein so far identified which induces neutralizing antibody [23], and elicits protective immunity [19, 33]. Our characterization and efficient expression of the gI homolog should lead to clarification for involvement of the glycoprotein in immunity against MDV infection in future.

ACKNOWLEDGEMENTS. We are grateful to Dr. K. Imai (National Institute of Animal Health, Japan) and Dr. Y. Matsuura (National Institute of Health, Japan) for providing MDV antisera of strains GA and SB-1, and the plasmid baculovirus transfer vector pAcYM1, respectively. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, and Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

1. Adler, R., Glorioso, J. C., Cossman, J., and Levine, M. 1978. Possible role of Fc receptors on cells infected and transformed by herpesvirus: escape from immune cytolysis. *Infect. Immun.* 21: 442–447.
2. Audonnet, J.-C., Winslow, J., Allen, G., and Paoletti, E. 1990. Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE. *J. Gen. Virol.* 71: 2969–2978.
3. Brunovskis, P., Chen, X., and Velicer, L. F. 1992. Analysis of Marek's disease virus glycoprotein D, I and E. pp. 118–122. *In*: Proceedings 19th World's Poultry Congress, vol. 1, Ponsen & Looijen, Wageningen, The Netherlands.
4. Brunovskis, P. and Velicer, L. F. 1995. The Marek's disease virus (MDV) unique short region: alphaherpesvirus-homologous, fowlpox virus-homologous, and MDV-specific genes. *Virology* 206: 324–338.
5. Buckmaster, A. E., Scott S. S., Sanderson, M. J., Boursnell, M. E. G., Ross, L. J. N., and Binns, M. M. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. *J. Gen. Virol.* 69: 2033–2042.
6. Calnek, B. W., Schat, K. A., Peckham, M. C., and Fabricant, J. 1983. Field trials with a bivalent vaccine (HVT and SB-1) against Marek's disease. *Avian Dis.* 27: 844–849.
7. Cebrian, J., Kaschka-Dierich, C., Berthelot, N., and Sheldrick, P. 1982. Inverted repeat nucleotide sequences in the genomes of Marek's disease virus and the herpesvirus of the turkey. *Proc. Natl. Acad. Sci., USA* 79: 555–558.
8. Davison, A. J. and Scott, J. E. 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* 67: 1759–1816.

9. Dingwell, K. S., Brunetti, C. R., Hendricks, R. L., Tang, Q., Tang, M., Rainbow, A. J., and Johnson, D. C. 1994. Herpes simplex virus glycoproteins E and I facilitate cell-to-cell spread *in vivo* and across junctions of cultured cells. *J. Virol.* 68: 834–845.
10. Frohman, M. A., Dush, M. K., and Martin, G. R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci., USA* 85: 8998–9002.
11. Fukuchi, K., Sudo, M., Lee, Y. S., Tanaka, A., and Nonoyama, M. 1984. Structure of Marek's disease virus DNA: detail restriction enzyme map. *J. Virol.* 51: 102–109.
12. Ghiasi, H., Kaiwar, R., Nesburn, A. B., Slanina, S., and Wechsler, S. L. 1994. Expression of seven herps simplex virus type 1 glycoproteins (gB, gC, gD, gE, gG, gH, and gI): comparative protection against lethal challenge in mice. *J. Virol.* 68: 2118–2126.
13. Ghiasi, H., Kaiwar, R., Nesburn, A. B., and Wechsler, S. L. 1992. Expression of herpes simplex virus type 1 glycoprotein I in baculovirus: preliminary biochemical characterization and protection studies. *J. Virol.* 66: 2505–2509.
14. Grose, C. 1990. Glycoproteins encoded by varicella-zoster virus: biosynthesis, phophorylation, and intracellular trafficking. *Annu. Rev. Microbiol.* 44: 59–80.
15. Hirai, K., Ikuta, K., Maotani, K., and Kato, S. 1984. Evaluation of DNA homology of Marek's disease virus, herpesvirus of turkeys and Epstein-Barr virus under varied stringent hybridization conditions. *J. Biochem.* 95: 1215–1218.
16. Hirai, K., Nakajima, K., Ikuta, K., Kirisawa, R., Kawakami, Y., Mikami, T., and Kato, S. 1986. Similarities and dissimilarities in the structure and expression of viral genomes of various virus strains immunologically related to Marek's disease virus. *Arch. Virol.* 89: 113–130.
17. Igarashi, T., Takahashi, M., Donovan, J., Jessip, J., Smith, M., Hirai, K., Tanaka, A., and Nonoyama, M. 1987. Restriction enzyme map of herpesvirus of turkey DNA and its collinear relationship with Marek's disease virus DNA. *Virology* 157: 351–358.
18. Ikuta, K., Ueda, S., Kato, S., and Hirai, K. 1981. Immunoprecipitation of Marek's disease virus-specific polypeptides with chicken antibodies purified by affinity chromatography. *Virology* 114: 227–281.
19. Jang, H.-K., Kitazawa, T., Ono, M., Kawaguchi, Y., Maeda, K.,Yokoyama, N., Tohya, Y., Niikura, M., and Mikami, T. 1996. Protection studies against Marek's disease using baculovirus-expressed glycoproteins B and C of Marek's disease virus type 1. *Avian Pathol.* 25: 5–24.
20. Jang, H.-K., Ono, M., Kato, Y., Tohya, T., Niikura, M., and Mikami, T. 1996. Identification of a potential Marek's disease virus serotype 2 glycoprotein gene with homology to herpes simplex virus glycoprotein D. *Arch. Virol.* (in press).
21. Johnson, D. C. and Feenstra, V. 1987. Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin. *J. Virol.* 61: 2208–2216.
22. Johnson, D. C., Frame, M. C., Ligas, M. W., Cross, A. M. and Stow, N. D. 1988. Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *J. Virol.* 62: 1347–1354.
23. Kato, S. and Hirai, K. 1985. Marek's disease virus. *Adv. Virus Res.* 30: 225–227.
24. Kitazawa, T., Ono, M., Maeda, K., Kawaguchi, Y., Kamiya, N., Niikura, M., and Mikami, T. 1993. Nucleotide sequence of the glycoprotein C (gC) homologous gene of Marek's disease virus (MDV) serotype 2 and comparison of gC homologous genes among three serotypes of MDV. *J. Vet. Med. Sci.* 55: 985–990.
25. Kozak, M. 1989. The scanning model for trranslation: an update. *J. Cell Biol.* 108: 229–241.
26. Lin, J. A., Kitagawa, H., Ono, M., Iwanaga, R., Kodama, H., and Mikami, T. 1990. Isolation of serotype 2 Marek's disease virus from birds belonging to genus Gallus in Japan. *Avian Dis.* 34: 336–344.
27. Lin, J. A., Kodama, H., Itakura C., Onuma M., and Mikami, T. 1990. Evaluation of pathogenicity efficacy of serotype 2 Marek's disease virus from birds belonging to genus *Gallus* in Japan. *J. Vet. Med. Sci.* 52: 329–337.
28. Litwin, V., Jackson, W., and Grose, C. 1992. Receptor properties of two varicella-zoster virus glycoproteins gpI and gpIV homologous to herpes simplex virus gE and gI. *J. Virol.* 66: 3643–3651.
29. Leung-Tack, P., Audonnet J.-C., and Riviere, M. 1994. The complete DNA sequence and the genetic organization of the short unique region (Us) of the bovine herpesvirus type 1 (ST strain). *Virology* 199: 409–421.
30. Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. L. 1987. Baculovirus expression vectors: the requirement for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* 68: 1233–1250.
31. McGeoch, D. J. 1990. Evolutionary relationships of virion glycoprotein genes in the S region of alphaherpesvirus genomes. *J. Gen. Virol.* 71: 2361–2367.
32. McGeoch, D. J., Dolan, A., Donald, S., and Rixon, F. J. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J. Mol. Biol.* 181: 1–13.
33. Nazerian, K., Lee, L. F., Yanagida, N., and Ogawa, R. 1992. Protection against Marek's disease by a fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus. *J. Virol.* 66: 1409–1413.
34. Niikura, M., Matsuura, Y., Endoh, D., Onuma, M., and Mikami, T. 1992. Expression of the Marek's disease virus (MDV) homolog of glycoprotein B of herpes simplex virus by a recombinant baculovirus and its identification as the B antigen (gp100, gp60, gp49) of MDV. *J. Virol.* 66: 2631–2638.
35. Niikura, M., Matsuura, Y., Hattori, M., Onuma, M., and Mikami, T. 1991. Expression of the A antigen (gp57–65) of Marek's disease virus by a recombinant baculovirus. *J. Gen. Virol.* 72: 1099–1104.
36. Ono, M., Jang, H.-K., Maeda, K., Kawaguchi, Y., Tohya, Y., Niikura, M., and Mikami, T. 1995. Preparation of monoclonal antibodies against Marek's diease virus serotype 1 glycoprotein D expressed by a recombinant baculovirus. *Virus Res.* 38: 219–230.
37. Ono, M., Jang, H.-K., Maeda, K., Kawaguchi, Y., Tohya, Y., Niikura, M., and Mikami, T. 1996. Detection of Marek's disease virus serotype 1 (MDV1) glycoprotein D in MDV1-infected chick embryo fibroblasts. *J. Vet. Med. Sci.* 58: 777–780.
38. Ono, M., Katsuragi-Iwanaga, R., Kitazawa, T., Kamiya, N., Horimoto, T., Niikura, M., Kai, C., Hirai, K., and Mikami, T. 1992. The restriction endonuclease map of Marek's disease virus (MDV) serotype 2 and collinear relationship among three serotypes of MDV. *Virology* 191: 459–463.
39. Ono, M., Kawaguchi, Y., Maeda, K., Kamiya, N., Tohya, Y.,

Kai, C., Niikura, M., and Mikami, T. 1994. Nucleotide sequence analysis of Marek's disease virus (MDV) serotype 2 homolog of MDV serotype 1 pp38, an antigen associated with transformed cells. *Virology* 201: 142–146.

40. Ono, M., Maeda, K., Kawaguchi, Y., Jang, H.-K., Tohya, Y., Niikura, M., and Mikami, T. 1995. Expression of Marek's disease virus (MDV) serotype 2 gene which has partial homology with MDV serotype 1 pp38 gene. *Virus Res.* 38: 219–230.
41. Pearson, W. R. and Lipman, D. J. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci., USA* 85: 2444–2448.
42. Petrovskis, E. A., Timmins, J. G., and Post, L. E. 1986. Use of λgt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. *J. Virol.* 60: 185–193.
43. Rigby, P. W., Dieckmann, M., Rhodes, C., and Berg, P. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113: 237–251.
44. Ross, L. J. N., Milne, B., and Biggs, P. M. 1983. Restriction endonuclease analysis of Marek's disease virus DNA and homology between amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487–491.
45. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. "Molecular Cloning: A Laboratory Manual." 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY.
46. Schat, K. A. and Calnek, B. W. 1978. Characterization of an apparently nononcogenic Marek's disease virus. *J. Natl. Cancer Inst.* 60: 1075–1082.
47. Silva, R. F. and Barnett, J. C. 1991. Restriction endonuclease analysis of Marek's disease virus DNA: differentiation of viral strains and determination of passage history. *Avian Dis.* 35: 487–495.
48. Spatz, S. J., Rota, P. A., and Maes, R. K. 1994. Identification of the feline herpesvirus type 1 (FHV-1) genes encoding glycoproteins G, D, I and E: expression of FHV-1 glycoprotein D in vaccinia and raccoon poxviruses. *J. Gen. Virol.* 75: 1235–1244.
49. Southern, M. E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503–517.
50. Tan, X. and Velicer, L. F. 1993. Transcriptional analysis of Marek's disease virus (MDV) homologs of HSV glycoproteins D, I, and E. pp. A145. *In*: 18th International Herpesvirus Workshop, University of Pittsburgh Medical Center, Pittsburgh, PA.
51. Yao, Z., Jackson, W., Forghani, B., and Grose, C. 1993. Varicella-zoster virus glycoprotein gpI/gpIV receptor: expression, complex formation, and antigenicity within the vaccinia virus-T7 RNA polymerase transfection system. *J. Virol.* 67: 305–314.
52. Yosida, S., Lee, L. F., Yanagida, N., and Nazerian, K. 1994. The glycoprotein B genes of Marek's disease virus serotypes 2 and 3: identification and expression by recombinant fowlpox virus. *Virology* 200: 484–493.
53. Zelnik, V., Darteil, R., Audonnet, J. C., Smith, G. D., Riviere, M., Pastorek, J., and Ross, L. J. N. 1993. The complete sequence and gene organization of the short unique region of herpesvirus of turkeys. *J. Gen. Virol.* 74: 2151–2162.
54. Zsak, L., Zuckermann, F. A., Sugg, N., and Ben-Porat, T. 1992. Glycoprotein gI of pseudorabies virus promotes cell fusion and virus spread via direct cell-to-cell transmission. *J. Virol.* 66: 2316–2325.
55. Zuckermann, F. A., Mettenleiter, T. C., Scheurs, C., Sugg, N., and Ben-Porat, T. 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. *J. Virol.* 62: 4622–4626.