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Identification and sequence analysis of the homologues of the herpes simplex virus type 1 glycoprotein H in Marek's disease virus and the herpesvirus of turkeys

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The glycoprotein H (gH) genes of two avian herpesviruses, Marek's disease virus and the herpesvirus of turkeys, have been cloned and sequenced and the coding regions found to be of 2439 and 2424 nucleotides respectively. The predicted primary polypeptide products of these open reading frames are 813 and 808 amino acids and correspond to M_rs of 90 800 and 91 100. Both amino acid sequences exhibit characteristic glycoprotein features such as hydrophobic signal and anchor sequences and potential sites for *N*-linked glycosylation. Polypeptide sequence comparison to the other eight available gH sequences revealed more similarity to the alphaherpesvirus subgroup than to either beta- or gammaherpesviruses.

Marek's disease viruses (MDVs) are highly cell-associated herpesviruses of gallinaceous fowl. Members of the group, though closely related serologically, display degrees of pathogenicity ranging from subclinal infections to the neuropathic and lymphoproliferative conditions of chickens with classical Marek's disease.

The disease has been effectively controlled for more than a decade using a live vaccine based on the non-pathogenic serotype 3 MDV, the herpesvirus of turkeys (HVT). In recent years, however, the world-wide emergence of 'highly oncogenic' strains of MDV amongst vaccinated flocks (Witter *et al.*, 1980; Eidson *et al.*, 1981; Powell & Lombardini, 1986), has led to a renewed interest in vaccine development. Increased vaccine efficacy can be attained using a multivalent system combining HVT with the naturally non-pathogenic serotype 2 viruses or laboratory-attenuated serotype 1 MDV, which suggests that viruses from the different serotypes possess different protective antigenic epitopes. Additionally, purified glycoproteins from MDV-infected cells have been shown to induce neutralizing antibody and to protect partially against the disease (Wyn-Jones & Kaaden, 1979). Consequently, the analysis of MDV glycoprotein sequences may prove invaluable in the determination of such epitopes, with a view to the production of synthetic peptide or recombinant vaccines.

Herpesviruses encode a number of glycoproteins, of which only glycoproteins B (gB) and H (gH) are conserved in all three subgroups. In addition, both these proteins appear to be essential for virus infectivity (Cai *et al.*, 1988; Desai *et al.*, 1988; Forrester *et al.*, 1991). gH was first identified in herpes simplex virus type 1 (HSV-1), following neutralization and immunoprecipitation studies with a series of monoclonal antibodies (MAbs) (Buckmaster *et al.*, 1984) and its sequence was presented by Gompels & Minson (1986) and McGeoch & Davison (1986). Furthermore, gH has been implicated in the cell-to-cell spread of virus during *in vitro* infections with HSV-1 (Showalter *et al.*, 1981; Buckmaster *et al.*, 1984; Gompels & Minson, 1986; Gompels *et al.*, 1991) and varicella-zoster virus (VZV) (Keller *et al.*, 1987). In addition, antibodies raised against the HSV-1, VZV, human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) gHs have been shown to be strongly neutralizing (Showalter *et al.*, 1981; Keller *et al.*, 1984; Rasmussen *et al.*, 1984; Strnad *et al.*, 1982). The inhibition of virus transfer between cells has also been noted in the presence of these antibodies for HSV-1, VZV and EBV (Desai *et al.*, 1988; Keller *et al.*, 1987; Miller & Hutt-Fletcher, 1988).

We have previously reported the full nucleotide sequences of homologues of four glycoprotein genes, gB, gC, gD and gE (Ross *et al.*, 1989, 1991; Binns & Ross, 1989), in a highly oncogenic strain of MDV, RB1B (Schat *et al.*, 1982). The sequence of the gC gene from the vaccine strain of HVT (FC126) has also been published recently, revealing an overall identity of 74·6% with the MDV sequence (Coussens & Velicer, 1988; Bandyopadhyay, 1989). This comparison also revealed regions of dissimilarity between the polypeptides, however, with

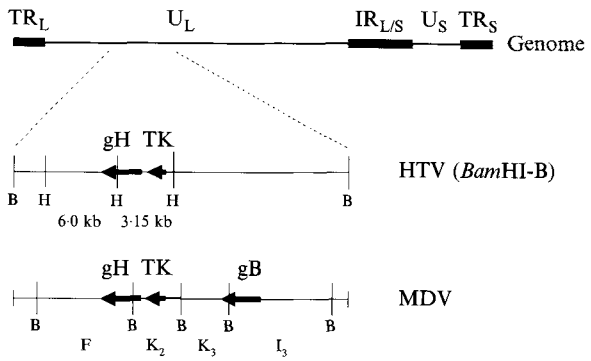

Fig. 1. Gene organization in the region of the MDV and HVT genomes encoding the homologues of the HSV-1 gH. U_L and U_S indicate the long and short unique regions of the genome, with the flanking internal and terminal repeats being denoted IR_L, IR_S, TR_L and TR_S respectively. Relevant *Hin*dIII (H) and *Bam*HI (B) restriction sites are shown. The lettered nomenclature of the *Bam*HI fragments is taken from Fukuchi *et al.* (1984) for MDV and from Igarashi *et al.* (1987) for HVT.

clusters of up to 17 different amino acids at some points. Some of these regions could belong to serotype-specific epitopes in the mature protein.

The HVT homologue of the HSV-1 gH gene was identified and mapped using an M13 clone probe (see Buckmaster *et al.*, 1988), and was found to span a 6 kbp and a 3·15 kbp *Hin*dIII fragment (Fig. 1). Sequencing of the latter (methodology as in Ross *et al.*, 1989) revealed the presence of two major open reading frames (ORFs). The smaller of these corresponded to the thymidine kinase (TK) gene (see Scott *et al.*, 1989) and the larger to the 5′-terminal three-quarters of the gH gene (Fig. 1). Primer extension sequencing within a cloned 1·2 kbp HVT *Bcl*I fragment was used to confirm the sequence across the *Hin*dIII junction and determine the extreme 3′-terminal 300 bp of the gene.

The mapping of serotype 1 MDV gH gene sequences to a 3·5 kb *Bam*HI fragment is described in Buckmaster *et al.* (1988). There are, however, two *Bam*HI fragments of this size in the MDV genome collinear with the HVT gH gene, denoted *Bam*HI-K_2 and -K_3 (Fukuchi *et al.*, 1984). Sequencing of *Bam*HI-K_3 revealed no gH gene sequences (Ross *et al.*, 1989). Thus the 3·5 kbp *Bam*HI fragment containing gH and TK gene sequences (Scott *et al.*, 1989) is -K_2. The remaining 3′-terminal three-quarters of the gH gene lies in the adjacent 8·9 kbp *Bam*HI-F fragment and was sequenced from pBluescript KS+ (Stratagene) nested deletion clones of a 1·6 kbp *Bam*HI–*Eco*RI subclone of *Bam*HI-F. Again, primer extension sequencing, this time within a cloned 2·2 kbp *Eco*RV fragment, was used to verify the *Bam*HI junction. The full sequences of the MDV and HVT gH genes and their predicted products are shown in Fig. 2.

Further primer extension sequencing of *Bam*HI-F itself, to provide data for the 500 bp downstream of the MDV gH gene, revealed no direct repeat elements or putative replication origin sequences, as has been found in this position for equine herpesvirus (EHV)-1 and -4 and pseudorabies virus (PRV) (Robertson *et al.*, 1991; Nicolson *et al.*, 1990; Klupp & Mettenleiter, 1991). The repeats in PRV are located 440 bp downstream of the gH stop codon and thus our results do not preclude the existence of an equivalent region further downstream in MDV. Indeed, Fukuchi *et al.* (1984) noted the presence of large direct repeats in the *Bam*HI-F fragment, which we now know to contain the 3′-terminal sequences of gH. It is also notable that in VZV and HSV-1, which do not possess an origin sequence in this region, the next gene is found within 200 bp of the gH stop codon. No such gene homologue was identified from the MDV sequence data produced.

Examination of the MDV and HVT gH amino acid sequences reveals 10 potential asparagine-linked glycosylation sites in the former and nine in the latter (Marshall, 1972; Kornfield & Kornfield, 1985). These are shown in bold in Fig. 2. This compares to 11 such sites in the VZV and EHV-1 and -4 gHs, eight in herpesvirus saimiri (HVS), seven in HSV-1, six in HCMV, five in EBV and three in PRV (Keller *et al.*, 1987; Robertson *et al.*, 1991; Nicolson *et al.*, 1990; Gompels *et al.*, 1988; Gompels & Minson, 1986; McGeoch & Davison, 1986; Cranage *et al.*, 1988; Baer *et al.*, 1984; Klupp & Mettenleiter, 1991). Only one of these sites shows conservation of position in all eight gH sequences. Gompels *et al.* (1988) noted that all five of the EBV gH glycosylation sites are conserved in the same position as in HVS. Indeed, three HVS sites are also conserved in HCMV. In contrast, the majority of the gH glycosylation sites in the alphaherpesviruses and MDVs are in different positions with respect to those in the EBV, HVS and HCMV polypeptides, both groups displaying higher conservation amongst themselves.

Assuming that each glycan contributes approximately 2500 to the M_r of a glycosylated protein (Klenk & Rott, 1980), the M_r of the primary glycosylated product of the MDV and HVT gH genes would be 115800 and 113600 respectively.

When hydropathy plots of MDV, HVT and HSV-1 gH polypeptides are produced using the HYDROPATH computer program of Staden (1984) (data not shown), a number of distinct hydrophobic regions can be seen, notably at both the N and C termini (see Fig. 2). These features are characteristic of glycoproteins and are likely to represent signal and anchor sequences involved in the transport to and subsequent insertion into cell membranes respectively. Other hydrophobic stretches within the gH sequences contain charged amino acids and are thus unlikely to be membrane-spanning. Some of these regions, however, may be located in the hydrophobic

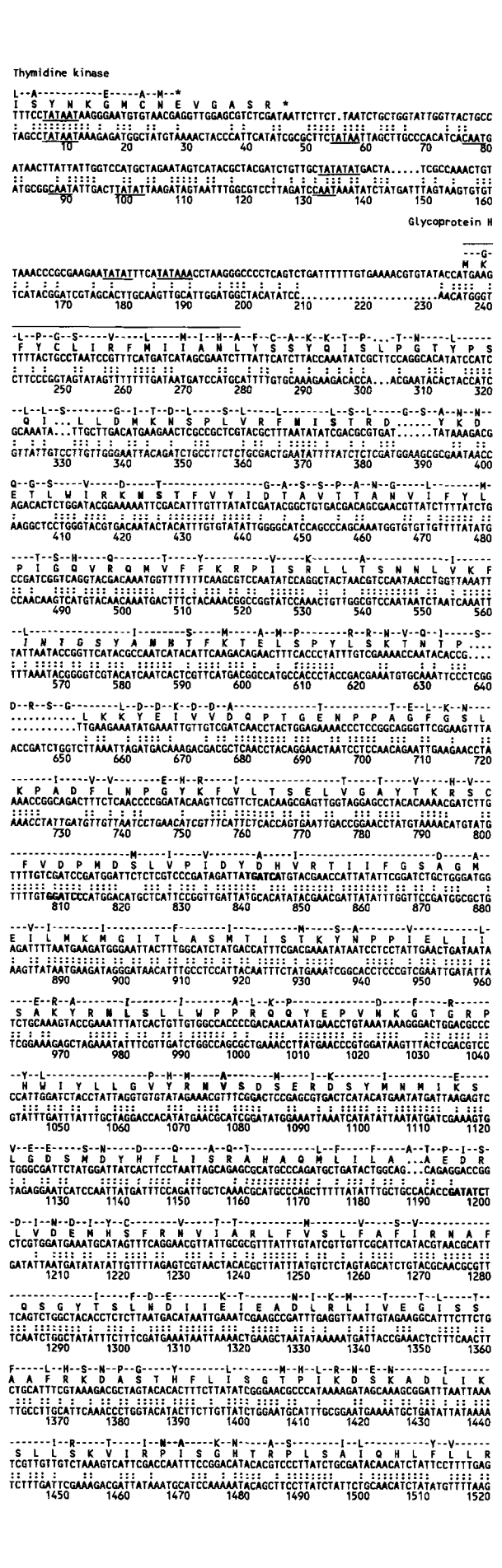

Fig. 2. DNA and predicted amino acid sequences of the MDV and HVT gH genes. Data derived from sequencing both DNA strands with the exception of the 3′-terminal 300 bp of both genes (see text for details). The probable initiation codons were defined by the method of Kozak (1981). Asterisks denote termination codons. Potential transcription/translation signal sequences (Benoist *et al.*, 1980; Corden *et al.*, 1980; Proudfoot & Brownlee, 1976) are underlined. Hydrophobic signal and transmembrane anchor sequences, as determined by the rules of McGeoch (1985) and von Heijne (1986) are overlined. Putative *N*-linked glycosylation sites are depicted in emboldened letters, as are relevant restriction sites for the enzymes *Bam*HI (GGATCC), *Bcl*I (TGATCA), *Eco*RV (GATATC) and *Hin*dIII (AAGCTT).

Table 1. *Similarity scores obtained by comparison of predicted amino acid sequences of the MDV and HVT gHs and those of eight other herpesviruses**

Virus and subfamily	MDV (γ_2)	HVT (γ_2)	VZV (α_2)	EHV-1 (α_1)	EHV-4 (α_1)	HSV-1 (α_1)	PRV (α_1)	HVS (γ_2)	EBV (γ_1)	HCMV (β)
MDV (γ_2)	3917									
HVT (γ_2)	2443	3959								
VZV (α_2)	707	707	4303							
EHV-1 (α_1)	629	662	1161	4113						
EHV-4 (α_1)	666	635	1188	3651	4134					
HSV-1 (α_1)	370	418	705	428	428	4126				
PRV (α_1)	327	307	391	403	406	331	3410			
HVS (γ_2)	152	170	149	135	139	111	121	3855		
EBV (γ_1)	160	136	157	82	92	155	113	850	3687	
HCMV (β)	115	98	56	123	136	98	160	317	212	3717

* Scores given refer to optimized similarity scores derived from amino acid sequence comparisons using the FASTP program of Lipman & Pearson (1985).

interior of the protein's tertiary structure, others being more loosely membrane-associated.

The two genes, at 2439 and 2424 bp respectively for MDV and HVT, are comparable in length with other gH sequences, which have a size range of 2118 to 2565 bp, though most notably with the larger, alphaherpesvirus gH homologues.

Results of the computer comparison between the gH sequences of the two Marek's disease viruses themselves and the other available gH sequences are shown in Table 1, with higher scores indicating a closer sequence similarity. As expected, MDV and HVT display the highest identity, at 55·9%. There are, however, regions of dissimilarity of up to 14 amino acids (see Fig. 2) which may represent serotype-specific epitopes. The raising of antipeptide antisera for subsequent Western blotting experiments would be a suitable route to determine this (see Ross *et al.*, 1989). Comparisons with the other herpesvirus gH polypeptides reveal that VZV and EHV-1 and -4 exhibit the most sequence similarity, at 23 to 25% identity. This is not thought to indicate a particularly close relationship to the MDVs, but rather a central position in the evolutionary tree, as evidenced by their sequence similarities to the other alphaherpesviruses.

In RNA mapping studies with MDV two transcripts of size 2·4 to 2·6 and 3·6 to 3·7 kb have been detected by probing Northern blots with cloned *Bam*HI-F and -K_2 fragments (Schat *et al.*, 1989). Because the ORF coding for gH is the only one spanning these two genomic fragments it is likely that one of these RNAs is the gH message. Furthermore, it is plausible that the larger RNA species may be a bicistronic TK/gH message, as has been suggested for the corresponding PRV transcript (Klupp & Mettenleiter, 1991).

The M_rs of the polypeptides following glycosylation and signal sequence cleavage at the predicted sites would be about 114000 and 111500 in MDV and HVT respectively. Unfortunately, these products are likely to comigrate with the viral gB in SDS–polyacrylamide gels [the MDV gB having been reported by Ross *et al.* (1989) as having an M_r of 115000], as was noted for their HSV-1 counterpart (Buckmaster *et al.*, 1984). Thus, gH-specific antisera would be needed to distinguish between these two glycoproteins using Western blotting. Again, antipeptide antisera may prove particularly useful in this regard.

The biological role of gH appears to be in virus entry into host cells and its subsequent cell-to-cell spread. Studies on a temperature-sensitive mutant of HSV-1, *ts*Q26 (Chu *et al.*, 1979; Desai *et al.*, 1988), revealed that a point mutation at a position corresponding to 828 in Fig. 2, causing an amino acid substitution of a cysteine (C) for a tryptophan (W), produced a non-infectious virus with no gH expressed on its virion surface when incubated at the non-permissive temperature. Interestingly, all wild-type alphaherpesviruses carry the tryptophan (and adjacent proline) residue at this position. In contrast, despite MDVs displaying obvious conservation with the alphaherpesviruses in this region of gH (including the possession of the aforementioned proline), MDV and HVT have isoleucine and valine at this position, respectively. Whether this has any bearing on the growth characteristics of these viruses, their tropism for avian rather than mammalian cells or their particularly cell-associated nature, for example, is not known. Specific site-directed mutagenesis of these MDV gH genes may help to elucidate this. Furthermore, the study of neutralizing MAb escape mutants of the MDV or HVT gHs, should any be isolated, would provide additional insight into the relevance of such differences.

Complement-independent neutralizing antibodies have been raised against gH that inhibit plaque formation *in vitro* (Buckmaster *et al.*, 1984; Gompels & Minson, 1986; Keller *et al.*, 1987) and protect experimental mice from further virus challenge following

passive immunization (Forrester *et al.*, 1991). gH is thus a promising target for inclusion in anti-herpesvirus vaccine development. However, expression of the HSV-1 gH in a recombinant vaccinia virus (Blacklaws *et al.*, 1990; Forrester *et al.*, 1991) or COS cell system (Gompels & Minson, 1989) does not result in the wild-type protein being produced. Consequently, vaccinia virus recombinants were unable to elicit neutralizing antibody let alone protection (Blacklaws *et al.*, 1990; Forrester *et al.*, 1991). Additionally, recent work on HSV-1 by Hutchinson *et al.* (1992) demonstrated the need for the presence of another glycoprotein, named gL, for the normal processing and transportion of gH. This glycoprotein is encoded by the HSV-1 UL1 gene, homologues of which have not, as yet, been identified in MDVs. Consequently, it may be important to present gH in a more native context in order that the authentic protein be expressed. An obvious choice of vector would be another herpesvirus. For MDV, expression of the gH gene in HVT would seem the most appropriate choice, and with the additional knowledge of gene sequence and arrangement in this virus group we may soon be able to apply the technology utilized in the production of mammalian virus recombinant vaccines to the control of avian disease.

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