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Epitopes of Glycoprotein G of Equine Herpesviruses 4 and 1 Located near the C Termini Elicit Type-Specific Antibody Responses in the Natural Host

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Specific serological diagnosis of equine herpesvirus 4 (EHV4; equine rhinopneumonitis virus) and EHV1 (equine abortion virus) hitherto has not been possible because of extensive antigenic cross-reactivity between these two closely related but distinct viruses. Recently, we identified EHV4 glycoprotein G (gG) and characterized it as a type-specific, secreted glycoprotein (B. S. Crabb, H. S. Nagesha, and M. J. Studdert, Virology 190:143–154, 1992). This paper shows that EHV1 gG also possesses type-specific epitopes and describes the localization of strong, type-specific epitopes to the apparently corresponding and highly variable regions comprising amino acids 287 to 382 of EHV4 gG and 288 to 350 of EHV1 gG. Fusion proteins expressing these variable regions reacted strongly and type specifically with sera from four foals, three of which were colostrum-deprived, specific-pathogen-free foals, whose history with respect to exposure to EHV4 or EHV1 was well-defined. These antigens provided the basis for the development of a single-well diagnostic enzyme-linked immunosorbent assay to distinguish horses infected with EHV4, EHV1, or both. Such a type-specific test provides for the first time the opportunity to differentiate antibodies to these viruses, and it has, therefore, important implications for understanding the epidemiology of these equine pathogens. Evidence for the existence of EHV1 in Australia 10 years prior to the first confirmed case of EHV1 abortion is presented.

Equine herpesvirus 4 (EHV4; equine rhinopneumonitis virus) and EHV1 (equine abortion virus) are alphaherpesviruses which are major causes of respiratory disease in young horses and of abortion in mares, respectively (1, 31). While these two viruses can be differentiated by their restriction endonuclease fingerprints, EHV4 and EHV1 antibodies in polyclonal serum cannot be differentiated because of extensive antigenic cross-reactivity between the two viruses (1, 6, 8). Accordingly, it has not been possible to undertake type-specific seroepidemiological studies to devise control measures particularly for EHV1, for which it is known that sporadic, devastating outbreaks of abortion are initiated either by latently infected horses or by infected horses which show no obvious clinical disease.

The antibody response of the horse to EHV4 and EHV1 is directed largely to the envelope glycoproteins (1, 3, 6). EHV1 glycoproteins gp2, gp10, gC (gp13), gB (gp14), gD (gp18), and gp21/22a have been definitively identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as have EHV4 glycoproteins gp2, gp10, gC (gp13), gB (gp14), gD (gp18), and gG (1, 4, 6, 7, 38–40). By using combinations of EHV1 and EHV4 monoclonal antibodies and polyclonal sera from horses infected with EHV1 only or EHV4 only, it has been shown that each of the glycoproteins possesses both type-common and type-specific epitopes (1, 5–7, 40, 41). The two exceptions are gp21/22a, which has been relatively poorly studied, and EHV4 gG, which appears to elicit a type-specific antibody response (7).

In this paper, we confirm that EHV4 gG possesses type-specific epitopes and show that EHV1 gG also possesses strong, type-specific epitopes. By using *Escherichia coli*-expressed gG fusion proteins, an epitope(s) responsible for eliciting type-specific responses to EHV4 gG and EHV1 gG was localized to the corresponding and highly variable regions located near the C termini of the molecules. EHV4 gG and EHV1 gG fusion proteins encompassing these variable regions were used to develop a single-dilution, type-specific enzyme-linked immunosorbent assay (ELISA).

Comparison of the sequences of EHV4 gG and EHV1 gG. The predicted amino acid sequences of EHV4 gG and EHV1 gG, which are derived from strains 405/76 (7) and Ab4p (32), respectively, were compared (Fig. 1) by means of the Geneworks software package (IntelliGenetics; Mountain View, Calif.). These sequences show an overall identity of 58%, with the C-terminal region showing considerably more divergence than the N-terminal region. Amino acids 1 to 286 of EHV4 gG and 1 to 287 of EHV1 gG show an identity of 75%, whereas the apparently corresponding regions comprising amino acids 287 to 382 (EHV4 gG) and 288 to 350 (EHV1 gG) have diverged widely and show only 21% amino acid identity after the insertion of five gaps into the EHV1 gG sequence (Fig. 1). A relatively high level of identity is once again evident in the sequences directly following these variable regions (Fig. 1). A second region of considerable diversity is a 25-amino-acid sequence comprising residues 32 to 57 of both viruses, in which only 9 amino acids (36%) are identical. The protein algorithm of Hopp and Woods (12) predicts that both divergent regions encompass antigenic sites (data not shown).

EHV4 gG and EHV1 gG each have nine cysteine residues in their respective predicted extracellular domains, all of which are conserved between the two viruses. Four cysteine residues located toward the N terminus are conserved with pseudorabies virus (PRV) gG (gX), herpes simplex virus type 2 (HSV2) gG, and infectious laryngotracheitis virus (ILTV) gG (gX) (Fig. 1). The predicted extracellular domain also contains five and six potential N-linked glycosylation sites for EHV4 gG and EHV1 gG, respectively. The three

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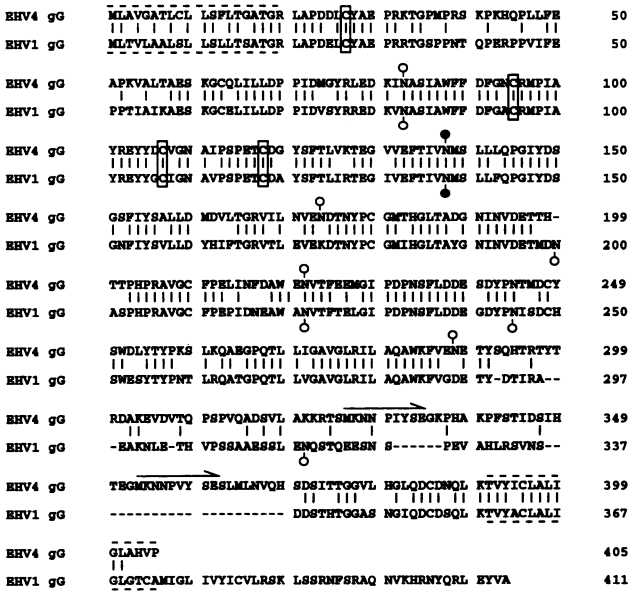

FIG. 1. Alignment of the amino acid sequences of EHV4.405/76 gG (7) and EHV1.Ab4p gG (32). Amino acid numbers are indicated on the right. The predicted signal sequence and the transmembrane sequence at the N and C termini, respectively, are indicated by the dashed lines. Potential N-linked glycosylation sites (N-X-S/T) (balloons) and a site conserved in the gG homologs of PRV (23), HSV2 (17), and ILTV (13) (filled balloons) are shown. The cysteine residues conserved between EHV4 gG and EHV1 gG and also in the gG homologs of PRV, HSV2, and ILTV (boxed) and a near-perfect repeated sequence in EHV4 gG (arrows), separated by 18 amino acids, are indicated.

N-linked glycosylation sites at positions 83, 138, and 221 (of EHV4) or 222 (of EHV1) that are conserved between EHV4 gG and EHV1 gG are also conserved in PRV gG, while the site at position 138 is also conserved in HSV2 gG and ILTV gG (gX) (Fig. 1). The C-terminal variable region of EHV4 gG (amino acids 287 to 382) also possesses repeated sequences of 9 amino acids, MKNNPXYSE, where X is an isoleucine in the first repeat and a valine in the second, which are separated by 18 amino acids (Fig. 1).

Expression of EHV4 and EHV1 gG fusion proteins. The bacterial expression plasmid pGEX-3X (AMRAD, Hawthorn, Australia) was used to express the EHV4 gG and EHV1 gG genes from strains 405/76 and 438/77, respectively. Virus strains EHV4.405/76 and EHV1.438/77 were grown in equine fetal kidney monolayer cell cultures and used at passages 5 and 3, respectively, as described elsewhere (28, 29). Restriction endonuclease digestion and ligation of DNA, as well as $CaCl_2$ transformation of competent *E. coli* (DH5α for pUC-19 clones and JM109 for pGEX clones), were carried out by the method of Sambrook et al. (25). For the derivation of clones pEG4var and pEG1var (see below), transformation was performed with the Gene-Pulser Electroporator (Bio-Rad). The EHV1.438/77 *Bam*HI D fragment, which encompasses much of the EHV1 unique short region (Fig. 2), was cloned into pUC-19 following purification of the fragment from an agarose gel slice with Prep A Gene matrix and buffers (Bio-Rad). The nucleotide sequences of the first and last 200 to 300 bases of each recombinant expression plasmid were determined to ensure correct identity, orientation, and codon usage of the cloned gene (data not shown). DNA sequencing and sequence analysis using the dideoxy chain termination method (26) and modified T7 DNA polymerase (Pharmacia, Uppsala, Sweden) and ^{35}S-dATP (Amersham) were performed as described previously (19).

The recombinant pGEX-3X plasmids pEG4var and pEG1var (Fig. 2) were constructed as follows. (i) pEG4var contains nucleotides encoding EHV4 gG amino acids 287 to 382 and was constructed by polymerase chain reaction (24) amplification (35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s) with pEG4.1, a plasmid which contains the entire EHV4 gG sequence (7), as the template DNA and the following primers: forward, 5′-GAAAATGAAACCTACAG-3′; reverse, 5′-TGGAGGCCATGCAACAC-3′. The frag-

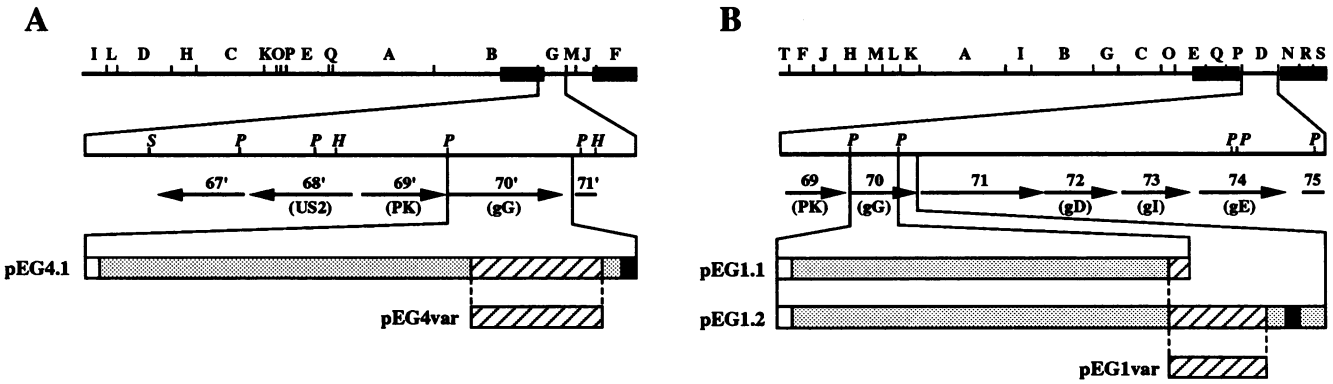

FIG. 2. Strategy for cloning EHV4 gG (A) and EHV1 gG (B). The *Eco*RI restriction endonuclease map of the EHV4 genome (10, 20) and the *Bam*HI restriction endonuclease map of the EHV1 genome (37) are shown at the top of panels A and B, respectively. The *Eco*RI G fragment of the EHV4 genome and the *Bam*HI D fragment of the EHV1 genome are expanded. *Sma*I (S), *Hin*dIII (H), and *Pst*I (P) cleavage sites are indicated for EHV4 *Eco*RI-G, while *Pst*I (P) cleavage sites are indicated for EHV1 *Bam*HI-D. The locations and orientations of the known open reading frames contained within these fragments are indicated by arrows. The EHV1 open reading frames are numbered according to the designations of Telford et al. (32), while their EHV4 homologs are given the same numbers but distinguished with a prime (19). HSV designations are given (in parentheses) where homology has been established. The bottom of each panel shows a schematic representation of the various gG segments that were expressed by the recombinant plasmids designated pEG4.1 and pEG4var (A) and pEG1.1, pEG1.2, and pEG1var (B). Predicted signal sequences and transmembrane domains (open and closed boxes, respectively) and the C-terminal variable regions of EHV4 gG (amino acids 287 to 382) and EHV1 gG (amino acids 288 to 350) (cross-hatched boxes) are indicated.

ment obtained was cloned into the *Sma*I site of pGEX-3X. (ii) pEG1var contains the nucleotides encoding EHV1 gG amino acids 288 to 350 and was constructed by polymerase chain reaction amplification as described above for pEG4var except that EHV1 *Bam*HI-D was used as the template DNA and the primers were as follows: forward, 5′-GGTGACG AAACATACGA-3′; reverse, 5′-TGGATGCCGTTCGACG C-3′.

E. coli strains containing the correct recombinant plasmids were identified, and glutathione *S*-transferase (GST) fusion protein was purified by a method based on that of Smith and Johnson (27). In this procedure, excess glutathione is used to elute GST fusion proteins bound to agarose beads coated with covalently attached, reduced glutathione (Sigma). Briefly, Luria-Bertani broth (10 ml) was inoculated with single colonies of bacteria transformed with the recombinant plasmid and incubated for 16 h at 37°C. The cultures were transferred to 1-liter flasks containing 100 ml of Luria-Bertani broth, incubated as described above for 1 to 2 h, and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Bacteria were lysed and GST fusion protein was prepared by the method of Smith and Johnson (27) except that the purification and washing (which was increased to six times) steps were carried out with phosphate-buffered saline (PBS) containing 1% Tween 20 (Sigma), 0.03% SDS, and 0.1 mM dithiothreitol. These more stringent conditions were used to reduce the possibility of *E. coli* proteins nonspecifically binding to the glutathione agarose beads. A quantity of 1 to 2 mg of GST fusion protein was obtained from each 100-ml culture of *E. coli* transformed with parental pGEX-3X, pEG4var, or pEG1var.

The sources of the equine sera used throughout this study were as follows. (i) Serum samples were obtained from three colostrum-deprived, specific-pathogen-free (SPF) foals following either a series of immunizations or experimental infection and virus challenges with EHV1 (SPF foals 1 and 2) or EHV4 (SPF foal 3) (8, 11). Serum samples were obtained from these foals at the following three time points: prior to exposure to EHV4 or EHV1 (sera a), 2 to 3 months after exposure to only one virus (sera b), and 1 to 2 months after exposure to both viruses, i.e., following cross-challenge (sera c) (see Table 1). (ii) Serum specimens were obtained from an 8-month-old foal before and after experimental infection with EHV4 (34). This foal, termed EHV4-infected foal 1, was considered seronegative for EHV4 prior to infection on the basis of serum neutralization assays (34). (iii) Serum samples were obtained from 11 Thoroughbred mares that aborted during an EHV1 abortion outbreak in Victoria, Australia, in 1989 (farm 1) (30), at times ranging from 1 day before to 6 weeks after abortion. EHV1 abortion was confirmed by isolation of the virus from each aborted fetus (30). Serum samples were obtained from 75 generally healthy, Thoroughbred mares and yearlings from at least 10 Thoroughbred farms in Victoria and South Australia. These serum samples were obtained between 1967 and 1974, a time prior to the first confirmed case of EHV1 in Australia, which occurred in 1977 (28–30). SDS-PAGE and Western blotting (immunoblotting) onto Immobilon polyvinylidene difluoride (Millipore) membranes were conducted as described previously (7, 8).

Previously, we showed that *E. coli* transformed with a recombinant pGEX plasmid, pEG4.1 (Fig. 2), expressed a GST fusion protein that included the entire EHV4 gG molecule (8). This fusion protein reacted type specifically in a Western blot, binding antibody present in sera obtained from horses known to have been exposed to EHV4 only

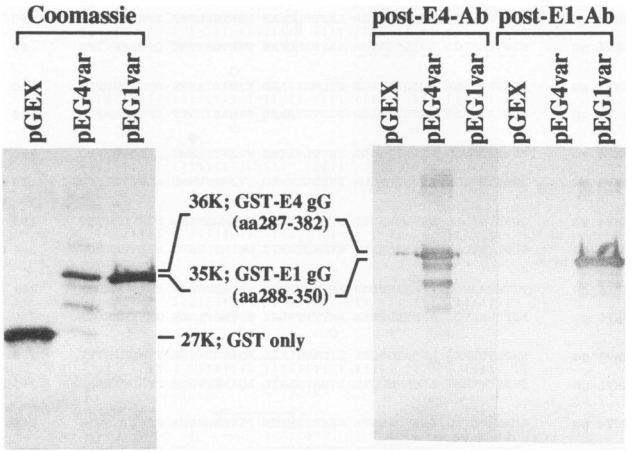

FIG. 3. Results of Coomassie brilliant blue staining and Western blotting of GST-gG fusion proteins expressed in *E. coli* transformed with parental pGEX-3X, pEG4var, or pEG1var. Membranes were probed with pooled SPF foal serum 3b and EHV4-infected-foal serum 1b (post-EHV4-Ab) or pooled SPF foal serum samples 1b and 2b (post-EHV1-Ab) diluted 1/100. The same loadings (approximately 3 μg of fusion protein) were used for both the Coomassie-stained gel and the Western blot. Samples for Western blotting were run on an SDS-PAGE gel different from those for Coomassie staining. Approximate M_rs of the major GST fusion protein species produced by pGEX (GST only), pEG4var [GST-E4 gG (aa287-382)], and pEG1var [GST-E1 gG (aa288-350)] are indicated. aa, amino acids.

(SPF foal sample 3b and EHV4-infected-foal sample 1b), but showed no detectable reaction with sera from horses known to have been exposed to EHV1 only (SPF foal serum specimens 1b and 2b) (7). Subsequently, we have shown that a GST fusion protein associated with the entire EHV1 gG molecule (411 amino acids), termed pEG1.2 (Fig. 2), reacted strongly with EHV1-only horse sera but not with EHV4-only horse sera in Western blots (9). Also, a GST fusion protein expressing only part of EHV1 gG (amino acids 1 to 310), termed pEG1.1 (Fig. 2), showed no reaction with any of the tested horse sera (9). These results suggested that EHV4 gG and EHV1 gG possessed type-specific, presumably continuous, epitopes and that these epitopes were located in the C-terminal regions of the molecules as a fusion protein containing amino acids 1 to 310 of EHV1 gG. This fusion protein was not antigenic, whereas a fusion protein containing amino acids 1 to 411 of the same protein was a strong antigen.

It was considered most likely that type-specific, continuous epitopes were located in the corresponding C-terminal variable regions of EHV4 gG (amino acids 287 to 382) and EHV1 gG (amino acids 288 to 350), which show little amino acid identity. Two pGEX-3X clones, termed pEG4var and pEG1var, which express these variable regions of EHV4 gG and EHV1 gG, respectively, were constructed (Fig. 2 and 3). The major fusion proteins, i.e., the most abundant species, produced by pEG4var and pEG1var had M_rs of approximately 36,000 and 35,000, respectively (Fig. 3). These sizes are similar to the predicted sizes of pEG4var and pEG1var fusion proteins of 36,000 and 33,000, respectively, which takes into account the M_r of GST of 26,000 (27).

The GST-gG fusion proteins produced by pEG4var and pEG1var reacted strongly and type specifically in Western blots using pooled EHV4-only foal serum samples (SPF foal serum 3b and EHV4-infected-foal serum 1b) or EHV1-only

TABLE 1. Reactivities of sera from foals with defined histories with regard to exposure to EHV4 and/or EHV1 against whole virus and against EHV4 gG and EHV1 gG fusion proteins

Source and serum	Virus exposure[a]	Reactivity in whole-virus ELISA[b]		Reactivity in single-dilution gG ELISA[c]	
		EHV4	EHV1	EHV4	EHV1
SPF foals					
1a	None (preexposure)	<30	<30	0.01	−0.01
1b	EHV1 only	4,000	21,000	0.03	1.05
1c	EHV1 and EHV4	ND[d]	ND	1.25	0.75
2a	None (preexposure)	<30	<30	0.01	0.00
2b	EHV1 only	630	4,000	−0.01	0.66
2c	EHV1 and EHV4	21,000	21,000	1.04	0.25
3a	None (preexposure)	<30	<30	0.06	0.07
3b	EHV4 only	6,300	13,000	0.86	0.01
3c	EHV4 and EHV1	ND	ND	0.65	0.36
EHV4-infected foal					
1a	None (preexposure)	<30	<30	0.04	0.01
1b	EHV4 only	6,300	6,300	1.25	0.05

[a] The SPF foals were exposed to either EHV1 (SPF foals 1 and 2) or EHV4 (SPF foal 3) before being cross-challenged with the heterologous virus (8, 11). EHV4-infected foal 1 was experimentally infected with EHV4 (34).

[b] Purified whole EHV4 or EHV1 (10 μg/ml) was used to coat wells. Results are titers determined from a titration curve and expressed as the reciprocal of the highest dilution of serum that gave an absorbance reading of twice the baseline reading.

[c] Wells were coated with fusion protein (0.5 μg/ml), and sera were added to the wells at a dilution of 1/100. Results are expressed as absorbance values after the absorbance value obtained for each serum against GST only has been subtracted.

[d] ND, not determined.

foal serum samples (SPF foal serum samples 1b and 2b), thereby confirming that these variable regions contained type-specific epitopes (Fig. 3). A pool of two EHV4-only and two EHV1 serum samples was used to reduce the possibility of not detecting a cross-reactive epitope due to the nonresponsiveness of individual horses. Contaminating *E. coli* proteins were not detected, as was evident from the absence of any bands in the GST-only lanes or in the Western blot lanes with heterologous gG fusion protein (Fig. 3). Some breakdown products were evident for each antigen, most particularly in the case of pEG4var. Faint bands are present at the top of the immunoreactive lanes; these probably represent oligomeric structures, as they are present in both immunoreactive lanes but are not present in the GST-only or nonreactive-antigen lanes (Fig. 3). Also, these high-M_r species migrate more slowly in the pEG4var lane than in the pEG1var lane, which is consistent with the slightly different M_rs of the two gG fusion proteins. Preexposure serum samples from the three SPF foals and the EHV4-infected foal showed no reactivity against either pEG1var or pEG4var fusion proteins in either Western blots (data not shown) or ELISAs (Table 1). The pooled serum samples used to produce the Western blot shown in Fig. 3 were tested individually in both Western blots (data not shown) and ELISAs (see below) (Table 1), and each serum reacted strongly and completely type specifically with the relevant gG fusion protein.

Development of a single-dilution type-specific ELISA. An ELISA using the recombinant gG fusion proteins produced by pEG4var and pEG1var was developed. ELISAs were carried out in 96-well polyvinyl chloride microtiter plates (Immunoplate Maxisorp; Nunc). The plates were washed four times between steps with PBS (pH 7.5) containing 0.05% (vol/vol) Tween 20 (PBST) and incubated with volumes of 100 μl per well on a shaker for 1 h. Wells were coated with 0.5 μg of GST fusion protein per ml diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. The dilution level was determined by checkerboard titration as the highest dilution of antigen that gave optimal binding to both SPF foal serum 1b (for pEG1var binding) and SPF foal serum 3b (for pEG4var binding). Unoccupied sites were blocked by incubation for 2 h with 10 mg of bovine serum albumin per ml in PBS. By using the SPF foal serum samples 1b and 3b (Table 1), it was determined that a dilution of 1/100 gave the best signal-to-noise ratio. Horseradish peroxidase-conjugated, affinity-purified goat anti-horse immunoglobulin G (Kirkegaard & Perry Laboratories Inc.), diluted 1/1,000 in antibody diluent, was added to each well. The assays were developed with a soluble 3,3′,5,5′-tetramethyl benzidine dihydrochloride (TMB) substrate (Sigma). The reaction was stopped after 7 min of incubation at room temperature by addition of 25 μl of 2 M HCl, and the plates were read spectrophotometrically at 450 nm with a Titertek Multiskan MC3 (Flow Laboratories).

All sera were tested against GST only and against the EHV4 gG and EHV1 gG fusion proteins at a dilution of 1/100. Each serum was tested in duplicate, and the mean of the two values was taken as the reading for that serum. Absorbance values for reactivity against GST only for 97 different horse serum samples tested (see Tables 1 and 2) ranged from 0.085 to 0.302, with a mean of 0.166 (data not shown). This indicated that while the level of reactivity to GST only was generally low, some variability in the reactivity of individual sera to GST only was evident. To account for the different background readings, the absorbance values obtained for each serum against the gG fusion proteins were corrected by subtracting from them the absorbance values obtained for GST only.

Table 1 shows the response in ELISA of serum samples from the four foals, which had defined histories with respect to exposure to EHV4 and EHV1, to whole virus, and to the gG fusion proteins. From the data shown in Table 1, the

TABLE 2. Reactivities of sera from selected Thoroughbred horses with EHV4 gG and EHV1 gG fusion proteins

Serum source[a]	n	Reactivity in ELISA[b]			
		EHV4		EHV1	
		Range	Mean	Range	Mean
1967–1974 Thoroughbreds	68	0.72–1.41	1.11	−0.06–0.09	0.01
	7	0.97–1.19	1.07	0.19–0.96	0.47
EHV1 abortion mares	11	1.22–1.74	1.49	0.39–1.73	1.12

[a] For the EHV1 abortion mares, sera were obtained at times ranging from 1 day before to 6 weeks after confirmed EHV1-caused abortion. The Thoroughbred sera obtained between 1967 and 1974 were from generally healthy horses which represented at least seven different Thoroughbred farms in Victoria and South Australia, Australia.

[b] Values as defined in Table 1, footnote *b*.

following is evident. (i) SPF foal preexposure serum samples had EHV4 gG and EHV1 gG absorbance readings of near zero (−0.01 to 0.07). (ii) Serum samples 1b and 2b, from SPF foals known to have been exposed to EHV1 only, and serum samples 3b and 1b, from an SPF foal and an EHV4-infected foal, respectively, both known to have been exposed to EHV4 only, had ELISA titers of between 4,000 and 21,000 in tests against the whole virus to which the horses were exposed and contained antibody which was cross-reactive with the heterologous whole virus. (iii) SPF foal serum specimens 1b and 2b have EHV1 gG absorbance readings of 1.05 and 0.66, respectively, while their EHV4 gG absorbance readings do not differ significantly from those of the preexposure serum samples. Conversely, SPF foal serum sample 3b and EHV4-infected-foal sample 1b have EHV4 gG readings of 0.86 and 1.25, respectively, while their EHV1 gG absorbance readings do not differ significantly from those of the preexposure serum samples. (iv) After cross-challenge with heterologous virus, each SPF foal seroconverted, with each serum showing a significant reaction with the heterologous gG antigen. It was also noted, in the case of each of the three SPF foals, that cross-challenge with heterologous virus did not boost the response to the homologous gG antigen; indeed, reactivity to the homologous gG antigen actually decreased.

Serum samples were obtained from 75 generally healthy Thoroughbred horses between 1967 and 1974. All sera showed EHV4 gG absorbance readings of 0.72 to 1.41, which suggested that each horse had been infected with EHV4 (Table 2). It was anticipated that these sera would be negative for EHV1 as they were obtained before 1977, the time of the first confirmed cases of EHV1 abortion in Australia. However, their EHV1 absorbance readings were separable into two groups. The first was a group of 68 specimens with seemingly negative EHV1 gG absorbance readings, which were normally distributed in the range −0.06 to 0.09, i.e., approximately the same range as the values obtained for the preexposure SPF foal sera (−0.01 to 0.07). The mean of the values obtained for the serum samples of this group was 0.01, and the standard deviation of these values was 0.03 (not shown in Table 2). This group of sera was considered negative for EHV1 gG fusion protein antibodies. The mean (0.01) plus 3 standard deviations of the EHV1 gG absorbance readings obtained for these 68 serum samples is 0.10. We propose that EHV1 gG absorbance readings of greater than 0.10 be considered positive for EHV1 gG antibodies. A second group of seven specimens taken between 1967 and 1974 had EHV1 gG absorbance readings of 0.19 to 0.96 (mean 0.47), all significantly greater than 0.10 and well outside the range of values in the first group. These EHV1 gG-positive sera were obtained from six different Thoroughbred farms in Victoria. The earliest positive serum was obtained in 1967.

Table 2 shows that serum samples from each of 11 mares that aborted EHV1-infected fetuses had EHV1 absorbance readings of 0.39 to 1.73, which were all significantly greater than the 0.10 cutoff. The 11 abortion mare serum samples also showed EHV4 absorbance readings of 1.22 to 1.74.

Essentially three main lines of evidence suggest strongly that the EHV4 gG and EHV1 gG fusion proteins produced by pEG4var and pEG1var are type-specific antigens in tests against serum derived from the natural host. (i) The serum samples from four foals known to have been exposed to only one virus (serum b for each foal; Table 1) produced antibody which was cross-reactive with whole virus as expected but was highly specific for the relevant gG fusion protein in both Western blots (Fig. 3) and ELISAs (Table 1). (ii) The 11 serum samples from mares that aborted as a result of confirmed EHV1 infection all had significant levels of antibody to the EHV1 gG fusion protein. (iii) Of the 75 serum samples obtained from Thoroughbred horses between 1967 and 1974, 68 (90.7%) had high levels of antibody to the EHV4 gG fusion protein but showed no significant reactivity against EHV1 gG fusion protein in ELISA.

On the basis of this evidence, the 7 of 75 serum samples (9.3%) obtained between 1967 and 1974 that did show significant levels of antibody against the EHV1 gG fusion protein were most probably from EHV1-positive horses. The data suggest that EHV1 was present in Australia at least 10 years prior to the first confirmed case of EHV1 in Australia (28–30). Significantly, each of the 75 serum samples from 1967 to 1974 and the serum samples from the 11 abortion mares had high levels of EHV4 gG antibody. This result highlights the ubiquitous nature of EHV4 infection in the general Thoroughbred population.

While we have designated 3 standard deviations from the mean of the 68 EHV1-negative serum samples as our cutoff in the above analysis, this value may be conservative and may lead to missing some low EHV1-positive results. As it is important in the context of an EHV1 diagnostic test to detect low-positive results, given the potentially devastating consequences of EHV1 infection in the Thoroughbred breeding industry, we are currently refining a Western blot procedure to test sera closer to and above 2 standard deviations from the mean which will, it is hoped, unambiguously distinguish EHV1-positive results from EHV1-negative results within this range. It may be necessary, however, to obtain subsequent serum samples from those horses giving low EHV1 gG absorbance readings.

The development of type-specific EHV4 and EHV1 anti-

gens has significant implications for our understanding of the epidemiology of EHV4 and EHV1, which should have an impact on current strategies for control of these economically important equine pathogens. It is now possible to undertake broad-based, contemporary seroepidemiological surveys, most easily pursued in countries such as Australia which have not introduced EHV1 vaccination, to determine the true prevalence and distribution of EHV4 and EHV1. It is presumed that most older horses will test positive for EHV4, as bouts of confirmed EHV4 respiratory disease in foals are common. In the case of EHV1, however, in which clinical disease is infrequently confirmed prior to the occurrence of the sporadic but devastating EHV1 abortion "storms," very little is known of its prevalence and distribution. It is possible, in Australia at least, that only a small population of horses will test positive for EHV1 antibodies since abortion outbreaks are relatively rare. Indeed, the current EHV4-EHV1 status of Thoroughbred horses in Australia may not be significantly different from the EHV4-EHV1 status of the 75 Thoroughbred horses tested between 1967 and 1974 described in this paper, of which only 7 (approximately 10%) tested positive for EHV1 while all 75 (100%) tested positive for EHV4. Such a result could pave the way for measures in addition to vaccination to be used for control of EHV1. For instance, segregation of EHV1 carrier mares from others would be a possible, although perhaps not always feasible, option.

It would be useful if future EHV4-EHV1 vaccines allowed distinction of vaccinated and infected horses. The deletion of the EHV4 and/or EHV1 gG gene, provided it did not prove to be an essential protective immunogen, could be used as a marker for this purpose. In the case of PRV, the gG (gX) gene has proved the most useful of several glycoprotein genes studied to date for such purposes since PRV mutants lacking gG do not appear to have reduced efficacy compared with that of parental PRV vaccines (22, 33, 35). It would also be useful to identify a location for the insertion of genes encoding important antigens from other equine pathogens. The PRV gG gene appears to possess a strong promoter (14, 18), and indeed the gG gene promoter, i.e., one in a deletion mutant from which almost the entire gG open reading frame had been removed, has been successfully used for the expression of the E1 envelope glycoprotein of hog cholera virus (36). For future EHV4 and/or EHV1 vaccines, gG homologs are clearly prime candidates for these purposes, as their deletion will not only allow the clear distinction of vaccinated horses from EHV4- and/or EHV1-infected horses but will probably also provide a suitable location for the insertion of foreign genes.

Evidence that gG homologs have some unusual features not found in other alphaherpesvirus glycoproteins is accumulating. Perhaps the most striking of these features is their considerable heterogeneity, particularly toward the C terminus of the molecules. It is apparent that the genes have diverged widely both by point mutation and by the deletion and/or insertion of segments of DNA (15, 17). HSV1 gG in particular appears to have suffered a large internal deletion. Consequently, the sizes of the predicted amino acid sequences are widely disparate compared with other alphaherpesvirus glycoprotein gene homologs; HSV1 gG comprises 238 amino acids (16), HSV2 gG comprises 699 (17), PRV gG comprises 498 (23), EHV4 gG comprises 405 (7), EHV1 gG comprises 411 (32), and ILTV gG (gX) comprises 298 (13).

The gG homologs of EHV4 and EHV1 show only 58% amino acid identity, considerably less than the next most divergent of the sequenced EHV4 and EHV1 glycoproteins, gC, which show 79% identity and which possess both shared and unique epitopes (2, 3, 5, 21). Significantly, epitopes responsible for eliciting a strong, type-specific response in the natural host are localized to the apparently corresponding, C-terminal variable regions of EHV4 gG (amino acids 287 to 382) and EHV1 gG (amino acids 288 to 350), regions which appear to have few constraints on either the number or the type of amino acid except perhaps that the amino acids in this region tend to be hydrophilic. It must also be considered likely that discontinuous epitopes that are probably not formed in the *E. coli*-expressed products are present on native gG molecules. Indeed, a second variable region comprising amino acids 32 to 57 of both EHV4 gG and EHV1 gG, which does not appear to be antigenic in the *E. coli*-expressed molecules, probably comprises part of a discontinuous epitope. If such an epitope(s) does exist, then it appears as though it is also largely type specific since native EHV4 gG, present as a secreted glycoprotein in cell culture supernatant, reacts type specifically with defined polyclonal horse sera in ELISAs (7).

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