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Coexpression by Vaccinia Virus Recombinants of Equine Herpesvirus 1 Glycoproteins gp13 and gp14 Results in Potentiated Immunity

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The equine herpesvirus 1 glycoprotein 14 (EHV-1 gp14) gene was cloned, sequenced, and expressed by vaccinia virus recombinants. Recombinant virus vP613 elicited the production of EHV-1-neutralizing antibodies in guinea pigs and was effective in protecting hamsters from subsequent lethal EHV-1 challenge. Coexpression of EHV-1 gp14 in vaccinia virus recombinant vP634 along with EHV-1 gp13 (P. Guo, S. Goebel, S. Davis, M. E. Perkus, B. Languet, P. Desmettre, G. Allen, and E. Paoletti, J. Virol. 63:4189–4198, 1989) greatly enhanced the protective efficacy in the hamster challenge model over that obtained with single recombinants. The inoculum doses (log_{10}) required for protection of 50% of hamsters were 6.1 (EHV-1 gp13), 5.2 (EHV-1 gp14), and <3.6 (vaccinia virus recombinant expressing both EHV-1 glycoproteins [gp13 and gp14]).

There are two subtypes of equine herpesvirus that, although cross-neutralizable, can be distinguished by their antigenic profiles, restriction endonuclease profiles, and pathogenicity for horses (3). Equine herpesvirus 1 (EHV-1) is associated with respiratory tract disease, central nervous system disorders, and classic herpetic abortions, whereas equine herpesvirus 4 (EHV-4) is predominantly associated with respiratory tract disease (3, 28). Equine herpesviruses are members of the alphaherpesvirus subfamily and display many of the typical biological and biochemical characteristics of human herpesviruses, such as genomic isomerization, regulation of gene expression, establishment of latent infections, generation of defective interfering virus particles, induction of neurological disorders, and in vitro oncogenic transformation (3, 5, 29). Thus, equine herpesviruses represent a useful system for studying the varied biological consequences of herpesvirus infections.

Herpesvirus glycoproteins mediate essential viral functions, such as cellular attachment and penetration and cell-to-cell spread of the virus, and, importantly, determine the pathogenicity profile of infection. Herpesvirus glycoproteins are critical components in the interaction with the host immune system (45, 46). Both subtypes of equine herpesvirus express six abundant glycoproteins (3, 4, 53). The genomic portions of the EHV-1 DNA sequences encoding glycoprotein 2 (gp2), gp10, gp13, gp14, gp17/18, and gp21/22a have been determined with lambda gt11 expression vectors and monoclonal antibodies (4). gp13 and gp14 were located in the same locations within the L component of the genome to which the gC and gB homologs, respectively, of herpes simplex virus map (4). Recently, the nucleotide sequence of the Kentucky T431 strain of the EHV-1 transcriptional unit encoding gp13 was reported (2, 18), and the glycoprotein was shown to be homologous to herpes simplex virus gC-1 and gC-2, to pseudorabies virus gIII, and to varicella-zoster virus gpV (2, 18). EHV-1 gp13 is thus the structural homolog of the herpesvirus gC glycoproteins.

EHV-1 gp13 was expressed in vaccinia virus (18). Inoculation of this recombinant vaccinia virus, vP483, into guinea pigs induced neutralizing antibodies to EHV-1. Protection against lethal EHV-1 challenge was demonstrated in hamsters immunized with the gp13-vaccinia virus recombinant vP483 (18). Thus, these studies demonstrated the contribution of EHV-1 gp13 to a potential vaccine against this herpetic infection.

We now report the expression of EHV-1 gp14 in vaccinia virus vectors either by itself or with EHV-1 gp13 and report the immunological responses to these EHV-1 glycoproteins elicited in laboratory animals upon inoculation with the recombinant vaccinia viruses.

Cloning and analysis of the EHV-1 gp14 gene. The EHV-1 gp14 coding sequence spans the junction between *Bam*HI restriction fragments a and i (4). DNA sequence (Fig. 1) analysis revealed an open reading frame extending from nucleotide positions 300 to 3239, reading from left to right relative to the EHV-1 genome; i.e., the ATG start codon was contained in the *Bam*HI a fragment, and the stop codon TAA was contained in the *Bam*HI i fragment (4, 56). The EHV-1 gp14 open reading frame encodes 980 amino acids and has a calculated molecular mass of 109.8 kilodaltons (kDa). Putative transcriptional regulatory signals were found in the region 5′ to the ATG initiation codon at position 300. A TATA box having the sequence AAATATAT (nucleotides 148 to 155) was located 70 nucleotides downstream from a putative CAT box at positions 71 to 77 and having the sequence GGTCAAT. A polyadenylation signal, AATAAA (nucleotides 3251 to 3256), was located 8 nucleotides downstream from the TAA termination codon (nucleotides 3240 to 3242). Nine of 11 nucleotides in the sequence 5′-TCCTG CGCGCA-3′ (nucleotides 218 to 228) are complementary to

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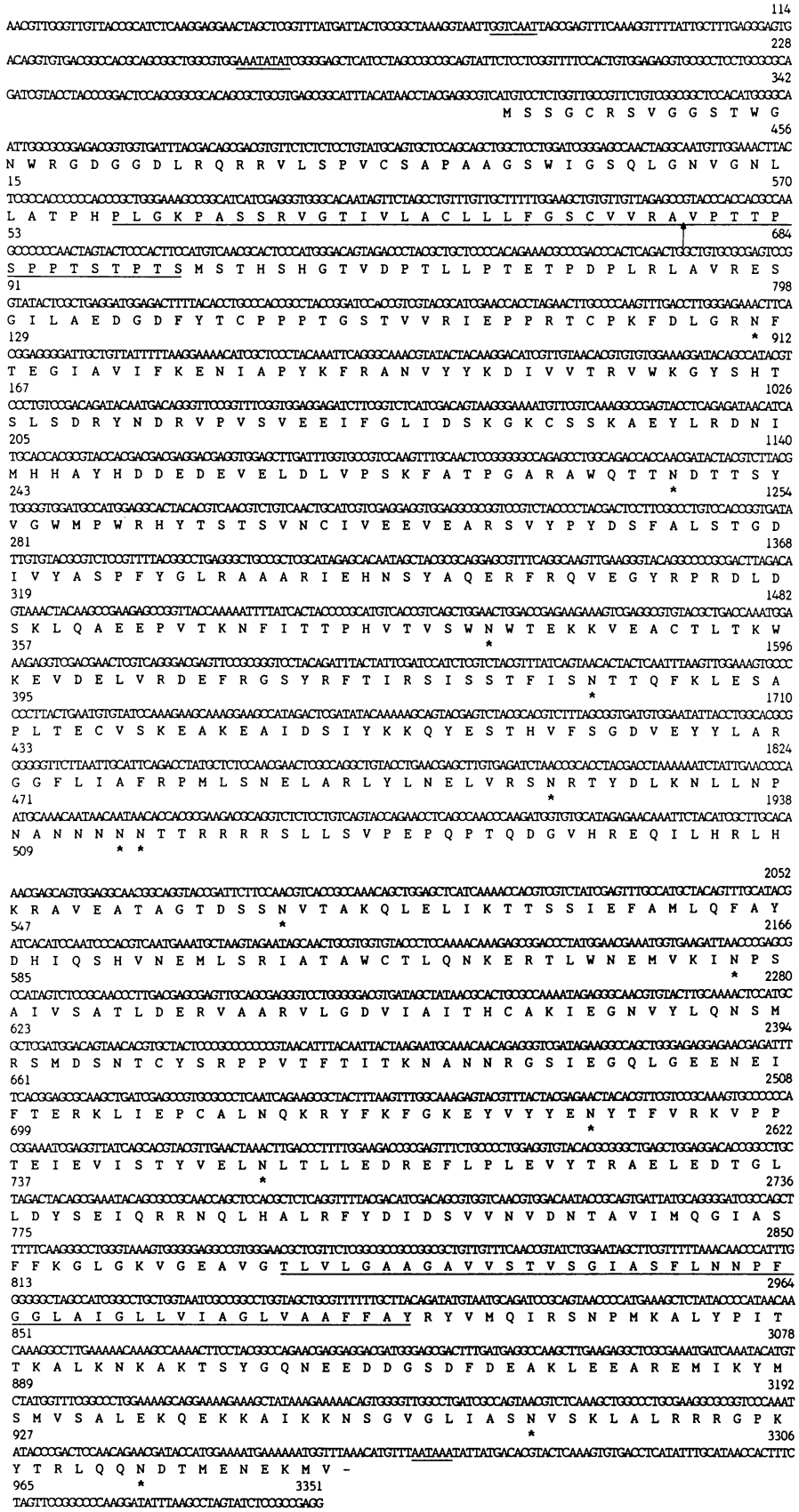

the 18S rRNA sequence 3'-AGGAAGGCGT-5' (19) and may serve as the ribosome-binding site.

Analysis of the amino acid sequence revealed a number of features common to membrane-associated glycoproteins. A region extending from amino acids 58 to 99 had a characteristic hydrophobicity profile and is proposed to be the signal sequence. An unusual feature of the EHV-1 gp14 gene is that the long hydrophobic signal sequence is preceded by a long hydrophilic sequence. This characteristic has also been noted for the pseudorabies virus gII (39) and bovine herpesvirus 1 gI (58) genes. A hydrophobic region consisting of 45 amino acids (amino acids 826 to 870) is predicted to function as a transmembrane anchor domain. The hydrophilic cytoplasmic domain contains 110 amino acids. There are 13 Asn-X-Thr/Ser (where X can be any amino acid except proline) sites for potential N-linked glycosylation (27). An unusual feature is that there are two potential glycosylation sites in the cytoplasmic domain (Fig. 1). A comparison of the amino acid compositions of the EHV-1 gp14 gene and other herpesvirus glycoprotein genes revealed extensive homology. Thus, EHV-1 gp14 is homologous to gII of pseudorabies virus (39), gI of bovine herpesvirus 1 (58), gII of varicella-zoster virus (14), gB of herpes simplex virus (9), glycoprotein BALF-4 of Epstein-Barr virus (32), and gB of human cytomegalovirus (12). These results are in good agreement with recent reports on the sequence analysis of gp14 of EHV-4 (38) and gp14 of isolate HVS-25A of EHV-1 (57). In a comparison of the amino acid sequence of EHV-1 gp14 reported here with that of EHV-4 gp14 (38), 83% overall identity was found. However, in a comparison of just the amino acids comprising the predicted mature protein, the homology was 93%. As would be expected, the derived amino acid sequence of EHV-1 gp14 reported here was even more similar to that reported for EHV-1 isolate HVS-25A gp14 (57). The DNA sequence was identical except for one short region. We found a second G at position 1074 and one less G at position 1102. The amino acids encoded between these two positions are V, P, S, K, F, A, T, P, and G instead of C, R, P, S, L, Q, L, R, and G. Significantly, the deduced amino acid sequence reported here over this region of EHV-1 was identical to the corresponding sequence reported for EHV-4 (38).

Lambda gt11 expression vectors and monoclonal antibodies have been useful in identifying the EHV-1 DNA sequences encoding the major EHV-1 glycoproteins (4). A lambda gt11 recombinant, 4a1, was shown to express an EHV-1 gp14 epitope recognized by the specific monoclonal antibody 3F6 (4). For determination of the identity of this epitope, the EHV-1 DNA contained within 4a1 was sequenced and compared with the DNA of the EHV-1 gp14 coding sequence (Fig. 1). The nucleotide sequence alignment indicated that this epitope was contained within the 66-amino-acid region corresponding to amino acids 107 (Thr) through 172 (Val) of the deduced primary translation product. The epitope is therefore located within the amino-terminal region of the deduced EHV-1 gp14 surface domain.

Construction of vaccinia virus recombinants vP458, vP613, and vP634. To insert the EHV-1 gp14 coding sequences into a vaccinia virus vector, we constructed a recombinant vaccinia virus, vP458, expressing the *Escherichia coli lacZ* gene at the M2L locus. We have previously reported deletions in the vaccinia virus (WR) genome extending leftward from the unique *Bgl*II site in the *Hin*dIII fragment M (34), indicating that these sequences are not essential for replication of the virus in tissue culture. We have now derived the DNA sequence of the *Hin*dIII M fragment from the Copenhagen strain of vaccinia virus (S. Goebel, G. P. Johnson, M. E. Perkus, S. Davis, J. Winslow, and E. Paoletti, unpublished data). An open reading frame reading right to left relative to the vaccinia virus genome and encoding a putative protein of 220 amino acids is located entirely within the *Hin*dIII M fragment to the left of the unique *Bgl*II site. According to convention (41), this gene, which is located immediately to the right of M1L (52), was designated M2L. Figure 2 details the construction of recombinant vaccinia virus vP458, in which the M2L gene is replaced by the *E. coli lacZ* gene under the control of the vaccinia virus 11-kDa promoter (8).

The gene for EHV-1 gp14 was cloned into the M2L deletion locus under the control of the vaccinia virus H6 promoter (18, 33). Several recombinants containing different amounts of the unusually long EHV-1 gp14 leader sequence were constructed. Vaccinia virus recombinant vP613 contains a truncated version of gp14 in which amino acids 2 to 34 are replaced by 4 amino acids derived from synthetic linkers (Fig. 3).

To obtain vaccinia virus recombinants containing both EHV-1 gp14 and EHV-1 gp13, we performed in vivo recombination with vP613 as the rescuing virus and donor plasmid pVHA6g13 (18). pVHA6g13 contains the EHV-1 gp13 gene under the control of the H6 promoter inserted at the vaccinia virus HA deletion locus. Recombination of pVHA6g13 with vaccinia virus recombinant vP613 generated the vaccinia virus recombinant vP634 containing both EHV-1 gp13 and EHV-1 gp14.

Confirmation of the presence of both EHV-1 genes was obtained by in situ hybridization of ^{32}P-labeled probes specific for either EHV-1 gp13 or EHV-1 gp14. Expression of the respective EHV-1 antigens was assessed by immunoprecipitation of metabolically radiolabeled polypeptides specifically reactive with monoclonal antibody 14H7 (gp13) or 3F6 (gp14). Immunoprecipitation analyses of vP613- and vP634-infected Vero cells with EHV-1 gp14-specific monoclonal antibody 3F6 (data not shown) revealed three predominant polypeptide species which migrated with apparent molecular masses of 116, 72 to 82, and 57 kDa. These molecular masses are in good agreement with those noted previously for EHV-1 gp14 by Meredith et al. (26) and

FIG. 1. Nucleotide sequence of EHV-1 gp14. Nucleotide sequence analysis was done with subclones of EHV-1 *Bam*HI fragments a and i in pUC-based plasmids. Sequence data for both strands were obtained with the modified T7 enzyme SEQUENASE (U.S. Biochemicals, Cleveland, Ohio) (51). Standard dideoxy chain termination reactions (43) were done with double-stranded plasmid templates which were denatured by treatment with 0.4 N NaOH. The M13 forward and reverse primers were used to obtain the initial sequence from each clone. Custom (16- or 17-mer) synthetic oligonucleotide primers were prepared (model 8700 synthesizer; Biosearch, San Rafael, Calif., model 3808 synthesizer; Applied Biosystems, Foster City, Calif.) to walk along the remaining fragment. The IBI/Pustell sequence analysis program was used in all sequence datum analyses (36). Numbering in the left and right hand margins pertains to amino acid and nucleic acid sequences, respectively. The putative CAT and TATA boxes are underlined. Amino acids in the signal and membrane-spanning regions are also underlined, with the arrow indicating a potential signal peptide cleavage site. The 13 potential glycosylation sites using the consensus sequence Asn-X-Ser/Thr are indicated by asterisks.

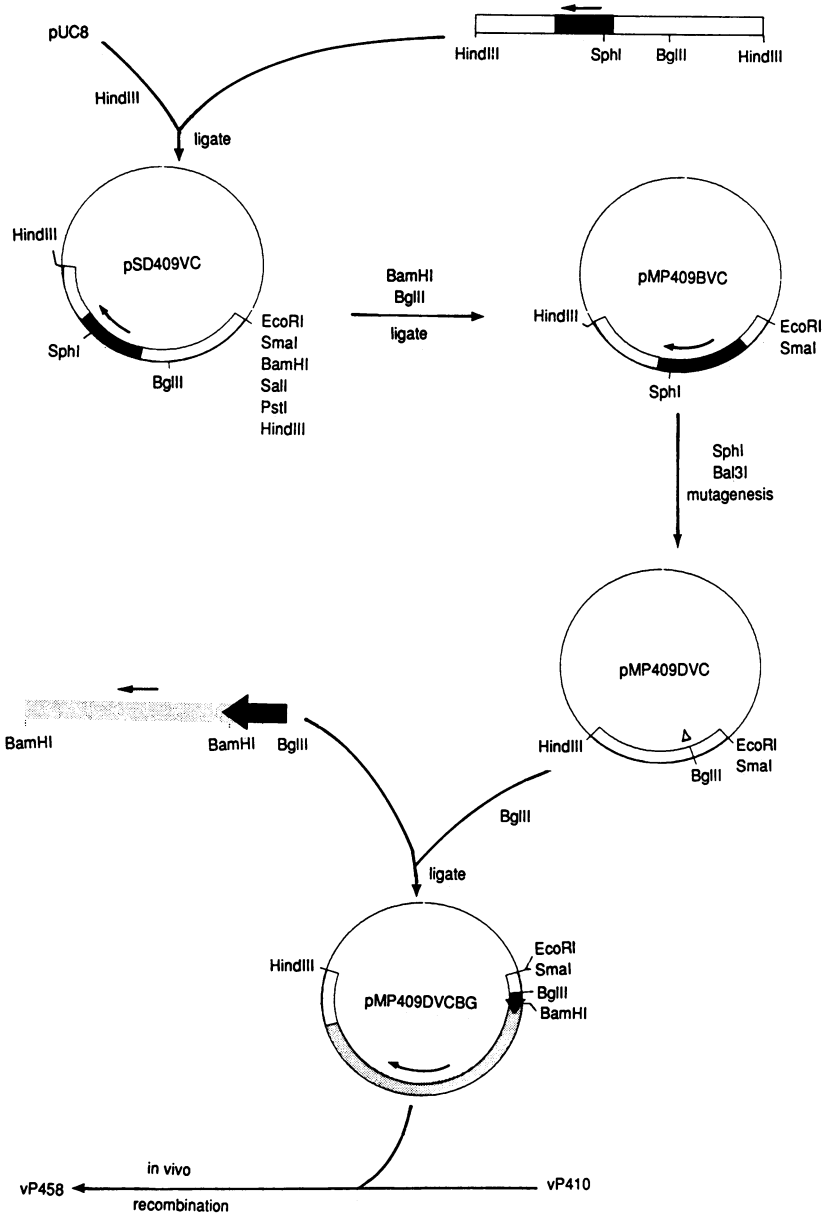

FIG. 2. Replacement of the M2L gene in vaccinia virus by the *E. coli* β-galactosidase gene to generate vP458. Shown at the top is a map of the vaccinia virus *Hin*dIII M fragment with the location of the M2L gene and relevant restriction sites indicated. The vaccinia virus *Hin*dIII M fragment was cloned into pUC8 cut with *Hin*dIII, resulting in plasmid pSD409VC. For removal of unwanted vaccinia virus DNA and restriction sites, the right vaccinia virus arm was shortened by digestion of pSD409VC with *Bam*HI-*Bgl*II and ligated, resulting in pMP409BVC. For removal of the M2L gene, pMP409BVC was digested with *Sph*I, followed by limited digestion with BAL-31 exonuclease and mutagenesis (23) with a synthetic 49-mer oligonucleotide (5′-TTTCTGTATATTTGCAACAATTTAGATCTTACTCAAAATATGTAA CAAT-3′; the *Bgl*II site is underlined). In the mutagenized plasmid, pMP409DVC, the M2L coding sequence was deleted from position +3 through the end of the open reading frame. The G of the initiation codon was changed to a C to create a unique *Bgl*II site (AGATCT) at the deletion junction. A *Bgl*II-*Bam*HI cassette containing the *E. coli* β-galactosidase gene (44) under the control of the vaccinia virus 11-kDa promoter (8) was inserted into pMP409DVC cut with *Bgl*II, resulting in plasmid pMP409DVCBG. pMP409DVCBG was used as a donor plasmid for in vivo recombination with the rescuing virus vP410 (18). The resulting vaccinia virus recombinant, vP458, contains the *E. coli* β-galactosidase gene under the control of the 11-kDa vaccinia virus promoter inserted into the M2L deletion locus in a right-to-left orientation relative to flanking vaccinia virus sequences. Vaccinia virus recombinant vP458 was detected by β-galactosidase gene expression with the chromogenic 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside substrate (11, 30) and purified by repeated plaque cloning. Symbols: solid blocks, vaccinia virus M2L gene (direction of transcription indicated by the arrow); open blocks, sequences in the vaccinia virus *Hin*dIII M fragment flanking the M2L gene; triangle, site of deletion of the M2L gene; stippled blocks, *E. coli* β-galactosidase gene (direction of transcription indicated by the arrow); large solid arrows, vaccinia virus 11-kDa promoter.

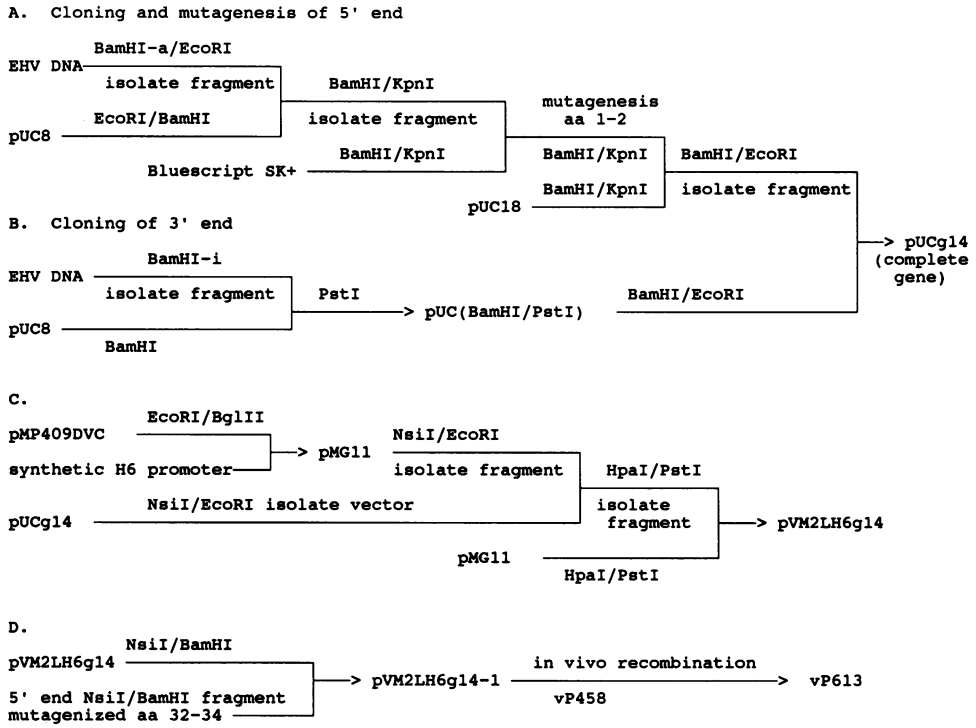

FIG. 3. Cloning of EHV-1 gp14 and construction of recombinant vaccinia virus vP613. The gene for EHV-1 gp14 is contained in *Bam*HI restriction fragments a and i. (A) Cloning and mutagenesis of the 5′ end of the gene. The *Bam*HI a fragment from EHV-1 was isolated and cut with *Eco*RI, and the 10-kilobase restriction fragment containing the 5′ end of the EHV-1 gp14 gene was cloned into pUC8 cut with *Bam*HI-*Eco*RI. For purposes of mutagenesis around the ATG initiation codon, a *Kpn*I-*Bam*HI fragment containing the 5′ end of the EHV-1 gp14 gene was subcloned into Bluescript SK+. Oligonucleotide-directed mutagenesis was performed by a modification of the procedure of Kunkel (20) with uracil-containing plasmid DNA produced in the *dut*, *ung* host *E. coli* CJ236. In the mutagenized plasmid, an *Nsi*I site was created at codons 1 and 2 of the EHV-1 gp14 gene, changing the sequence ATG/TCC (Met/Ser) to ATG/CAT (Met/His). The *Kpn*I-*Bam*HI fragment from the mutated plasmid was transferred to *Kpn*I- *Bam*HI-digested pUC18. (B) Cloning of the 3′ end of the gene. The 7.1-kilobase EHV-1 *Bam*HI i fragment containing the 3′ end of the EHV-1 gp14 gene was cloned into pUC8 cut with *Bam*HI. Extraneous DNA was removed by digestion of the plasmid with *Pst*I, followed by self-ligation, resulting in plasmid pUC (*Bam*HI-*Pst*I). For assembly of the entire EHV-1 gp14 gene in one plasmid, a *Bam*HI-*Eco*RI fragment from panel A containing the mutated 5′ end of the EHV-1 gp14 gene was ligated into pUC (*Bam*HI-*Pst*I) cut with *Bam*HI-*Eco*RI, resulting in plasmid pUCg14. (C) Insertion of the EHV-1 gp14 gene into vaccinia virus flanking arms. The synthetic H6 promoter (33) containing an *Nsi*I site at the ATG initiation codon was inserted into the Copenhagen M2L deletion vector plasmid pMP409DVC, generating plasmid pMG11. An *Nsi*I-*Eco*RI fragment containing the promoter and right vaccinia virus flanking arm was isolated from pMG11 and ligated into pUCg14 cut with *Nsi*I-*Eco*RI. An *Hpa*I-*Pst*I fragment containing the right vaccinia virus arm and the EHV-1 gp14 gene under the control of the H6 promoter was ligated into the pMG11 vector plasmid cut with *Hpa*I-*Pst*I. The resulting plasmid, pVM2LH6g14, contained the H6 promoter–EHV-1 gp14 gene flanked by vaccinia virus arms. (D) Truncation of the EHV-1 gp14 gene and generation of vaccinia virus recombinant vP613. An *Nsi*I site was added to the Bluescript SK+-based clone containing the 5′ end of the EHV-1 gp14 gene at codons 32 to 34 by mutagenesis as described for panel A. An *Nsi*I-*Bam*HI fragment containing the 5′ end of the gp14 gene in pVM2LH6g14 was replaced with an *Nsi*I-*Bam*HI fragment containing the newly mutagenized truncated 5′ end of the gene. Synthetic *Nsi*I linkers were added to bring the ATG initiation codon in-frame with the gene. In the resulting plasmid, pVM2LH6g14-1, the sequence of the truncated 5′ end of EHV-1 gp14 is ATG/CAT/GCA/TGC/ATT/GCT, encoding Met/His/Ala/Cys/Ile/Ala, where GCT (Ala) is codon 35 of EHV-1 gp14. In vivo recombination was performed with donor plasmid pVM2LH6g14-1 and rescuing virus vP458, generating vaccinia virus recombinant vP613.

Sullivan et al. (47), corresponding to the full-length glycosylated gp14 gene product and its cleavage products (76 and 58 kDa). The EHV-1 gp14 amino acid sequence (Fig. 1) contains the Arg/Arg/Ser tripeptide sequence at amino acids 520 through 522 which has been proposed to specify cleavage after Arg (amino acid 521) of the EHV-1 gp14 gene product (57). Glycosylation of the primary gp14 translation product, accompanied by cleavage after amino acid 521, would generate the 57- and 72- to 82-kDa polypeptides observed. These polypeptides are known to be disulfide linked (26, 47) and thus coprecipitated by monoclonal antibody 3F6 in the absence of reducing reagents. Further, both EHV-1 antigens were localized on the membrane of vP634-infected cells, as detected by immunofluorescence (data not shown).

Immunogenic potential of EHV-1 glycoproteins expressed by vaccinia virus recombinants. Guinea pigs were immunized to evaluate their response to EHV-1 gp14 expressed by vaccinia virus recombinant vP613. vP613 induced levels of EHV-1 serum neutralizing antibodies (Table 1) similar to those induced by vaccinia virus recombinant vP483 expressing EHV-1 gp13 (18). Although EHV-1 serum neutralizing antibodies were detectable 3 weeks after primary vaccination, more significant levels were observed 2 weeks after secondary immunization (Table 1). In all immunized animals, responses were obtained when vaccinia virus antibodies were assayed by an enzyme-linked immunosorbent assay (data not shown). To determine the protective efficacy of a vaccinia virus recombinant expressing EHV-1 gp14 alone or

TABLE 1. Serum neutralizing antibodies present in guinea pigs inoculated with a vaccinia virus recombinant expressing EHV-1[a]

Inoculum	Serum neutralization titer (log_{10}) on day:		
	0	21	35
vP613	0.4	0.7	1.3
	0.2	0.7	1.2
	0.2	0.7	1.7
	0.2	1.1	1.6
	0.2	1.0	1.6
None	0.2		0.4
	0.6		0.4
	0.7		0.8
	0.6		0.2
	0.4		0.4

[a] Guinea pigs weighing approximately 450 g received 10^8 50% tissue culture infective doses (1 ml) of vaccinia virus recombinant vP613 by the subcutaneous route on days 0 and 21. Guinea pigs were bled on days 0, 21, and 35, and sera were prepared and assayed for EHV-1 antibodies. Neutralization tests were performed on swine testicular cells against 50 50% tissue culture infective doses of EHV-1 strain Kentucky.

in combination with EHV-1 gp13, we performed challenge studies on vaccinated hamsters. The EHV-1 gp14 gene as expressed by the vaccinia virus recombinant vP613 gave good protection on challenge (Table 2). The calculated 50% protective dose (log_{10}) was somewhat better (5.2) than that (6.1) obtained with the EHV-1 gp13-expressing vaccinia virus recombinant vP483. Surprisingly, the coexpression of EHV-1 gp13 and gp14 gave significantly enhanced protective vaccine efficacy as compared with that given by expression of the EHV-1 glycoproteins singly. The calculated 50% protective doses (log_{10}) were 6.1 for vP483, 5.2 for vP613, and <3.6 for vP634 (Table 2).

The well-characterized glycoproteins of herpes simplex virus include gB, gC, gD, gE, gG, gH, and gI (1, 15, 16, 22, 37, 45, 46, 50, 61). A number of studies have indicated the importance of herpes simplex virus glycoproteins in eliciting immune responses. Hence, it has been reported that gB and gD can elicit important immune responses (7, 10, 13, 21, 24, 25, 31, 35, 40, 55, 59, 60). gC can stimulate class I-restricted cytotoxic lymphocytes (17, 42), whereas gD can stimulate class II cytotoxic T-cell responses (24, 25, 55, 59, 60). gG has been shown to be a target for complement-dependent antibody-directed virus neutralization (48, 49). A number of glycoproteins from other herpesviruses have also been shown to elicit important immune responses (6, 12, 18, 45, 54).

TABLE 2. Protection of hamsters against EHV-1 challenge after vaccination with vaccinia virus recombinants expressing EHV-1 gp13 and/or gp14[a]

Expt	Inoculum	EHV-1 protein(s)	Vaccination dose ($TCID_{50}$/ml [log_{10}])	No. of survivors	50% Protective dose (log_{10})
A	vP483	gp13	8	5	6.1
			6	2	
			4	0	
	None			0	
B	vP613	gp14	8.4	5	5.2
			6.4	5	
			4.4	1	
	vP634	gp13 + gp14	7.6	5	<3.6
			5.6	5	
			3.6	5	
	Vaccinia virus		8	0	
	None			0	

[a] Twenty one-day-old Syrian hamsters weighing approximately 60 g were inoculated subcutaneously with control vaccinia virus or with recombinant vaccinia viruses expressing EHV-1 gp13 (vP483) gp14 (vP613), and gp13 + gp14 (vP634). Primary vaccination was followed by an identical vaccinating dose on day 14. All hamsters, including noninoculated controls, were challenged 14 days after the last immunization with an intraperitoneal injection of 200 50% lethal doses of EHV-1 hamster-adapted strain Kentucky. The number of survivors in groups of five hamsters were calculated 14 days postchallenge, at which point the experiment was terminated. The dose of inoculum giving 50% protection of the hamsters was evaluated as log_{10} 50% tissue culture infective dose ($TCID_{50}$) per milliliter of inoculant.

In this communication, we have reported the expression of the herpesvirus gB homolog EHV-1 gp14 in vaccinia virus recombinants either by itself or coexpressed with the herpesvirus gC homolog EHV-1 gp13. The form of EHV-1 gp14 expressed in vP613 and vP634 had amino acids 2 to 34 deleted from the amino-terminal hydrophilic region preceding the putative hydrophobic signal sequence. Additional vaccinia virus recombinants either expressing the intact gp14 gene or having further deletions (amino acids 2 to 62) gave results qualitatively and quantitatively similar to those reported here (unpublished data).

Inoculation of vP613 into guinea pigs elicited the production of neutralizing antibodies against EHV-1. vP613 was also shown to be efficacious in protecting hamsters against an EHV-1 challenge. The immunization of hamsters with vaccinia virus recombinant vP634 expressing both EHV-1 gp13 and gp14 and subsequent challenge gave surprising results. Coexpression of EHV-1 gp13 and gp14 by vaccinia virus recombinants demonstrated that the protective immunity obtained was significantly potentiated by the associated coexpression of the two genes. Hence, the amount of virus inoculum needed to achieve a 50% protection of the vaccinated hamsters was significantly decreased when EHV-1 gp13 and gp14 were coexpressed in the same vaccinia virus recombinant. These data suggest that coexpressed gC and gB analogs of herpesvirus glycoproteins in vaccinia virus recombinants are promising vaccine candidates.

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