JOURNAL OF VIROLOGY, Oct. 1989, p. 4189–4198
0022-538X/89/104189-10$02.00/0

Vol. 63, No. 10

Expression in Recombinant Vaccinia Virus of the Equine Herpesvirus 1 Gene Encoding Glycoprotein gp13 and Protection of Immunized Animals

PEIXUAN GUO,[1]† SCOTT GOEBEL,[1] STEPHEN DAVIS,[1] MARION E. PERKUS,[1] BERNARD LANGUET,[2] PHILIPPE DESMETTRE,[2] GEORGE ALLEN,[3] AND ENZO PAOLETTI[1,4]*

Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, Albany, New York 12201[1]; Rhone Merieux, 69007 Lyon, France[2]; Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099[3]; and Virogenetics Inc., Empire State Plaza, Albany, New York 12220[4]*

Received 17 April 1989/Accepted 22 June 1989

The equine herpesvirus 1 (EHV-1) gene encoding glycoprotein 13 (gp13) was cloned into the hemagglutinin (HA) locus of vaccinia virus (Copenhagen strain). Expression of the gp13 gene was driven by the early/late vaccinia virus H6 promoter. Metabolically radiolabeled polypeptides of approximately 47 and 44 kilodaltons and 90 kilodaltons (glycosylated form) were precipitated with both polyclonal and gp13-specific monoclonal antibodies. Presentation of gp13 on the cytoplasmic membrane of cells infected with the recombinant gp13 vaccinia virus was demonstrated by immunofluorescence of unfixed cells. Inoculation of the recombinant gp13 vaccinia virus into guinea pigs induced neutralizing antibodies to both EHV-1 and vaccinia virus. Hamsters vaccinated with the recombinant gp13 vaccinia virus survived a lethal challenge with the hamster-adapted Kentucky strain of EHV-1. These results indicate that expression in vaccinia virus vectors of EHV-1 gp13, the glycoprotein homolog of herpes simplex virus gC-1 and gC-2, pseudorabies virus gIII, and the varicella-zoster virus gpV may provide useful vaccine candidates for equine herpesvirus infections.

Equine herpesvirus 1 (EHV-1) is associated with respiratory tract disease, abortion, and central nervous system disorders in horses (2). EHV-1 is a member of the alphaherpesviruses and shares some of the typical biological and biochemical properties of the group. Thus, EHV-1 displays genomic isomerization, alpha-beta-gamma gene regulation, the potential to generate defective particles, the potential for establishing latent infections, induction of neurological disorders, ability to transform cells in vitro, and an array of pathological responses to infection (2, 4, 21). There are two subtypes of EHV-1 that, although cross-neutralizing, can be distinguished by antigenicity profile, restriction endonuclease fingerprints, and pathogenicity for horses (2).

The glycoproteins of herpesviruses mediate essential viral functions such as cellular attachment, penetration into cells, and, importantly, the pathogenicity profile of infection. The herpesvirus glycoproteins are critical components in the interaction of the virus with the host immune system (35, 36). A number of studies, predominantly with the well-characterized glycoproteins of herpes simplex virus, have demonstrated the importance of herpesvirus glycoproteins in both antibody and cellular immune responses (5, 6, 8, 10, 11, 13, 18–20, 24, 25, 29, 31, 35, 37, 38, 43, 45, 46). Both subtypes of EHV-1 express six abundant glycoproteins, designated gp2, gp10, gp13, gp14, gp17/18, and gp21/22a (2, 3, 42). The genomic portions of the DNA sequences encoding the six major glycoproteins have been determined by using lambda gt11 expression vectors and monoclonal antibodies (3). Recently, the nucleotide sequence of the Kentucky T431 strain of the EHV-1 transcriptional unit encoding gp13 has been reported (1). An open reading frame encodes a 468-amino-acid primary translation product of 51 kilodaltons (kDa). The protein has the characteristic features of a membrane-spanning protein, with nine potential N-linked glycosylation sites (Asn-X-Ser/Thr) present in the surface domain between the putative signal and transmembrane anchor portions of the protein (1). Significant amino acid homology was observed in the carboxy half of gp13 and corresponding regions of herpes simplex virus gC-1 and gC-2, pseudorabies virus gIII, and varicella-zoster virus gpV, suggesting that EHV-1 gp13 is the structural homolog of the herpesvirus gC-like glycoproteins (1).

In this communication, we report the cloning and expression of the EHV-1 gp13-coding sequences in a vaccinia virus vector and the biological properties of this recombinant vaccinia virus in eliciting EHV-1-neutralizing antibodies in guinea pigs and protective immunity in hamsters against a lethal EHV-1 challenge.

MATERIALS AND METHODS

Cells and viruses. The Copenhagen strain of vaccinia virus, obtained from Rhone Merieux, Inc. (Athens, Ga.), was propagated from a purified plaque isolate on either VERO (ATCC CCL81) or MRC-5 (ATCC CCL171) cells in Eagle minimal essential medium plus 10% fetal bovine serum. A derivative of the wild-type virus from which the entire coding sequence for the thymidine kinase gene was deleted by standard methods (23, 26) was isolated and designated vP410. This thymidine kinase deletion mutant was used for further manipulations (see below).

Identification of DNA sequences encoding EHV-1 gp13. The DNA sequence encoding EHV-1 gp13 resides in the 7.5-kilobase-pair (kbp) *Bam*HI H fragment (3). Nucleotide sequence data for both strands were obtained from the pUC

* Corresponding author.
† Present address: Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD 20892.

(*Bam*HI-H) region, using overlapping subclones and treatment with the modified T7 enzyme Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) (39). Standard dideoxy-chain termination reactions (32) were performed on double-stranded plasmid templates that had been denatured in alkali. The M13 forward and reverse primers were used to obtain the initial sequence of each clone. Custom 16- and 17-mer primers, synthesized by using standard chemistries (Biosearch 8700, San Rafael, Calif.; Applied Biosystems 380B, Foster City, Calif.), were used to walk along the remaining fragment. The IBI Pustell sequence analysis program was used in all sequence data analyses (27).

Replacement of the HA gene of vaccinia virus with the *Escherichia coli* β-galactosidase gene. The 13-kbp *Sal*I F fragment of vaccinia virus that spans the *Hin*dIII A-B fragment junction was ligated into *Sal*I-digested pUC8, generating pSD419VC (Fig. 1). The right arm of pSD419VC, corresponding to the *Hin*dIII B portion of the *Sal*I F fragment, was removed by digestion with *Hin*dIII and religation, generating pSD456VC (Fig. 1). pSD456VC thus contains the right end of the *Hin*dIII A fragment, within which is the complete coding region for the hemagglutinin (HA) gene (34) flanked by approximately 0.4 kbp of additional vaccinia sequences on each side. To generate a plasmid vector virtually devoid of HA-coding sequences, pSD456VC was cut (partial digest) at the *Rsa*I site upstream of the HA gene and at the *Eag*I site 80 bp from the 3′ end of the HA gene. The approximately 3.5-kbp *Rsa*I-*Eag*I fragment was isolated from an agarose gel. Synthetic oligonucleotides MPSYN59 -62 were prepared to replace the region from the *Rsa*I site through position 2 upstream of the HA-coding sequence, immediately followed by *Bgl*II, *Sma*I, and *Pst*I restriction sites and an *Eag*I sticky end (see sequence presented below). The annealed mixture of MPSYN59-62 was ligated into the 3.5-kbp *Rsa*I-*Eag*I fragment from pSD456VC, generating pSD466VC (Fig. 1). Thus, in pSD466VC, the HA gene was replaced by a polylinker region.

A 3.2-kbp *Bgl*II-*Bam*HI (partial) fragment containing the *E. coli* β-galactosidase gene from pMC1871 (33) under the transcriptional control of the vaccinia virus 11-kDa promoter (7) was cloned into pSD466VC that had been digested with *Bgl*II. A plasmid containing the 11-kDa promoter–β-galactosidase gene cassette in a left-to-right orientation relative to flanking vaccinia virus arms was designated pSD466VCBGA and recombined into vaccinia virus vP410, generating the vaccinia virus recombinant vP425, expressing β-galactosidase (Fig. 1). To generate an HA deletion mutant of vaccinia virus devoid of extraneous genes for use as a control, the 11-kDa promoter–β-galactosidase unit was removed from vP425 by in vivo recombination with pSD466VC. The resulting HA-minus vaccinia virus was isolated as a colorless plaque, purified, and designated vP452.

To facilitate future cloning steps (see below), the *Sma*I site derived from the pUC8 multicloning region was eliminated by digestion of pSD466VC with *Bam*HI-*Eco*RI, blunt ending with the Klenow fragment of *E. coli* polymerase, and religation. Thus, the single *Sma*I site remaining in the resulting plasmid, pSD467VC, was in the polylinker region of the HA deletion (Fig. 2).

Cloning of the EHV-1 gp13 gene into a vaccinia virus donor plasmid. An early/late vaccinia virus promoter, H6, was used for expression of the EHV-1 gp13. This promoter element corresponds to the DNA sequences immediately upstream of the H6 open reading frame in the vaccinia virus *Hin*dIII H fragment (30) and has previously been used for expression of foreign genes in fowlpox virus vectors (40, 41).

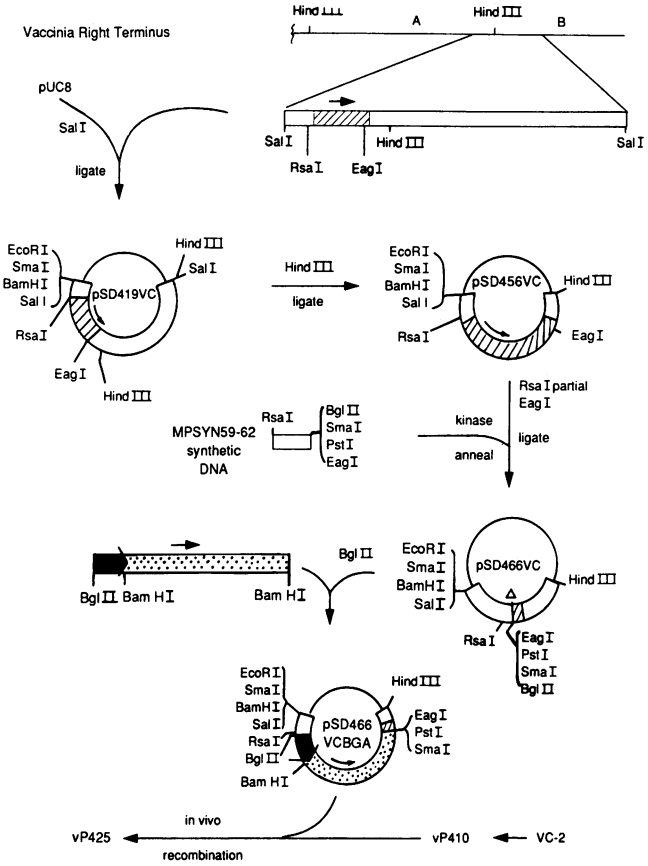

FIG. 1. Replacement of the HA gene in vaccinia virus by the *E. coli* β-galactosidase gene. Cloning is described in the text. The right terminus of the vaccinia virus *Hin*dIII map is indicated, with the *Sal*I F fragment overlapping the *Hin*dIII A-B junction expanded. Symbols: □, vaccinia virus sequences flanking the HA gene; ▨, vaccinia virus HA gene (direction of transcription is indicated by an arrow); △, site of deletion of the vaccinia virus HA gene; ■, vaccinia virus 11-kDa promoter; ▩, *E. coli* β-galactosidase gene (direction of transcription is indicated by an arrow). VC-2 is a plaque-purified isolate of the Copenhagen strain of vaccinia virus. The MPSYN59-62 synthetic DNA sequence and in vivo recombination procedures are described in the text. Final product vP425 is a recombinant vaccinia virus containing the *E. coli* β-galactosidase gene under the control of the 11-kDa vaccinia virus promoter inserted into the HA deletion in a left-to-right orientation relative to flanking vaccinia virus sequences.

To mutate and insert the H6 promoter into pSD467VC, H6SYN oligonucleotides (oligos) A to D were synthesized (sequence below). The underlined bases denote modification from the native H6 promoter sequence. The 130-bp full-length, double-stranded DNA formed by the annealing of H6SYN oligos A to D was purified by electroelution from an agarose gel and ligated to 0.5-kbp *Sma*I-*Hin*dIII and 3.1-kbp *Bgl*II-*Hin*dIII fragments derived from pSD467VC. The resulting plasmid, pTP15, has the ATG initiation codon modified to CCC as part of the *Sma*I site, which is immediately followed by a *Pst*I site (Fig. 2). An *Nsi*I linker, 5′-TGCATG CATGCA-3′ (New England BioLabs, Inc., Beverly, Mass.), was inserted into the *Sma*I site of pTP15 to generate plasmid pNSI (Fig. 2).

An EHV-1 *Eco*RI-*Nar*I fragment in which the *Eco*RI site is 120 bp upstream of the ATG initiation codon and the *Nar*I site is 23 bp upstream of the TAG termination codon of the

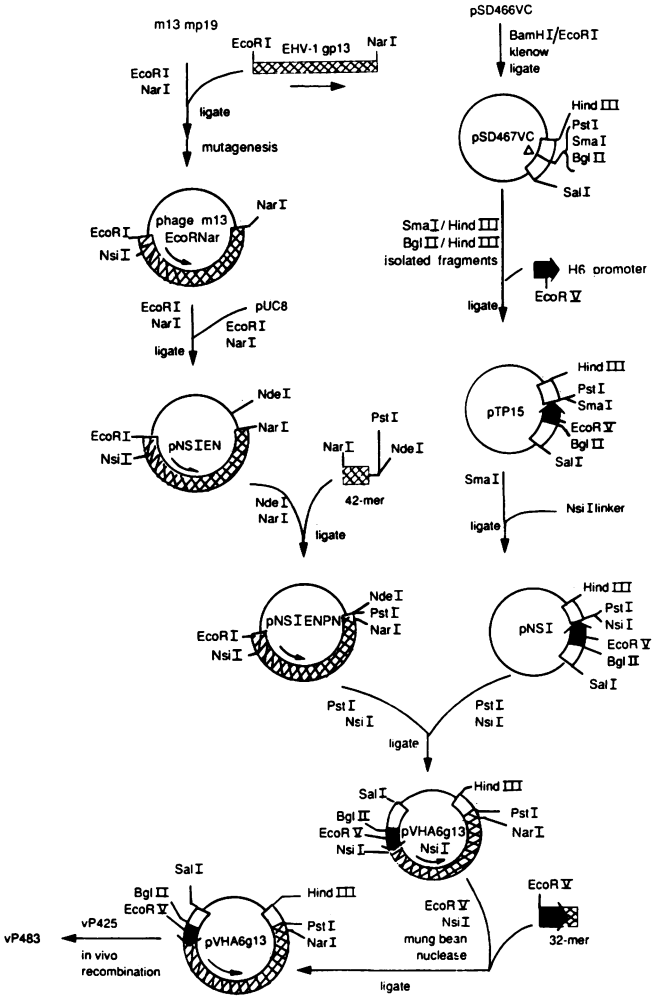

FIG. 2. Replacement of the β-galactosidase gene in vaccinia virus vP425 by the EHV-1 gp13 gene. Cloning is described in the text. An EHV-1 *Eco*RI-*Nar*I fragment containing the bulk of the gp13 gene was cloned into the M13 system for initial mutagenesis near the ATG initiation codon of gp13. The mutagenized EHV-1 *Eco*RI-*Nar*I fragment was cloned into the pUC system for subsequent manipulations. Symbols: ▩, EHV-1 DNA (direction of gp13 transcription is indicated by an arrow); □, vaccinia virus sequences flanking the HA gene; △, site of deletion of the HA gene; ■, synthetic vaccinia virus H6 promoter. Sequences of H6SYN oligos A to D, sequences of synthetic DNA 42- and 32-mers, and in vivo recombination procedures are described in the text. Final product vP483 is a recombinant vaccinia virus containing the EHV-1 gp13 gene under the control of the vaccinia virus H6 promoter inserted into the HA deletion in a left-to-right orientation relative to flanking vaccinia virus sequences.

EHV-1 gp13 gene was cloned into bacteriophage M13mp19, generating the recombinant phage M13*Eco*R*Nar* (Fig. 2). By using oligonucleotide-directed mutagenesis (16), an *Nsi*I site was introduced by changing the sequence TTGCCT (bases 130 to 135 in Fig. 3) in the EHV-1 gp13 gene to ATGCAT. The *Eco*RI-*Nar*I fragment from mutant phage M13*Eco*R*Nar* was cloned into pUC8 at *Eco*RI-*Nar*I sites, generating plasmid pNSIEN (Fig. 2).

Two 42-mer oligonucleotides were synthesized (see below). The double-stranded DNA fragment obtained by annealing the pair of 42-mers contained an *Nar*I sticky end, followed by the 3′ end of the coding sequence for the EHV-1 gp13 gene, as well as a vaccinia early transcription termination signal (44), a *Pst*I site, and an *Nde*I sticky end. This fragment was inserted between the *Nar*I-*Nde*I sites of pNSIEN, generating pNSIENPN (Fig. 2).

The *Nsi*I-*Pst*I fragment from pNSIENPN was isolated and cloned into the *Nsi*I-*Pst*I sites of plasmid pNSI, generating plasmid pVHA6g13*Nsi*I (Fig. 2). pVHA6g13*Nsi*I was cut at the *Eco*RV site in the H6 promoter and the *Nsi*I site that had been introduced near the beginning of the EHV-1 gp13 gene. This vector fragment was blunt ended with mung bean nuclease. Two complementary 32-mers were synthesized (see below). These oligonucleotides were annealed and ligated into the pVHA6g13*Nsi*I vector fragment, producing plasmid pVHA6g13, which contains a precise junction at the ATG (underlined in 32-mer sequence shown below) of the H6 promoter and EHV-1 gp13 gene (Fig. 2). pVHA6g13 was transfected into vP425-infected cells to generate the vaccinia virus recombinant vP483, containing the EHV-1 gp13 gene (Fig. 2).

Construction of vaccinia virus recombinants. Procedures for transfection of recombinant donor plasmids into tissue culture cells infected with a rescuing vaccinia virus and identification of recombinants by in situ hybridization on nitrocellulose filters were as previously described (23, 26). To construct vP425, in which the *E. coli* β-galactosidase gene replaces the vaccinia HA-coding sequences, plasmid DNA (25 μg of pSD466VCBGA in HeBS [14]) was electroporated (Gene Pulser, capacitance 960, 200 volts; Bio-Rad Laboratories, Richmond, Calif.) into VERO cells. Subconfluent monolayers of cells were infected with 10 PFU of vP410 per cell 1 h before use. The infected cells were harvested with trypsin and washed with HeBS before electroporation. Cells were incubated in minimal essential medium plus 5% fetal bovine serum at 37°C for 24 h and harvested, and progeny virus were plated on VERO monolayers. Recombinant virus expressing β-galactosidase was detected as blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate (9, 22). To generate recombinant vaccinia virus in which the EHV-1 gp13 gene replaced the β-galactosidase gene in vP425, a similar protocol was followed except that the donor plasmid was pVHA6g13 and the rescuing virus was vP425. The vaccinia virus recombinant vP483, containing EHV-1 gp13, was detected as a colorless plaque in the presence of X-Gal and confirmed as a true recombinant by DNA hybridization after three cycles of plaque purification.

Immunofluorescence of EHV-1 gp13 expression. BSC-40 cells were seeded on 22-mm-diameter glass cover slips in 35-mm-diameter dishes at 5×10^5 cells per dish. At approximately 80% confluency, the cells were infected at 2 PFU per cell. After a 1-h adsorption period, the virus inoculum was removed and minimal essential medium plus 2% fetal bovine serum was added. At 20 h postinfection, the cover slips were washed with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin and 0.1% NaN_3 (PBS+) and exposed to 0.1 ml of anti-gp13 monoclonal antibody 14H7 (3) diluted 1:1,000 in PBS+. After 1 h in a humidified chamber at room temperature, the cells were washed three times in PBS+. This procedure was repeated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. Finally, the cells were fixed for 20 min in 2% paraformaldehyde in PBS. The cover slips were mounted in 80% glycerol in PBS containing 3% *n*-propyl gallate. Fluorescence was observed with a Nikon Optiphot microscope, and photographs taken with Kodak Tri-X film (Eastman Kodak Co., Rochester, N.Y.).

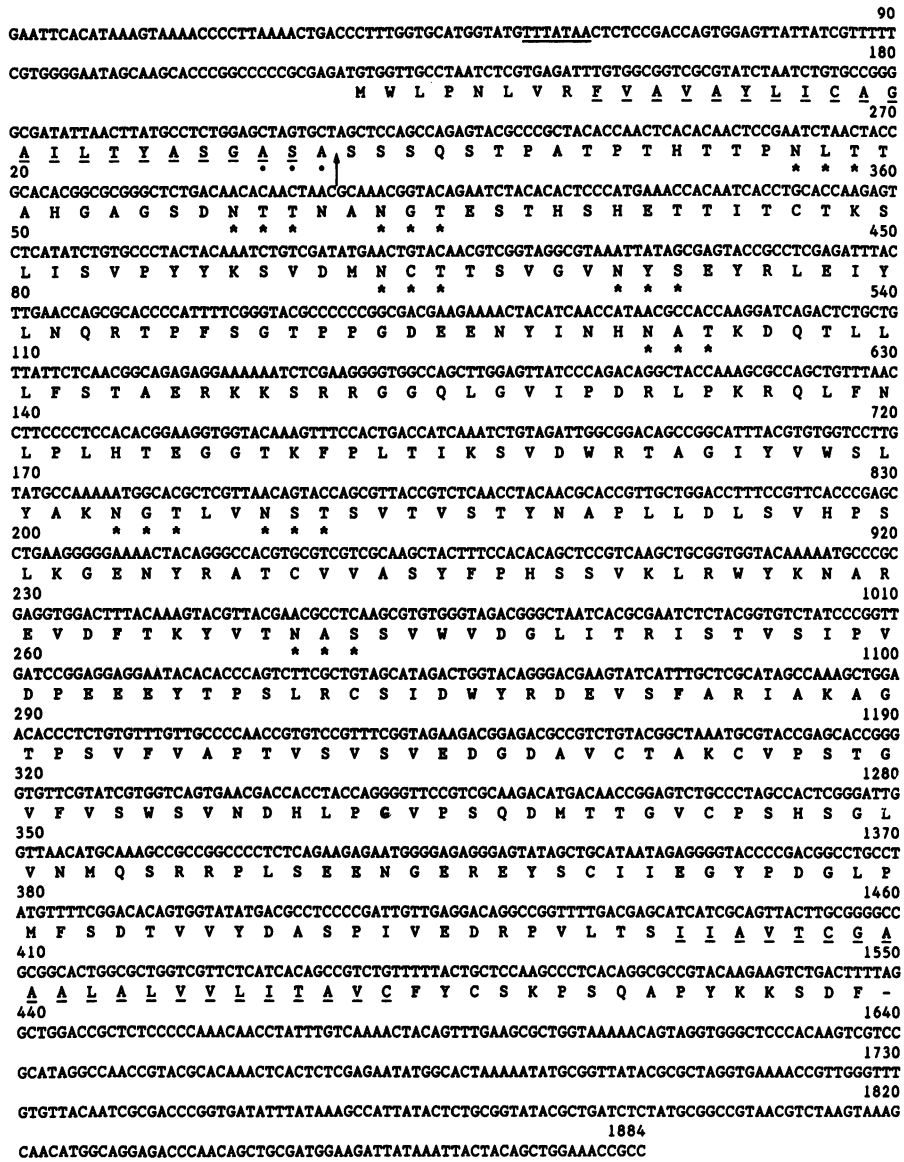

FIG. 3. Nucleotide sequence of an EHV-1 1,884-bp fragment containing the gp13-coding sequences. A presumptive TATA box and amino acids comprising putative signals and membrane anchor elements are underlined. The potential cleavage site of the signal sequence (↑) is noted after the cleavage signal Ala-Ser-Ala (○). There are potentially nine N-linked glycosylation sites between the signal and anchor sequences, as defined by the Asn-X-Ser/Thr motif (*). The sequence data were derived independently of the data presented in reference 1 and are identical to those data except at amino acid 43, where ACA (Thr) instead of CCA (Pro) is found.

Immunoprecipitation of EHV-1 gp13 from recombinant vaccinia virus-infected cells. A total of 2×10^6 cells forming a confluent monolayer in a 60-mm-diameter dish were infected at 10 PFU per cell. The inoculation was performed in methionine-free medium. After the adsorption period, the inoculum was removed, and 2 ml of methionine-free medium containing 20 μCi of [^{35}S]methionine per ml was added. The infection was allowed to proceed for 24 h, when cells were lysed by the addition of 1 ml of 3× buffer A, containing 3% Nonidet P-40, 30 mM Tris (pH 7.4), 450 mM NaCl, 3 mM EDTA, 0.03% sodium azide, and 0.6 mg of phenylmethylsulfonyl fluoride per ml. The lysed cells and supernatant were harvested, vortexed, and clarified by centrifugation at 10,000 × *g* for 15 min.

Protein A-Sepharose CL-4B (catalog no. 17.0780.01; Pharmacia, Inc., Piscataway, N.J.) was prepared as a 1:1 slurry in 1X buffer A. A rat anti-mouse conjugate (catalog no. 605 500; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was diluted to 1:100 in the slurry and bound to the beads at room temperature for 4 h with rocking. The beads were then washed thoroughly with six 1-ml washes in buffer A to remove unbound conjugate. A monoclonal antibody specific to gp13 was then bound to the beads at room temperature for 4 h. Excess antibody was removed by thorough washing. A 1-ml amount of clarified infected cell lysate was precleared by incubation with protein A-Sepharose beads to which normal mouse serum had been bound. These beads were removed by centrifugation. A 1-ml amount of the clarified precleared lysate was then mixed with 100 μl of the beads to which the specific monoclonal antibody had been bound. This mixture was rocked at room temperature for 4 h. The beads were then removed by

centrifugation and washed thoroughly by four washes in 1X buffer A and two washes in 10 mM Tris (pH 7.4) containing 0.2 M LiCl and 2 M urea. The antibody-antigen complex was then removed from the beads and disrupted by the addition of 50 μl of 2× Laemmli disrupting solution (17). The sample was then boiled for 5 min before electrophoresis.

Immunization of guinea pigs. Fifteen guinea pigs weighing approximately 450 g each were divided into groups of five. One group received 1 ml of the vaccinia recombinant vP483 (10^8 50% tissue culture infective doses [$TCID_{50}$]/ml) on day 0, followed by a 1-ml booster on day 21 by subcutaneous inoculation. The second group received similar inoculations but with the control HA-deleted vaccinia virus, vP452 (10^8 $TCID_{50}$/ml). The third group remained unvaccinated. All of the guinea pigs were bled before the primary vaccination and on days 21 and 35. Sera were prepared and tested for the presence of neutralizing antibodies to both vaccinia virus and EHV-1 (Kentucky), using 50 $TCID_{50}$ of virus assayed on swine testicular cells.

Vaccination of hamsters and challenge with EHV-1. Forty Syrian hamsters (40 days old, weighing between 55 and 65 g each) were separated into four groups. Group A received a single subcutaneous 1-ml inoculation of 10^8, 10^6, or 10^4 $TCID_{50}$ of the vaccinia virus recombinant vP483. Five animals were used for each dose. Group B was vaccinated with vP483 on day 0, followed by a booster on day 14. The 1-ml primary and booster doses were administered subcutaneously to groups of five animals, using 10^8, 10^6, and 10^4 $TCID_{50}$. Group C consisted of five hamsters that received two subcutaneous injections (10^8 $TCID_{50}$ per injection) of the control vaccinia virus, vP452, on days 0 and 14. Five hamsters in group D were left as unvaccinated controls. All of the hamsters received a challenge of 200 50% lethal doses of a hamster-adapted Kentucky strain of EHV-1 by the intraperitoneal route 14 days after the last immunization. Survivors were counted 7 days after challenge.

Synthetic DNA. Oligonucleotides were synthesized by using standard chemistries (Biosearch 8700; Applied Biosystems 380B). Sets of complementary oligonucleotides were annealed at 65°C for 20 min, cooled slowly to room temperature, and ligated into plasmid vectors as described above. In sets composed of four synthetic oligonucleotides (MPSYN59-62; H6SYN), internal 5′ ends (MPSYN60 and MPSYN62; H6SYN oligos B and D) were kinase treated (T4 polynucleotide kinase; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) before annealing.

(i) MPSYN59-62. Restriction sites are indicated.

```
5′-ACACGAATGATTTTCTAAAGTATTTGGAAAGTTTTATAGGTAGTTGATAGAACAAAATACATAATTTTGTAAAAATAAAT
3′-TGTGCTTACTAAAAGATTTCATAAACCTTTCAAAATATCCATCAACTATCTTGTTTTATGTATTAAAACATTTTTATTTA

CACTTTTTATACTAAGATCTCCCGGGCTGCAGC-3′
GTGAAAAATATGATTCTAGAGGGCCCGACGTCGCCGG-5′
          BglII  SmaI  PstI  EagI
```

(ii) H6SYN oligos A to D. The modified base is underlined, and restriction sites are indicated.

```
  BglII
5′-GATCTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAA
3′-    AGAAATAAGATATGAATTTTTCACTTTTATTTATGTTTCCAAGAACTCCCAACACAATTTAACTTTCGCTCTTTATT

TCATAAATTATTTCATTATCGCGATATCCGTTAAGTTTGTATCGTACCC-3′
AGTATTTAATAAAGTAATAGCGCTATAGGCAATTCAAACATAGCATGGG-5′
                                              SmaI
```

(iii) 42-mer. The termination codon (TAG) is immediately followed by a vaccinia virus early transcription terminator (ATTTTTAT). Restriction sites are indicated.

```
  NarI      gp13  3′ end                        NdeI
5′-CGCCGTACAAGAAGTCTGACTTTTAGATTTTTATCTGCAGCA-3′
3′-  GGCATGTTCTTCAGACTGAAAATCTAAAAATAGACGTCGTAT-5′
                                    PstI
```

(iv) 32-mer. The initiation codon is underlined, and the restriction site is indicated.

```
  EcoRV
5′-ATCCGTTAAGTTTGTATCGTAATGTGGTTGCC-3′
3′-TAGGCAATTCAAACATAGCATTACACCAACGG-5′
      H6 promoter          gp13  5′  end
```

RESULTS

Nucleotide sequence of the EHV-1 gp13 gene. The 7.5-kbp *Bam*HI H fragment of EHV-1 has been shown to contain the sequences encoding the glycoprotein gp13 (3). A 5.6-kbp *Eco*RI-*Bam*HI region of the 7.5-kbp fragment was sequenced as described in Materials and Methods. DNA sequence analysis revealed an open reading frame of 1,404 bp encoding 468 amino acids with a predicted primary translation product of 50.9 kDa. Significant amino acid homology in the carboxy half of the putative gp13 open reading frame was observed to gC of herpes simplex virus types 1 and 2, gIII of pseudorabies virus, and gpV of varicella-zoster virus, suggesting that gp13 was a member of the gC-like glycoproteins of herpesviruses. Further detailed analysis of the EHV-1 gp13 open reading frame was presented in a previous publication (1). To facilitate our description of the cloning and expression of EHV-1 gp13 in vaccinia

virus vectors, the gp13 open reading frame plus additional 5′ and 3′ sequence is shown in Fig. 3.

Construction of recombinant vaccinia viruses. A thymidine kinase deletion mutant, vP410, of the Copenhagen strain of vaccinia virus was used as parental virus to replace the vaccinia virus HA-coding sequences with the β-galactosidase gene promoted by the 11 kDa late vaccinia virus regulatory element (Fig. 1). Eighty base pairs at the carboxy terminus of the HA gene were retained to preserve a short potential open reading frame transcribed right to left relative to the vaccinia virus genome (data not presented). The recombinant vaccinia virus, vP425, was identified on the basis of blue plaque formation in the presence of the chromogenic substrate X-Gal, as described by others (9, 22). Substitution of the β-galactosidase gene by yet another foreign gene in subsequent vaccinia recombinants could be readily scored by isolating colorless plaques instead of blue plaques. Construction of the recombinant vaccinia virus vP425 is shown in Fig. 1.

To construct the chimeric donor plasmids to introduce EHV-1 gp13 into vP425, we inserted two synthetic double-stranded DNA fragments into plasmids pNSIEN and pVHA6g13*Nsi*I at the 3′ and 5′ termini, respectively, of the EHV-1 gp13 gene (Fig. 2). The 42-mer (*Nar*I-*Nde*I) fragment (Fig. 2) contained the exact 23 bp of the gp13 gene from the *Nar*I site to the termination codon, followed by the early transcription termination consensus signal (44) 5′-ATTTTT AT-3′. Inclusion of a *Pst*I site in the 42-mer facilitated the transfer of an *Nsi*I-*Pst*I fragment into the pNSI cloning vector, which contains *Nsi*I and *Pst*I sites (Fig. 2).

When the *Nsi*I site was created in phage M13*Eco*R*Nar* by oligonucleotide-directed mutagenesis, two nucleotides were changed in amino acids 3 and 4 of the gp13 gene (TTGCCT to ATGCAT). The mutated H6 promoter-gp13 junction was excised by *Eco*RV-*Nsi*I cleavage of pVHA6g13*Nsi*I, followed by treatment with mung bean nuclease to remove the nucleotides TGCA at the *Nsi*I sticky end. Insertion of the second synthetic DNA fragment (32-mer) at the blunt-ended sites regenerated the correct sequence. Thus, the chimeric donor plasmid, pVHA6g13, contains the full-length EHV-1 gp13-coding sequences precisely joined to the vaccinia virus early/late H6 promoter and flanked by sequences from the *Hin*dIII A fragment of vaccinia virus (Fig. 2). Transfection of pVHA6g13 into cells infected with vP425 allowed for the identification of colorless plaques, indicating replacement of the β-galactosidase gene in vP425 with the EHV-1 gp13 gene. The recombinant virus, vP483, was further plaque purified, and the presence of the EHV-1 gp13 gene was confirmed by nucleic acid hybridization.

Expression of EHV-1 gp13 on the surface of cells infected with the recombinant vaccinia virus vP483. The EHV-1 gp13 translation product predicted from the DNA sequence has the typical features of a membrane-spanning glycoprotein (12). In a productive EHV-1 infection, gp13 is incorporated into the various membrane systems of the cell, is transported into the cytoplasmic membrane, and is detectable on the external surface of the infected cell. EHV-1 gp13 is also a component of the EHV-1 virion. Therefore, immunofluorescence studies were performed to determine whether EHV-1 gp13 expressed by the vaccinia virus recombinant vP483 was similarly presented on the cytoplasmic membrane of infected cells. Treatment with the anti-gp13 monoclonal antibody followed by fluorescein-conjugated goat anti-mouse immunoglobulin G revealed a strong membrane immunofluorescence in vP483-infected cells but not in vaccinia virus vP410-infected cells (Fig. 4). This finding suggested that the EHV-1 gp13 expressed by the recombinant vaccinia virus vP483 was present on the cytoplasmic membrane, as expected for authentic synthesis of a membrane-spanning glycoprotein.

Immunoprecipitation of EHV-1 gp13 products synthesized in recombinant vaccinia virus vP483-infected cells. Three dominant metabolically radiolabeled polypeptides were specifically precipitated by anti-gp13 monoclonal antibody from recombinant virus-infected cells (Fig. 5). The smaller polypeptides of apparent molecular sizes 47 and 44 kDa were smaller than the expected size of the primary translation product (51 kDa) as predicted from the DNA sequence. Migration of a more diffuse polypeptide with an apparent molecular size of 85 to 90 kDa is consistent with the presence of a fully glycosylated form of the translation product. No equivalent polypeptides were precipitated from vP452 vaccinia virus-infected cells (Fig. 5).

Immunization of guinea pigs with the vaccinia virus recombinant vP483. To determine the immunogenicity of the EHV-1 gp13 gene product expressed by the vaccinia virus recombinant vP483, guinea pigs were inoculated with the virus, and the presence of serum neutralizing antibodies against both vaccinia virus and EHV-1 was assayed. The vaccinia virus recombinant elicited an obvious seroconversion in guinea pigs (Table 1). Both vaccinia virus and EHV-1 serum neutralizing antibodies were detectable 21 days after the primary inoculation, and a significant increase in the titer of serum neutralizing antibodies was obtained by 2 weeks after a second inoculation of virus on day 21. It should be noted that the serum vaccinia virus-neutralizing titers obtained in guinea pigs inoculated with the recombinant virus were significantly higher ($t = 7.2$) than the titers obtained from guinea pigs inoculated with control vaccinia virus, vP452. This observation is reproducible, but the reason for the difference is not apparent.

Protection of vaccinated hamsters from challenge with EHV-1. To assess the protective efficacy of the vaccinia virus recombinant vP483, expressing EHV-1 gp13, hamsters were given either a primary or primary-plus-booster vaccination, and they, along with an uninoculated control group

TABLE 1. Serum neutralizing antibodies present in guinea pigs inoculated with a recombinant vaccinia virus expressing EHV-1 gp13 or with a control vaccinia virus[a]

Inoculum virus	Animal no.	Serum neutralizing titer (log_{10}) on days:		
		0	21	35
None	26	0.24 (0.35)		0.24 (0.70)
	27	0.24 (0.35)		0.56 (1.05)
	28	0.24 (0.35)		0.80 (0.70)
	29	0.24 (0.35)		0.40 (0.70)
	30	0.24 (0.35)		0.32 (0.35)
Vaccinia virus vP452 (control)	191	0.24 (0.35)	0.36 (0.47)	0.72 (1.75)
	192	0.24 (0.35)	0.21 (0.93)	0.24 (2.30)
	193	0.24 (0.35)	0.48 (0.58)	
	194	0.24 (0.35)	0.24 (0.82)	0.24 (2.10)
	195	0.24 (0.35)		
Recombinant vP483	186	0.24 (0.35)	0.48 (1.28)	1.20 (2.57)
	187	0.24 (0.35)	0.72 (1.63)	1.68 (2.57)
	188	0.24 (0.35)	0.24 (1.52)	1.68 (2.57)
	189	0.24 (0.35)	0.36 (1.40)	1.56 (2.22)
	190	0.24 (0.35)	0.48 (1.63)	1.56 (3.00)

[a] Guinea pigs were vaccinated with vaccinia virus as indicated. Serum neutralizing antibodies were evaluated by a neutralization test on swine testicle cells, using 50 $TCID_{50}$ of the Kentucky strain of EHV-1. Serum neutralization titers obtained with vaccinia virus are shown in parentheses.

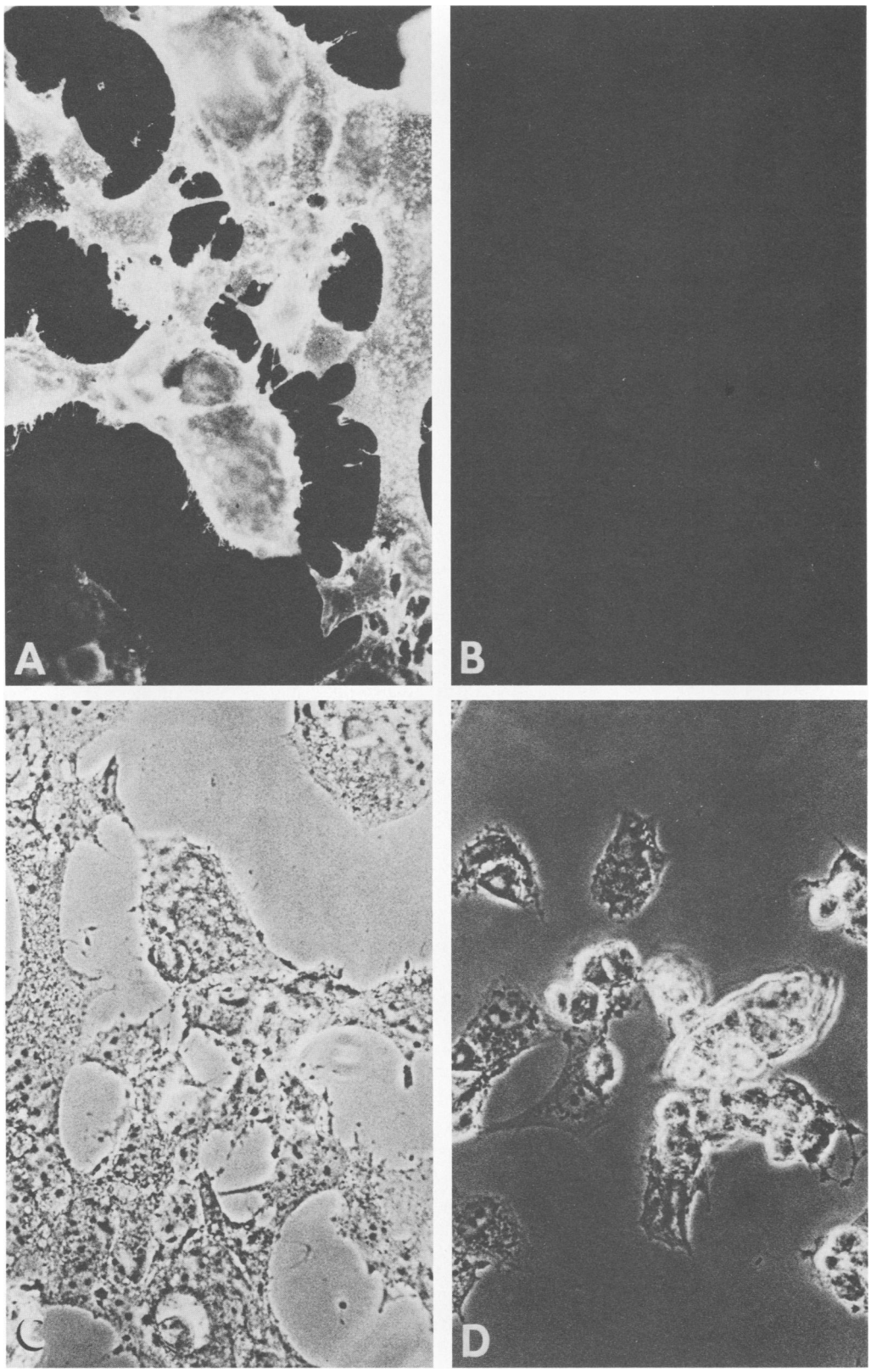

FIG. 4. Expression of EHV-1 gp13 on the surface of BSC-40 cells infected with the recombinant vaccinia virus vP483. BSC-40 cells infected with vaccinia virus vP483 (A and C) or vP410 (B and D) were probed with an anti-EHV-1 gp13 monoclonal antibody, followed by fluorescein-conjugated goat anti-mouse antibody. Panels C and D are photographs taken under phase-contrast microscopy. Membrane fluorescence is observed only on the surface of vP483 cells expressing EHV-1 gp13 (A).

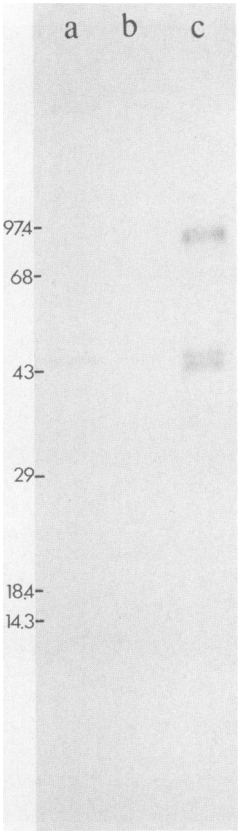

FIG. 5. Immunoprecipitation of EHV-1 gp13 synthesized in cells infected with the recombinant vaccinia virus vP483. [^{35}S]methionine-labeled polypeptides of approximately 44, 47, and 85 to 90 kDa were precipitated with EHV-1 gp13-specific monoclonal antibody from cells infected with the recombinant vaccinia virus vP483 (lane c) but not from uninfected cells (lane a) or cells infected with the control vaccinia virus, vP452 (lane b). Details are given in Materials and Methods.

or a group inoculated twice with the control vaccinia virus vP452, were challenged intraperitoneally with a hamster-adapted Kentucky strain of EHV-1. All unvaccinated and vaccinia virus vP452-vaccinated hamsters died within 5 days of challenge. Significant levels of protection against EHV-1 challenge were observed in hamsters vaccinated with the vaccinia virus recombinant vP483 expressing EHV-1 gp13. No significant differences in protection levels were observed in hamsters immunized with either primary or primary-plus-booster doses. Of five animals given primary doses of 10^8, 10^6, and 10^4 $TCID_{50}$, four, one, and two, respectively, survived 7 days after challenge; comparable numbers of survivors from five hamsters given equivalent primary-plus-booster doses were five, two, and zero. The 50% protective doses were similar: 6.32 $\log_{10}$ (primary) and 6.12 $\log_{10}$ (primary plus booster). Nevertheless, in this small pilot study, 100% protection was observed only in the group receiving two doses of 10^8 $TCID_{50}$ of recombinant virus.

DISCUSSION

The study reported here is part of a long-term research effort designed to understand the contribution of various herpesvirus glycoproteins in defining protective immunity against herpesvirus infections and disease. The unique nature of recombinant poxviruses allows the elucidation of how glycoproteins individually or in combination contribute to immunity to herpesviruses.

Although it has been shown that the gC-like glycoproteins are not essential for viral replication in tissue culture cells for either herpes simplex virus type 1 or type 2 or for pseudorabies virus (15, 28, 47), little is known about the specific functions of these glycoproteins in in vivo situations. Certainly, however, the contributions of the gC-like herpesvirus glycoproteins in eliciting immunological responses are well known. Induction of cytotoxic T-cell responses in mice are considered to be a function of the herpes simplex virus type 1 gC (13, 31). The majority of virus-neutralizing activity present in pseudorabies-infected convalescent serum of swine is directed against glycoprotein gIII, the pseudorabies virus gC analog (5). Likewise, the presence of anti-gp13 antibody detectable in the serum of convalescing EHV-infected horses and the finding that several anti-gp13 monoclonal antibodies neutralize viral infectivity (G. Allen, in press) indicate an important role in immune reactivity of the gC-like glycoproteins in vivo.

In this study, we have expressed the EHV-1 glycoprotein gp13 in a vaccinia virus vector. This structural homolog of the herpes simplex virus gC was demonstrated to be recognizable by polyclonal (data not presented) as well as monoclonal antibody preparations in immunoprecipitation studies and to be localized on the plasma membrane of cells infected in vitro with the recombinant virus.

By immunoprecipitation, three polypeptides were demonstrated. The larger polypeptide, migrating with an apparent molecular size of 85 to 90 kDa, is consistent with the fully glycosylated mature form of the EHV-1 glycoprotein. The reason for the appearance of the two smaller polypeptides (44 and 47 kDa), which are somewhat smaller than the predicted size of 51 kDa as determined by DNA sequence, is not immediately clear. Possible explanations are anomalous migration on sodium dodecyl sulfate-polyacrylamide gels, the presence of primary translation products with or without the signal sequence, or additional posttranslation processing events. To fully appreciate the significance of these two smaller polypeptides, it would be useful to know the N-terminal amino acid sequence.

Immunogenicity was demonstrated in vivo by eliciting EHV-1-neutralizing antibody production in guinea pigs. Protective immunity in hamsters was demonstrated. Hamsters vaccinated with the recombinant virus survived challenge with a significant burden (200 50% lethal doses) of hamster-adapted EHV-1 live virus.

This study clearly demonstrates the potential significance of gC-like herpesvirus glycoproteins as components of vaccines resulting in protective immunity against herpesvirus infections.

ACKNOWLEDGMENTS

We thank E. Norton, J. Taylor, A. Eastman O'Neill, C. Molnar, H. Martinez, G. Johnson, J. Tartaglia, and S. Pincus for their contributions to various aspects of this study and K. Dombrowski for typing the manuscript.

LITERATURE CITED

1. **Allen, G., and L. D. Coogle.** 1988. Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein C. J. Virol. **62**:2850–2858.
2. **Allen, G. P., and J. T. Bryans.** 1986. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus 1 infec-

tions, p. 78–144. *In* R. Pandey (ed.), Progress in veterinary microbiology and immunology, vol. 2. S. Karger, Basel.
3. **Allen, G. P., and M. R. Yeargan.** 1987. Use of lambda gt11 and monoclonal antibodies to map the genes for the six major glycoproteins of equine herpesvirus 1. J. Virol. **61**:2454–2461.
4. **Baumann, R. P., D. C. Sulivan, J. Staczek, and D. J. O'Callaghan.** 1986. Genetic relatedness and colinearity of the genomes of equine herpesvirus types 1 and 3. J. Virol. **57**:816–825.
5. **Ben-Porat, T., J. DeMarchi, B. Lomniczi, and A. Kaplan.** 1986. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. Virology **154**:325–334.
6. **Berman, P. W., D. Dowbenko, L. A. Lasky, and C. C. Simonsen.** 1983. Detection of antibodies to herpes simplex virus with a continuous cell line expressing cloned glycoprotein D. Science **222**:524–527.
7. **Bertholet, C., R. Drillien, and R. Wittek.** 1985. One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. Proc. Natl. Acad. Sci. USA **82**:2096–2100.
8. **Cantin, E. M., R. Eberle, J. L. Baldick, B. Moss, D. E. Willey, A. L. Notkins, and H. Openshaw.** 1987. Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. Proc. Natl. Acad. Sci. USA **84**:5908–5912.
9. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
10. **Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith.** 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. **5**:3057–3063.
11. **Cremer, K. J., M. Mackett, C. Wohlenberg, A. L. Notkins, and B. Moss.** 1985. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science **228**:737–740.
12. **Eisenberg, D.** 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. **53**:595–623.
13. **Glorioso, J., U. Kees, G. Kumel, H. Kirchner, and P. Krammer.** 1985. Identification of herpes simplex virus type 1 (HSV-1) glycoprotein gC as the immunodominant antigen for HSV-1-specific memory cytotoxic T lymphocytes. J. Immunol. **135**: 575–582.
14. **Graham, F. L., and A. J. Van der Eb.** 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology **54**:536–539.
15. **Holland, T. C., F. L. Homa, S. D. Marlin, M. Levine, and J. Glorioso.** 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. **52**:566–574.
16. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lasky, L. A., D. Dougenko, S. Christian, and P. W. Berman.** 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with a secreted form of cloned glycoprotein D. Bio/Technology **2**:527–532.
19. **Martin, S., B. Moss, P. W. Berman, L. A. Lasky, and B. T. Rouse.** 1987. Mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T cells. J. Virol. **61**:726–734.
20. **Martin, S., and B. T. Rouse.** 1987. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J. Immunol. **138**:3431–3437.
21. **O'Callaghan, D. J., B. E. Henry, J. H. Wharton, S. A. Dauenhauer, R. B. Vance, J. Staczek, and R. A. Robinson.** 1981. Equine herpesviruses: biochemical studies on genomic structure, DI particles, oncogenic transformation, and persistent infection, p. 387–418. *In* Y. Decker (ed.), Developments in molecular virology, vol. 1. Herpesvirus DNA. Martinus Nijhoff, The Hague, The Netherlands.
22. **Panicali, D., A. Grzelecki, and C. Huang.** 1986. Vaccinia virus vectors utilizing the β-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. Gene **47**:193–199.
23. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA **79**:4927–4931.
24. **Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali.** 1984. Construction of live vaccines using genetically engineered poxvirus: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **81**:193–197.
25. **Perkus, M. E., A. Piccini, B. R. Lipinskas, and E. Paoletti.** 1985. Recombinant vaccinia virus: immunization against multiple pathogens. Science **229**:981–984.
26. **Piccini, A., M. E. Perkus, and E. Paoletti.** 1987. Vaccinia virus as an expression vector. Methods Enzymol. **153**:545–563.
27. **Pustell, J., and F. C. Kafatos.** 1984. A convenient and adaptable package of computer programs for DNA and protein sequence management, analysis, and homology determination. Nucleic Acids Res. **12**:643–655.
28. **Robbins, A. K., M. E. Whealy, R. J. Watson, and L. W. Enquist.** 1986. Pseudorabies virus gene encoding glycoprotein gIII is not essential for growth in tissue culture. J. Virol. **59**:636–645.
29. **Rooney, J. F., C. Wohlenberg, K. J. Cremer, B. Moss, and A. L. Notkins.** 1988. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. J. Virol. **62**:1530–1534.
30. **Rosel, J. L., P. L. Earl, J. P. Weir, and B. Moss.** 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J. Virol. **60**:436–449.
31. **Rosenthal, K. L., J. R. Smiley, S. South, and D. C. Johnson.** 1987. Cells expressing herpes simplex virus glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes. J. Virol. **61**:2438–2447.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Shapira, S. K., J. Chou, F. V. Richaud, and M. J. Casadaban.** 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to lacZ gene sequences encoding an enzymatically active carboxyl-terminal portion of β galactosidase. Gene **25**:71–82.
34. **Shida, H.** 1986. Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology **150**:451–462.
35. **Spear, P. G.** 1985. Antigenic structure of herpes simplex virus, p. 425–443. *In* M. H. V. Van Regenmoutel and A. R. Neurath (ed.), The basis for serodiagnosis and vaccines, vol. 2. Immunochemistry of viruses. Elsevier Science Publishing Co., Inc., New York.
36. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Publishing Corp., New York.
37. **Sullivan, V., and G. L. Smith.** 1987. Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: neutralization of HSV-1 infectivity with anti-gG antibody. J. Gen. Virol. **8**:2587–2589.
38. **Sullivan, V., and G. L. Smith.** 1988. The herpes simplex virus type 1 US7 gene product is a 66K glycoprotein and is a target for complement-dependent virus neutralization. J. Gen. Virol. **69**: 859–867.
39. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
40. **Taylor, J., R. Weinberg, Y. Kawaoka, R. Webster, and E.**

Paoletti. 1988. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine **6**:504–508.

41. **Taylor, J., R. Weinberg, B. Lanquet, P. Desmettre, and E. Paoletti.** 1988. A recombinant fowlpox virus induces protective immunity in non-avian species. Vaccine **6**:497–503.
42. **Turtinen, L. W., and G. P. Allen.** 1982. Identification of the envelope surface glycoproteins of equine herpesvirus type 1. J. Gen. Virol. **63**:481–485.
43. **Wachsman, M., L. Aurelian, C. C. Smith, B. R. Lipinskas, M. E. Perkus, and E. Paoletti.** 1987. Protection of guinea pigs from primary and recurrent herpes simplex virus (HSV) type 2 cutaneous disease with vaccinia virus recombinants expressing HSV glycoprotein D. J. Infect. Dis. **155**:1188–1197.
44. **Yuen, L., and B. Moss.** 1987. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. USA **84**:6417–6421.
45. **Zarling, J. M., P. A. Moran, R. L. Burke, C. Pachl, P. W. Berman, and L. A. Lasky.** 1986. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. IV. Recognition and activation by cloned glycoproteins gB and gD. J. Immunol. **136**:4669–4673.
46. **Zarling, J. M., P. A. Moran, L. A. Lasky, and B. Moss.** 1986. Herpes simplex virus (HSV)-specific human T-cell clones recognize HSV glycoprotein D expressed by a recombinant vaccinia virus. J. Virol. **59**:506–509.
47. **Zezulak, K. M., and P. G. Spear.** 1984. Mapping of the structural gene for the herpes simplex virus type 2 counterpart of herpes simplex virus type 1 glycoprotein C and identification of a type 2 mutant which does not express this glycoprotein. J. Virol. **49**:741–747.