Transcript analysis of the equine herpesvirus 1 glycoprotein B gene homologue and its expression by a recombinant vaccinia virus

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Transcript mapping of the equine herpesvirus 1 (EHV-1) glycoprotein B (gB) gene homologue by Northern blot, S1 nuclease and primer extension analyses indicated that two overlapping transcripts of 3.4 and 4.6 kb originated from the same strand and were transcribed from left to right between coordinates 0.40 and 0.43 of the EHV-1 genome. The 3.4 kb transcript encoded EHV-1 gB and the 5' RNA terminus was located approximately 30 bases downstream from a probable TATA element. The coding region of the gB gene homologue was reconstructed from two subclones using oligonucleotide mutagenesis and inserted into vaccinia virus by homologous recombination. Cells infected with the recombinant virus synthesized EHV-1 gB antigen, which was detectable in the cytoplasm and on the cell surface by immunofluorescence using an EHV-1 neutralizing horse serum and EHV-1 monoclonal antibodies. On Western blots, bands of 138K to 143K, 80K to 90K and 55K to 57K were identified in recombinant virus-infected cells, by both EHV-1 monoclonal antibodies and the polyclonal horse serum. These were similar in Mr to bands identified by these sera in EHV-1-infected cells. Mice vaccinated with the recombinant virus produced antibodies which recognized proteins of the same Mr as EHV-1 gB, on Western blots, but did not have in vitro neutralizing activity.

Introduction

The alphaherpesvirus equine herpesvirus type 1 (EHV-1) is an important cause of abortion in pregnant mares, respiratory tract infection and neurological disease in young horses. Immunity to EHV-1, either as the result of natural infection or vaccination, is weak and short-lived (Bryans, 1969). Since the efficacy of present vaccines in preventing the disease symptoms is uncertain (Mitchell, 1983; Baker, 1983; Bryans & Allen, 1986) we and others have sought to increase the understanding of the horse's protective immune response by identifying antigens of EHV-1 that activate this response.

The glycoproteins of herpesviruses have been shown to have a major role in the infectious process and in strain variation, and are the most significant targets for the immune system. EHV-1 synthesizes several major and minor glycoprotein antigens which are localized both in the envelope of the virus and in the plasma membrane of infected cells (Allen & Bryans, 1986; Turtinen & Allen, 1982). Recently, several glycoprotein genes of both EHV-1 and the closely related EHV-4 have been identified and sequenced, namely the gC homologue (gp13) and the gB homologue (gp14) (Allen & Coogle, 1988; Whalley et al., 1989; Riggio et al., 1989). Purified and recombinant-expressed herpes simplex virus (HSV) glycoprotein B (gB) have both been shown to invoke circulating antibody and cell-mediated immune responses which protect mice against lethal challenge with the virus (Chan et al., 1985; Cantin et al., 1987; Willey et al., 1988; McDermott et al., 1989). The high degree of amino acid homology between the gB homologue glycoproteins of EHV-1, HSV and several other herpesviruses (Whalley et al., 1989) indicates a conservation of function which makes it likely that immunity to the major glycoprotein antigens could protect against EHV-1 infection. The vaccine potential of EHV-1 glycoproteins has also been clearly demonstrated by the protection of hamsters against EHV-1-induced disease following passive immunization with individual and pooled monoclonal antibodies (MAbs) to glycoproteins including gp14 (Stokes et al., 1989).

Vaccinia virus has been used as an expression vehicle for a number of eukaryotic genes because their products undergo post-translational modification (e.g. glycosylation) and can be expressed on the surface of infected cells. This system has been a valuable tool in defining and expressing protective antigens of many pathogens (Tomley et al., 1987; Mackett et al., 1984). Genes for several viral glycoproteins have been incorporated into
vaccinia virus to investigate how the expression of individual antigens affects the host’s immune response to the pathogen as a whole (Cranage et al., 1986; Smith et al., 1983; Wiktor et al., 1984). Recently EHV-1 gp13 has been expressed in vaccinia virus and shown to provide protective immunity against EHV-1 infection in hamsters (Guo et al., 1989). We report here the transcriptional mapping of the EHV-1 gB homologue mRNA and the construction of a recombinant vaccinia virus that expresses this gene.

**Methods**

**Nomenclature.** The position of the gene in EHV-1 with homology to the gB gene of HSV has been identified as spanning the BamHI A and I fragments (Whalley et al., 1989) indicating that this is the same region as that coding for the EHV-1 gp14 glycoprotein mapped by Allen & Yeagian (1987). Pending a review of EHV glycoprotein nomenclature, for convenience the EHV-1 gB homologue will be referred to here as EHV-1 gB.

**Virus and cell culture.** EHV-1 (isolate HVS 25A; Whalley et al., 1981) was propagated in BHK-21 and RK-13 cells in Glasgow modified MEM (Flow Laboratories) with 10% foetal calf serum (FCS) (Commonwealth Serum Laboratories; CSL). For RNA preparations, monolayers of BHK-21 cells were infected with EHV-1 at 10 TCID50/cell and RNA was extracted at 16 h post-infection. Purified EHV-1 virions were prepared by infecting BHK-21 cells with 0.01 TCID50/cell, and following incubation at 35 °C for 30 to 40 h, the cells were frozen and thawed twice and then clarified at 10000 g. The supernatant was spun at 80000 g for 1 h and the pellet resuspended overnight in TE buffer (10 mM-Tris Cl, 1 mM-EDTA, pH 8.0). This was then layered onto preformed 25 to 40% (w/v) potassium tartrate gradients and centrifuged at 65000 g for 2 h. The virus-containing band was removed, diluted with TE, centrifuged down at 80000 g and resuspended in a small volume of TE buffer.

Vaccinia virus strain WR (VW-WR) was propagated in human 143B thymidine kinase-negative (TK−) cells grown on Auto-Pow MEM (Flow Laboratories) with 5% FCS and 25 μg/ml of 5-bromodeoxyuridine. Virus was prepared from vaccinia virus-infected 143B cells and DNA was extracted as described by Nakano et al. (1982).

**RNA preparation and Northern blot analysis.** Total cytoplasmic RNA was prepared from mock- and virus-infected cells, denatured with glyoxal and electrophoresed in 1% agarose according to the methods of Maniatis et al. (1982). The RNA was then transferred to a nylon membrane and hybridized with nick-translated 32P-labelled DNA (Rigby et al., 1977) or with M13 strand-specific gB probes (Whalley et al., 1989) made using the M13 universal primer (MSR) or the hybridization probe primer (HPP) (Holland et al., 1984). Probes were either denatured by heat prior to use (MSR and nick translation) or used without denaturation (HPP). Size standards on Northern blots consisted of separate restriction digestes of M13 replicative form (RF) DNA which had been combined to give a range of markers from 7.25 to 0.19 kb and which hybridized with the probes used. Single-stranded forms of both RNA and DNA have been shown to migrate similarly in agarose following glyoxalation (Carmichael & McMaster, 1980).

Single-stranded probes used in transcript mapping (Table 1) were prepared by the prime-cut method (Davis et al., 1986) using the C2/11 oligonucleotide (Fig. 4) with the exception that purification was carried out on a polyacrylamide gel as described by Kouzarides et al. (1987).

**Table 1. Probes used in transcript mapping and Southern blotting experiments**

<table>
<thead>
<tr>
<th>Probe</th>
<th>EHV-1 sequence* position</th>
<th>Map coordinates</th>
<th>Length (bases)</th>
</tr>
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<tbody>
<tr>
<td>C2/11-KpnI</td>
<td>450-1058</td>
<td>0.403-0.407</td>
<td>608</td>
</tr>
<tr>
<td>C2/11-Avi</td>
<td>893-1058</td>
<td>0.405-0.407</td>
<td>165</td>
</tr>
<tr>
<td>C1/60-66</td>
<td>1-351</td>
<td>0.400-0.402</td>
<td>350</td>
</tr>
<tr>
<td>H21+</td>
<td>1384-4039</td>
<td>0.410-0.429</td>
<td>2655</td>
</tr>
<tr>
<td>C138</td>
<td>1384-1789</td>
<td>0.410-0.413</td>
<td>405</td>
</tr>
</tbody>
</table>

* Numbering according to Fig. 3 of Whalley et al. (1989).
† Single-stranded probe made by primed second strand synthesis on M13. Resulting double-stranded molecule cut with indicated restriction enzyme and purified.
‡ Double-stranded probe made from M13 replicative form by nick translation.
§ Single-stranded probe specific for the leftward strand (MSR) or the rightward strand (HPP).

For transcript analyses, two M1 size markers were used. The first was a 32P-end-labelled HinfI/EcoRI digest of pBR322 giving a range from 990 to 75 bases and the other was a sequencing ladder generated with the C2/11 oligonucleotide.

**SI nuclease mapping.** Prime-cut probe (approx. 200 000 c.p.m.) was ethanol-precipitated with 40 μg of total RNA and annealed at 75 °C for 10 min in 40 μl of hybridization buffer (80% deionized formamide, 0.4 M- NaCl, 40 μm-PIPES pH 6.4, 1 mM-EDTA); the temperature was then reduced to 37 °C and incubation continued overnight. To the annealed mixture was added 400 μl of chilled SI digestion buffer (30 mM-sodium acetate pH 4.6, 250 mM-NaCl, 1 mM-ZnSO4, 5% glycerol; 0.014 μg/ml salmon sperm DNA, 0.2 units/ml SI nuclease) and incubation continued at 14 °C for 1 h. The nuclease-resistant fragments were extracted with phenol-chloroform, precipitated with ethanol, denatured in formamide and resolved on a 6% acrylamide-8M-urea gel.

**Primer extension analysis.** The procedure used was essentially that of Kouzarides et al. (1987) except that the probe (10 0000 c.p.m.) was precipitated along with 3 μg of total RNA and resuspended in 15 μl of hybridization buffer (80% deionized formamide, 0.5 μM-PIPES pH 6.4, 1 mM-EDTA). This was heated to 75 °C for 10 min and then the incubation was continued overnight at 42 °C.

**Molecular cloning.** All DNA manipulations were as described in Maniatis et al. (1982) unless otherwise stated. Restriction enzymes (Boehringer Mannheim) were used according to the manufacturer’s conditions. Recombinant plasmids pMAC209 and pMAC201 contained the EHV-1 BamHI I and BamHI A fragments, respectively, inserted in pBR322 (Robertson & Whalley, 1985). pMAC209 was digested with restriction enzymes BamHI and Clal followed by ligation into M13mp18 and mp9 and selection of a subclone containing a 2.9 kbp insert. pMAC201 was digested with BamHI and PstI, ligated into the same vectors and a subclone was selected which contained a 1.38 kbp insert. Several series of deletion mutants were generated from the subclones using the method of Dale et al. (1985). These were used in the sequencing of the EHV-1 gB gene which spans the two BamHI fragments (Whalley et al., 1989). H21 (mp18, from pMAC209), APBS (mp18, from pMAC201), C13 (mp18, from pMAC209) and C1/60-66 (mp9, from pMAC201) were individual deletion subclones derived from these series. To clone the EHV-1 gB gene a PstI site was inserted at position 858 in APBS and the internal HindIII site present at position 3694 was eliminated from H21 by oligonucleotide mutagenesis according to the Mutagenekit (Bio-Rad). The mutant 21-mers and the
16-mer (C2/11), listed in Fig. 4, were obtained from Bioquest Ltd., and purified on denaturing polyacrylamide gels. Mutagenesis was confirmed by sequencing and subsequent restriction enzyme cleavage. M13 replicative form DNA from the mutagenized subclones, APBS and H21, was digested with BamHI-PstI and BamHI-HindIII respectively, and the inserts were recovered. These were ligated into the polynicker of vaccinia virus insertion vector pBCB07 (Andrew et al., 1987) to yield the plasmid pGBC3/28-1.

**Construction of recombinant vaccinia virus.** A TK+ recombinant vaccinia virus carrying the EHV-1 gB gene was generated by transfection and homologous recombination of VV-WR-infected 143B cells with pGBC3/28-1 DNA (Mackett et al., 1984; Boyle et al., 1985). Recombinant virus plaques were identified by hybridization to 32P-labelled H21 DNA and were carried through two cycles of plaque purification to yield the virus VV-027. DNA from the recombinant virus was analysed by Southern blots.

**Western blots.** Samples of purified EHV-1- VV-027- and VV-WR-infected 143B cells and mock- and EHV-1-infected BHK-21 cells were solubilized in buffer containing 1% 2-mercaptoethanol, 1.6% (w/v) SDS, 0.5 mM-Tris–HCl pH 7.6 and electrophoresed using a modified Laemmli buffer system in gradient mini gels (Hoeler) of 5 to 11% polyacrylamide as described by Mattick et al. (1981). Prestained markers (Bethesda Research Laboratories) were included on each gel and approximately equal amounts of antigen were loaded as judged by Coomassie blue staining. Following electrophoresis, proteins were transferred to nitrocellulose using the semi-dry method of Kyhse-Anderson (1984). After transfer the blots were incubated for 1 h in blocking buffer. The sheets were washed in blocking buffer, then incubated for 1 h with either horseradish peroxidase (HRP)-conjugated anti-horse serum (prepared by the method of Wilson & Nakane, 1978) or HRP-conjugated anti-mouse serum (Tago) diluted in blocking buffer. The chromogen used was 4-chloro-1-naphthol.

**Serum samples.** Neutralizing sera obtained from horses which had been infected with EHV-1 were pooled and designated C3/85. Mabs 3F6 and 12D12, specific for EHV-1 gp14 were a generous gift from Dr George Allen, University of Kentucky, Lexington, Ky., U.S.A. Antisera were derived from groups of five CBA/H mice which had been inoculated intravenously with 1 × 107 p.f.u. of VV-027/mouse. Serum was collected 21 days post-vaccination, mice were revaccinated with the same dose of virus and the final serum was collected 42 days post-vaccination. The sera were heat-inactivated at 56 °C for 30 min.

**Immunofluorescence.** Indirect immunofluorescence tests were carried out on monolayers of 143B and BHK-21 cells grown on eight-chambered tissue culture slides (Lab Tek, Miles Scientific) infected with VV-027 or EHV-1. Cells were fixed 16 h post-infection with cold methanol for 5 min or with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, then blocked with 4% bovine serum albumin (BSA) in PBS for 1 h. Some slides were permeabilized by treatment with 1% Triton X-100, 10% sucrose, 1% BSA for 5 min followed by washing with PBS–BSA. The cells were then incubated with either C3/85 serum, MAb 3F6 or normal rabbit serum for 30 min at 37 °C, washed in PBS–BSA, incubated with fluorescein-conjugated sheep anti-species serum (Silusen Laboratories) for 30 min at 37 °C, washed again and photographed under u.v. illumination.

**Neutralization assays.** Neutralization titres of equine and mice sera were determined using twofold serum dilutions, 100 TCID50 of EHV-1 and RK-13 cells as the indicator, both in the presence and absence of guinea-pig complement (CSL), according to the method of Willey et al. (1988). The neutralization titres were expressed as the reciprocal of the dilution giving a 50% endpoint.

**Results**

The sequence of a 4.3 kbp PstI–Clal fragment containing the EHV-1 gB gene and the 3' end of the ICP 18.5 gene equivalent has been reported and all sequence positions reported here refer to Fig. 3 from Whalley et al. (1989).

**Transcript analysis**

To identify transcripts originating from the EHV-1 gB gene, a Northern blot of total cytoplasmic RNA prepared from EHV-1-infected cells was probed initially with a double-stranded probe containing the 3' end of the coding sequence (H21). Two transcripts of 3.4 and 4.6 kb were detected in RNA made late in infection (Fig. 1, lane 1). There was no hybridization to uninfected cell RNA. In blots hybridized with a probe specific for the left strand (C13-MSR) the same bands were identified, whereas no hybridization was seen using a probe specific for the right strand (C13-HPP, data not shown). On Northern blots hybridized with a strand-specific probe (C1/60-66) from sequence upstream of the gB open reading frame (ORF) but within the EHV-1 homologue of HSV-1 ICP 18.5 ORF, only a 4.6 kb band was detected (Fig. 1, lane 3). Again this was observed only when the probe was specific for the left strand. This indicated that both the 3.4 and 4.6 kb transcripts are transcribed from left to right (leftward), that there are no transcripts from the right strand between coordinates 0.40 and 0.43 and that the 5' end of the smaller transcript is located between the BamHI site at 1384 and the end of the C1/60-66 clone at position 351.

The 5' terminus of the gB mRNA was mapped by S1 nuclease digestion of the C2/11-KpnI probe annealed to total RNA. Three major S1-resistant fragments of 222, 225, and 252 to 254 bases were generated (Fig. 3b) indicating that 5' RNA–DNA discontinuities exist at positions 836, 833, and 806 to 804 (Fig. 4). The presence of a band representing full-length protection of the probe (Fig. 3b, lane 2) was ascribed to the binding of a proportion of the probe to the larger transcript. To investigate the presence of what appeared to be multiple initiation sites for transcription, a primer extension experiment was carried out in which the purified C2/11-Nci probe (165 bases long) was annealed to total RNA and extended with reverse transcriptase. The single 232 base extension product (Fig. 3a) obtained confirmed that a major 5' RNA terminus exists at position 826 (Fig. 4). The band at 806 to 804 from the S1 analysis was considered artefactual.

The accuracy of the bands generated by S1 and primer extension analysis was estimated to be within 3 bases of the size shown. This places the start site of transcription for the gB mRNA between positions 823 and 839, with
Fig. 1. Northern blot analysis. Total cytoplasmic RNA from EHV-1-(lanes 1 and 3) or mock-infected (lane 2) cells was glyoxalated, separated on agarose and transferred to nitrocellulose. The probes used were H21 (lanes 1 and 2) and C1/60-66 (MSR) (lane 3). The position of markers and transcripts (arrowed) is indicated and the sizes are given in kb.

Fig. 2. EHV-1 BamHI map showing the positions of the gB and ICP 18.5 ORFs within the genome (a). The positions of probes in Table 1 (b) and the direction and size of the mapped transcripts (c) are indicated. Restriction sites (a) are denoted: p, PstI; b, BamHI; h, HindIII; c, ClaI.

the most likely starting bases being the A residues at positions 831 and 834, which are 29 and 32 bases, respectively, downstream from the proposed TATA element. These conform to established rules for the distance between eukaryote mRNA 5' ends and the TATA box and for the initiation site consensus sequence which is an A surrounded by pyrimidines (Corden et al., 1980).

Recombinant vaccinia virus containing the EHV-1 gB gene

The vaccinia virus vector used, pBCB07, has the polylinker from pUC8 adjacent to the 7.5K vaccinia promoter and is flanked on either side by part of the vaccinia virus HindIII J fragment (which contains the TK gene). The EHV-1 gB gene was present in two M13mp18 subclones, derived from adjacent BamHI genomic fragments (Fig. 2). Reconstruction of the gene was facilitated by using oligonucleotide mutagenesis of deletion subclones, which allowed the use of the universal primer in sequencing reactions to verify the position of the mutations. The start site for translation of gB was placed as close as possible to the 7.5K promoter by inserting a PstI restriction site at position 858 (Fig. 4b). The first, and therefore likely, initiating methionine codon of the gB gene then occurs approximately 145 bp downstream from the vaccinia virus promoter early mRNA start site. A HindIII site present near the 3' end of the gene at position 3694 was also eliminated by designing an oligonucleotide which altered the base sequence but did not change the amino acid sequence at this point (Fig. 4c). The inserts from correctly mutagenized subclones were cloned into pBCB07 to form the insertion vector pGBC3/28-1. This was used to prepare the recombinant vaccinia virus VV-027 by transfection and homologous recombination. The position of the EHV-1 gene within VV-027 was confirmed by restriction enzyme digestion and Southern blot hybridizations.

Expression of EHV-1 gB

Several approaches were used to test for the expression of the EHV-1 gB by the recombinant virus. In the first of these, an indirect immunofluorescence test, VV-027-infected cells were fixed with methanol or paraformaldehyde and reacted with polyclonal horse serum (C3/85) or MAb 3F6, which is specific for EHV-1 gp14. These were compared to similarly treated EHV-1-infected cells (Fig. 6). Both antibodies reacted positively with the recombinant-infected cells. With methanol fixation, strong immunofluorescence was observed in discrete areas of the cytoplasm of infected cells in the monolayer (Fig. 6c and d). Following paraformaldehyde fixation to preserve the cell membrane, immunofluorescence was observed on the cell surface, giving the indication that the gB product was transported to the cell membrane (Fig. 6g). When cells were permeabilized with Triton X-100 to enable internal antibody binding, the immunofluorescence
Expression of vaccinia–EHV-1 gB

remained associated with the cytoplasm (Fig. 6h). Similar patterns of cytoplasmic fluorescence were observed in EHV-1-infected cells (Fig. 6e and f). The background staining of the polyclonal serum was slightly greater than that of the MAb, but no immunofluorescence was observed with the negative controls (Fig. 6a and b) or in VV-WR-infected cells (data not shown).

Secondly, Western blots were probed with several specific sera. The C3/85 polyclonal serum identified two major immunodominant bands of 121K and 76K, along with numerous other minor bands in EHV-1-infected cells (Fig. 7a, lane 4). Major bands of 74K and 57K were also identified in preparations of purified virus (Fig. 7a, lane 5). In VV-027-infected cells, two major bands of 138K and 80K were seen (Fig. 7a, lane 2) which were not present in cells infected with VV-WR. A number of other bands occurred which were common to both VV-027 and VV-WR and probably represented cross-reactive antigens of vaccinia virus or 143B cells. The bands were detected at a 1:200 dilution of the primary antibody. Blots were also probed with two MAbs directed against epitopes located on the disulphide-linked heterodimer of EHV-1 gB (gp14) (3F6, Allen & Yeargan, 1987; 12D12, Sullivan et al., 1990). Using 3F6 two major bands of 135K and 86K and two minor bands of 76K and 72K were seen in EHV-1-infected cells (Fig. 7b, lane 4) and a single diffuse 82K band in purified EHV-1 (Fig. 7b, lane 5). In VV-027-infected cells two bands of 143K and 90K were identified (Fig. 7b, lane 2). When blots were probed with the 12D12 MAb a single band of 55K to 72K was seen in purified EHV-1 (Fig. 5, lane 3). In both EHV-1- and VV-027-infected cells a major band of this size also occurred (Fig. 5, lanes 4 and 6), whereas less reactive bands of 180K and 124K were also seen in the EHV-1-

Fig. 3. Nuclease S1 digestion and primer extension analysis. The probe (lane 3) was annealed with total cytoplasmic RNA from mock-infected (lane 1) or EHV-1-infected (lane 2) cells and extended with reverse transcriptase (a) or digested with S1 nuclease (b). The size of bands (arrowed) and of end-labelled markers (lane 4) is given in bases. A sequence ladder generated from the C2/11 oligonucleotide is also included (TCGA).
infected cells. The intensity of staining with 12D12 was much less than that of 3F6. MAb 3F6 showed no binding to VV-WR-infected cells but 12D12 reacted with a 64K protein in both VV-WR- and VV-027-infected cells.

**Mice immunized with recombinant vaccinia virus produce antibodies that recognize EHV-1**

To determine whether the recombinant virus could induce EHV-1-specific antibodies, CBA/H mice were immunized twice with live VV-027. Sera from the mice, taken after each immunization, were individually assayed by Western blot. The cumulated results are given in Table 2 and a Western blot from one of these sera is shown in Fig. 7(c). Preimmune sera did not react against any of the antigens. All immune sera produced an equally strong anti-vaccinial response against VV-WR- and VV-027-infected cells (Fig. 7c, lanes 1 and 2). In addition one of five mice after a single immunization and four of five mice after two immunizations had a detectable response
Expression of vaccinia–EHV-1 gB

Fig. 5. Western blot analysis. Samples of purified EHV-1 (lane 3), EHV-1- and mock-infected cells (lanes 4 and 5) and VV-027- and VV-WR-infected cells (lanes 6 and 7) were electrophoresed in an 8% denaturing gel, transferred to nitrocellulose and incubated with MAb 12D12. Bands discussed in Results are indicated by arrows and the Mr of markers (lanes 1, 2 and 8) is shown on the left.

against either EHV-1-infected cells, purified EHV-1 or both. Three major bands were identified by the immune sera, in the ranges of 123K to 135K, 86K to 94K and 67K in EHV-1-infected cells and 78K to 84K and 56K to 60K in purified EHV-1 (Table 2). The Western blot in Fig 7(c) shows the reaction of a mouse given two immunizations with VV-027. In the EHV-1-infected cells, several bands were identified, with two major bands of 123K and 86K and minor reactions to bands of 66K and 53K (Fig. 7c, lane 4). In purified EHV-1 this serum identified a single 84K band (Fig. 7c, lane 5). There was no reaction to uninfected cells.

Virus neutralization test

The sera from mice immunized with VV-027 were pooled and tested in a virus neutralization assay. No neutralizing antibody response was detected against EHV-1 either with or without complement. Positive control sera gave neutralization titres of 120 and 60 in the presence and absence, respectively, of complement.

Fig. 6. Immunofluorescence. Cells (143B) infected with VV-027 (b to d, g and h) and BHK-21 cells infected with EHV-1 (e and f) or mock-infected (a) were fixed at 16 h post-infection with methanol (c and d) or paraformaldehyde (a, b, e to h). Some cells were permeabilized with Triton X-100 (e, f and h) before being reacted with C3/85 horse serum (d and f), 3F6 MAb (a, c, e, g and h) or normal rabbit serum (b) and stained for immunofluorescence. Bar markers represent 25 μm (b, c, d, g and h) or 50 μm (a, e and f).
Fig. 7. Western blot analysis. Samples of VV-WR- and VV-027-infected 143B cells (lanes 1 and 2), mock- and EHV-1-infected BHK-21 cells (lanes 3 and 4) and purified EHV-1 (lane 5) were electrophoresed in 5 to 11% gradient gels and transferred to nitrocellulose. They were incubated with C3/85 horse serum (a), monoclonal antibody 3F6 (b) and serum from a mouse given two immunizations with VV-027 (c). Bands discussed in Results are indicated by arrowheads and the size of prestained Mr markers (M) is shown on the right.

Table 2. Mr of major bands identified on Western blots by sera from groups of five mice vaccinated with VV-027

<table>
<thead>
<tr>
<th>Time post-vaccination (days)</th>
<th>EHV-1-infected cells</th>
<th>Purified EHV-1</th>
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</thead>
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<tr>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>135K (1/5)</td>
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<td></td>
<td>94K (1/5)</td>
<td>84K (1/5)</td>
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<td></td>
<td>67K (1/5)</td>
<td>60K (1/5)</td>
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<tr>
<td>42</td>
<td>123K–129K (4/5)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>86K (1/5)</td>
<td>78K–84K (3/5)</td>
</tr>
<tr>
<td></td>
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<td>56K (1/5)</td>
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Discussion

EHV-1 gB (gp14) is one of the six most abundant glycoprotein antigens of the virus. Its significance in the immune response of the horse is demonstrated by the presence of immunoprecipitating antibodies against it in convalescent equine sera (Allen & Bryans, 1986). We have attempted to define this candidate protective antigen of EHV-1, first by identification and determination of the sequence encoding this major glycoprotein (Whalley et al., 1989) and secondly by transcript analysis and expression in a recombinant vaccinia virus, as described here. Individual glycoproteins from several other herpesviruses have been expressed in vaccinia virus and have been shown to induce neutralizing antibodies and protect animals against lethal infection, in particular gB and gD of HSV (Cantin et al., 1987; Willey et al., 1988; Paoletti et al., 1984) and gp50, the gD equivalent in pseudorabies virus (PRV) (Marchioli et al., 1987).

Transcript mapping of the EHV-1 gB mRNA revealed several features. From Northern blot analysis two mRNAs were identified between coordinates 0-40 and 0-43 and both were transcribed in a leftward direction. The hybridization pattern with the single-stranded probes was consistent with the occurrence of two overlapping transcripts as shown to occur commonly with HSV-1 (Holland et al., 1984) when the promoter for one mRNA is located within the interior of an upstream mRNA. The nucleotide sequence of this region (Whalley et al., 1989) contains two overlapping ORFs with the 5' ORF (ICP 18.5 gene homologue) containing the promoter and regulatory regions proximal to the gB ORF. The 3-4 kb transcript is most likely to represent the RNA coding for gB for two reasons: the size of the gB ORF is 2.94 kb and the mRNAs for the gBs of related herpesviruses are similar to this, being 3.3, 3.0 and 3.0 kb for HSV-1 gB, PRV gII and Epstein–Barr virus BALF4, respectively (Holland et al., 1984; Robbins et al., 1987; Gong et al., 1987). It is proposed that the larger 4-6 kb transcript encodes the EHV-1 ICP 18-5, with the 5' region of each mRNA encoding the unique polypeptide. These two transcripts are probably equivalent to the 3-6 and 4-4 kb transcripts identified by Gray et al. (1987) as coming from a similar region of the genome of EHV-1 (Kentucky A strain). No obvious poly(A) signal is evident downstream from the EHV-1 ICP 18-5 ORF, whereas a polyaadenylation signal occurs a few nucleotides from the termination codon of the EHV-1 gB ORF, suggesting that the two transcripts have a common 3' terminus. Fine structure mapping of the 5' terminus of the gB mRNA by S1 nuclease and primer extension analysis located a single start site for transcription at either nucleotide 831 or 834. The base sequence...
The preceding nucleotide 831 has a match of seven in nine to the proposed HSV-1 gB mRNA start site (Beik et al., 1986). The first downstream AUG codon occurs at position 951 which agrees with the predicted start site of the EHV-1 gB gene (Whalley et al., 1989).

Reconstruction of the EHV-1 gB gene from subclones enabled the generation of a recombinant vaccinia virus, VV-027, which contained the coding region of this gene interrupting the vaccinia virus TK gene. Cells infected with the recombinant virus expressed the EHV-1 gB protein within their cytoplasm and at the cell membrane as detected by immunofluorescence with MAb 3F6 and serum from EHV-1-infected horses. From Western blot analysis the diffuse nature of the majority of bands was consistent with the expression of glycoproteins by the recombinant virus. An endoproteolytic cleavage site is present within the TK gene in vaccinia virus, enabling the generation of a recombinant vaccinia virus, consistent with the expression of glycoproteins by the analysis of cell lysates. The majority of bands was due to a 145K complex composed of disulphide-linked dimers of 75K to 77K and 53K to 58K (Meredith et al., 1989; Sullivan et al., 1990). Our results were consistent with this finding, in that major bands of 74K and 57K (polyclonal serum) and 82K (3F6) and 55K to 57K (12D12) were detected in purified EHV-1. MAbs 3F6 and 12D12 are directed against gp14 of the strain of EHV-1 and both immunoprecipitate three species of polypeptide, of 138K, 75K to 77K and 53K to 55K, from the Kentucky A strain of EHV-1 (Sullivan et al., 1990). The results presented here indicate that these MAbs recognize native and recombinant-expressed gp14 from the geographically distant HSV-1 strain of EHV-1, which does appear however, from DNA restriction enzyme patterns, to be identical to the American 1P strain (Allen et al., 1985; Allen & Bryans, 1986; Whalley et al., 1981). Indeed the gp14 epitopes so far examined with MAbs are common to all isolates of EHV-1, EHV-4 and EHV-3 (Sullivan et al., 1990) although the amino acid homology of 88% between EHV-1 and EHV-4 gB shows that there has been some divergence (Riggio et al., 1989; Whalley et al., 1981). From the Western blot analyses, the epitope on native EHV-1 gB specific for the 3F6 MAAb appears to be different from that for 12D12. This difference was also observed in the reaction of these antibodies with EHV-1 gB expressed by vaccinia virus. A probable explanation for these Western blot results (Fig. 5 and 7b) is that the 3F6 MAAb detects the larger subunit of EHV-1 gB (gp14) which has a reported Mr of 87K to 90K and that the 12D12 MAAb recognizes the smaller EHV-1 gB subunit which is likely to have been called gp18 previously and had a reported Mr of 60K to 63K (Turtinen & Allen, 1982; Allen & Bryans, 1986). In both EHV-1- and VV-027-infected cells the high Mr, band of 121K to 143K is likely to be the mature glycosylated gB present in the cell membrane prior to cleavage and its size is approximately the sum of the two smaller bands. An analogous situation occurs with the processing of the PRV gB homologue, gII, where a higher Mr, form of 120K is cleaved into 68K and 55K forms (Lukacs et al., 1985). The presence of immunologically reactive bands in VV-027-infected cells which were similar in number and size to those identified in EHV-1-infected cells indicated that the recombinant EHV-1 gB protein undergoes proteolytic cleavage similar to that of the native EHV-1 gB.

The expression of other glycoprotein genes in vaccinia virus has usually produced proteins of Mr identical to the native form (discussed by Mackett & Smith, 1986). However the protein produced by VV-027 was consistently of a higher apparent Mr than that seen in EHV-1-infected BHK-21 cells. This did not appear to alter its reactivity with either polyclonal horse serum or MAbs to gp14 nor the ability to generate an EHV-1-specific immune response in mice. This higher Mr, may indicate extra glycosylation occurring in the 143B cells. Smith et al. (1987) noted that expression of the influenza virus HA gene in vaccinia virus produced a glycosylated product slightly larger than the native HA but that this did not alter the immunogenicity or antigenicity of the protein. Andrew et al. (1987) found that a truncated version of rotavirus VP7 protein was expressed in vaccinia virus with a higher Mr than intracellular VP7. Both groups inferred that the size changes were due to differences in the glycosylation pattern.

The immunogenicity of the recombinant gB protein was demonstrated by the response of vaccinated mice whose sera specifically bound to bands corresponding to purified EHV-1 and EHV-1-infected cells. These were very similar in Mr to the bands identified by polyclonal horse serum and MAbs to EHV-1 gB. A band of 55K to 57K identified by the 12D12 MAAb in EHV-1-infected cells was not seen in the VV-027-infected cells, but a band of this Mr, was detected in purified EHV-1 by sera from two of the vaccinated mice (Table 2).

An in vitro neutralizing antibody response to EHV-1 was not stimulated in mice immunized with the recombinant virus. Blacklaws et al. (1987) found that a murine cell line constitutively expressing HSV-1 gB failed to elicit the production of neutralizing antibodies in mice, although a degree of protection against latent infection was obtained. Stokes et al. (1989) found that the MAAb to EHV-1 gp14 we used in this study did not neutralize EHV-1 in vitro, but when given passively was nonetheless able to protect hamsters against infection with EHV-1.

These results indicate that vaccinia virus is able to
process and present the EHV-1 gB protein in an immunologically recognized form and that VV-027 could act as an efficient live viral vector for delivery of this antigen. This provides a basis for further studies to assess the ability of the gB antigen to protect against EHV-1 infection. A similar approach with other individual glycoproteins of EHV-1 should provide additional information for the development of more efficient vaccines for this equine disease.

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