Identification and characterization of the protein product of gene 71 in equine herpesvirus 1

Yi Sun,1 Alasdair R. MacLean,1 Derrick Dargan2 and S. Moira Brown2*

1Institute of Virology and 2MRC Virology Unit, University of Glasgow, Church Street, Glasgow G11 5JR, U.K.

Equine herpesvirus 1 (EHV-1) strain Ab4 gene 71 is predicted to encode a primary product with a M_r of 80-1K. We have previously constructed a deletion/lacZ insertion mutant, ED71, and demonstrated that gene 71 is dispensable for growth of virus in cell culture. We have now constructed a gene 71 revertant, Re71. To identify and characterize the product of gene 71, we produced a specific antiserum, anti-71, against a fl-galactosidase fusion protein containing the carboxy terminus of the gene 71 polypeptide. Using the anti-71 serum, mutant ED71 and the revertant Re71, we have demonstrated that gene 71 encodes a 192K polypeptide. Experiments with glycosylation inhibitors revealed that the protein product of gene 71 is N-glycosylated and heavily O-glycosylated. When the 192K polypeptide is synthesized in the presence of monensin, the M_r of the polypeptide is reduced to 80K, the predicted unmodified M_r of the gene 71 polypeptide. The gene 71 product is found in virions and L particles in a fully processed form that runs as a diffuse band in electrophoresis, with a M_r in excess of 200K. Immunofluorescence and virion surface labeling experiments showed that the polypeptide product of gene 71 is located on cellular membranes and the virion envelope. A time course of infection confirmed that gene 71 is regulated as a leaky late gene in infected cells. Finally, using wild-type EHV-1 Ab4, mutant ED71, revertant Re71 and two antibodies (P19 against EHV-1 glycoprotein gp300, and anti-71) we conclusively demonstrated that gene 71 encodes gp300. This contradicts published results with P19 alone, which indicated gp300 was the product of EHV-1 gene 28.

Introduction

Equine herpesvirus 1 (EHV-1), a member of the subfamily Alphaherpesvirinae, is a significant viral pathogen of horses, causing a variety of clinical problems including respiratory disease, abortion and neurological disorders (Bryans & Allen, 1989). The EHV-1 genome is a linear dsDNA molecule of approximately 150 kbp in size that consists of two covalently linked components, the long and short regions (Whalley et al., 1981). The complete DNA sequence of strain Ab4, a pathogenic U.K. isolate of EHV-1, has recently been determined (Telford et al., 1992). The genome is 150223 bp in size and contains 76 distinct open reading frames (ORFs). At least 10 of these ORFs have been predicted to encode membrane glycoproteins. To date up to 13 glycoproteins have been found in EHV-1 virions, ranging in M_r, from 24000 to 260000 (Turtinen & Allen, 1982; Meredith et al., 1989). Some have been characterized and mapped to ORFs. Herpesvirus glycoproteins play an important role in essential viral functions such as absorption, penetration and egress. Consequently they play a significant role in viral pathogenesis (Spear, 1993). They are also the main targets of neutralizing antibodies.

Gene 71 is located in the short unique region of the EHV-1 genome and is predicted to encode a 797 amino acid polypeptide. The gene corresponds in position to herpes simplex virus type 1 (HSV-1) gene US5, which encodes a potential glycoprotein, gJ, that also contains a single putative N-linked glycosylation site (McGeoch et al., 1985). However no similarity was detected between the EHV-1 and HSV-1 amino acid sequences (Telford et al., 1992). The predicted amino acid sequence of EHV-1 gene 71 contains a single putative N-linked glycosylation site and an extensive region encoding a high proportion of serine and threonine residues, which is likely to be heavily O-glycosylated. Two equivalent ORFs, EUS4 and ORF1, have been identified in the Ky A and D strains respectively of EHV-1 (Audsonnet et al., 1990; Colle et al., 1992). These encode 383 amino acid polypeptides with a predicted M_r of 40K and have the same amino and carboxy termini as the gene 71 protein, but they differ in having an internal deletion and two potential N-linked glycosylation sites. Their amino acid sequence is also rich in serine and threonine residues.

We have previously demonstrated that gene 71 of EHV-1 Ab4 is non-essential for virus growth in cell culture by the construction of a viable mutant, ED71, in...
which the gene 71 ORF has been deleted (Sun & Brown, 1994). In this paper, we have identified and characterized the protein product of gene 71 using an antisera raised against its carboxy terminus (amino acids 465 to 797). The specificity of this antisera was confirmed using the deletion/insertion mutant ED71 and its revertant Re71.

Methods

Cells and virus. Baby hamster kidney clone 13 cells [BHK-21 (C13); Macpherson & Stoker, 1962] were propagated as previously described (Brown et al., 1973). EHV-1 strain Ab4p (kindly supplied by Dr E. A. R. Telford) was the wild-type virus strain used in this study. Stock preparation of the virus at passage 13 was made by low multiplicity passage in equine dermal NBL-6 cells maintained in MEM with 1% fetal calf serum. The construction of mutant ED71, in which the gene 71 ORF was removed and substituted with lacZ has been previously described (Sun & Brown, 1994).

Construction of a revertant of mutant ED71. To isolate a revertant of ED71, the cloned 5.8-kbp BamHI-EcoRI fragment (pUT1) (Sun & Brown, 1994) was cotransfected as described by Stow & Wilkie (1976) with intact viral DNA of mutant ED71. Individual plaques lacking lacZ were isolated in medium containing X-Gal (0.05 mg/ml) and their DNA analysed for a wild-type profile (Sun & Brown, 1994). Revertant viruses were plaque-purified a further three times before a virus stock was prepared.

Plasmids. pUEH71, which expresses amino acids 454 to 797 of gene 71 fused to the carboxy terminus of β-galactosidase in pUR278 (Rüther & Müller-Hill, 1983) was constructed by inserting a HindIII fragment (120487 to 131594) into the pUR278 HindIII site at the 3' end of the lacZ gene.

Preparation of a bacterial fusion protein and rabbit antiserum against the gene 71 product. The β-galactosidase fusion protein was prepared by the method of Harlow & Lane (1988). Two New Zealand white rabbits were each injected subcutaneously with 0.6 mg of total protein dissolved in 0.25 ml of water and emulsified with an equal volume of Freund's complete adjuvant. Each rabbit was boosted on days 14, 30, 60 and 90 with an emulsion of 0.2 mg of fusion protein dissolved in 0.25 ml of water plus an equal volume of Freund's incomplete adjuvant. Final antisera were collected on day 100.

Preparation of radiolabelled cell extracts. Confluent BHK-21 (C13) cell monolayers in 35 mm Petri dishes were infected with virus at 20 p.f.u./cell in Eagle's MEM containing 2% calf serum. Infected cells in buffer adjusted to 150 mM-NaCl were separated into supernatant (cytosol) and total membrane fractions. A sample of the total membrane fraction was then adjusted to 1 M-NaCl and separated into a supernatant fraction containing those proteins that bound only at low ionic strength and a pellet fraction containing proteins that bound to membranes at high ionic strength.

Immunoprecipitation. Infected cell polypeptides were harvested and immunoprecipitations carried out essentially as described by MacLean et al. (1992) except that 200 μl of the supernatant was incubated with 20 μl of antiserum overnight at 4 °C and then transferred to ice for a further 2 h in the presence of 60 μl of a 50% (v/v) suspension of Protein A-Sepharose.

Surface labelling of virion proteins. EHV-1 virion proteins were labelled in vitro by enzymatic labelling specific for exposed carbohydrate residues on the virion envelope surface (Wallenfels, 1979). Briefly, the reaction mixture containing (in a total volume of 460 μl) 0.25 M-Tris–HCl pH 7.4, 400 μg of purified virions, 2 μCi UDP-[14C]galactose (270 mCi/mmol), 0.28 units of galactosyl transferase and 0.02 mM-MnCl2 was incubated with shaking for 1 h at 37 °C, followed by 5 min at 0 °C. The labelled virions were washed by centrifugation in 10 mM-Tris–HCl pH 7.5 and 1 mM-EDTA to remove unincorporated UDP-[14C]galactose.

Immunoblotting. Confluent BHK-21 (C13) cell monolayers were infected at a multiplicity of 20 p.f.u./cell and maintained in medium containing 2% calf serum. To inhibit viral DNA synthesis, cells were maintained from 1 h before and then throughout infection in medium containing 300 μg/ml phosphonoacetic acid (PAA; Sigma). Infected cell polypeptides were harvested up to 24 h p.i. in 0.15 ml of extraction buffer per 106 cells and immunoblotting was carried out as described by Frame et al. (1987). Anti-71 serum was diluted 100-fold. Monoclonal antibody (MAb) P19 against EHV-1 gp300 has been previously described (Whittaker et al., 1992) and was kindly supplied to Dr D. Meredith. The reactivities on Western blots of MAb 6989 and rabbit antiserum 14327, used to detect expression of UL42 and US11, were characterized respectively by Partis et al. (1988) and MacLean et al. (1987).

Treatment with glycosylation inhibitors. Confluent monolayers of BHK-21 (C13) cells were infected with virus at 20 p.f.u./cell or mock-infected. The cells were treated with 2 μg/ml tunicamycin (Sigma) or 1 μM-monensin (Sigma) from the end of the absorption period (1 h) until 20 h p.i.

Treatment with glycosidases

(i) N-glycanase. Purified EHV-1 virions (10 μl) were incubated with 10 μl of 1% (v/v) Triton X-100 at 0 °C for 10 min and centrifuged at 10000 g for 10 min. The supernatant was treated with 15 μl of 0.2 M-sodium phosphate buffer pH 8.6, 3 μl of 100 mM-1,10-phenanthroline and 10 μl/ml N-glycosidase [peptide-N-4-(N-acetyl-β-glucomosaminyl) asparagine amidase; Genzyme] and incubated at 37 °C for 6 h.

(ii) Neuraminidase. Purified EHV-1 virions were treated with N-glycanase as above and adjusted to pH 5 with 0.1 M-citric acid. Next 0.2 units of neuraminidase (acytelneuraminyl) hydrolase; Sigma) was added and incubated at 37 °C for 4 h.

(iii) Exoglycanidases. Purified EHV-1 virions were digested with N-glycanase followed by neuraminidase as above. β-N-acetyllhexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucosylhydrolase; Sigma) 0.01 unit, 0.01 unit of l-fucose fucosidase (Sigma) and 0.01 unit of β-galactosidase (β-D-galactoside galactohydrolase; Boehringer Mannheim) were added and incubated at 25 °C for 4 h.

Subcellular fractionation. Subcellular fractionation was based on the method described by Bryant & Rafter (1990) as modified by MacLean et al. (1992). Infected cells in buffer adjusted to 150 mM-NaCl were separated into supernatant (cytosol) and total membrane fractions. A sample of the total membrane fraction was then adjusted to 1 M-NaCl and separated into a supernatant fraction containing those proteins that bound only at low ionic strength and a pellet fraction containing proteins that bound to membranes at high ionic strength.

Purification of virus particles. The procedure used was essentially that described by Szilagyi & Cunningham (1991). Briefly, monolayers of BHK-21 (C13) cells in 80 oz roller bottles were infected with 1000 p.f.u. of EHV-1 strain AB4 and incubated until c.p.e. was complete. Supernatant virus was separated into two fractions, a virion preparation and an L particle preparation, which consisted of nucleocapsid-free tegument–envelope structures.

Immunofluorescence. For detection of cell surface antigens, cells infected with EHV-1 AB4 for 16 h were washed twice in PBS and fixed in PBS containing 4% formaldehyde at room temperature for 10 min.
and immunofluorescence carried out as described by Sodora et al. (1989). Anti-71 was used at a 1:100 dilution.

Results

Synthesis of β-galactosidase fusion proteins for immunization of rabbits

Plasmid pUEH71, which contains the sequence encoding the carboxy-terminal 333 amino acids of the gene 71 ORF fused to the carboxy terminus of the pUR278 lacZ gene (Fig. 1) was constructed. The junction between the lacZ and EHV-1 gene 71 sequences was checked by the double-strand sequencing method of Masahira & Yoshiyuki (1988) to ensure that the EHV-1 insert was in the correct frame (data not shown). Plasmid pUEH71 was transfected into Escherichia coli XL1 for fusion protein synthesis. After induction by IPTG at a final concentration of 1 mM, the lysates were analysed by 6% SDS-PAGE. The fusion protein migrated at the expected position with a M<sub>r</sub> of 160K (data not shown). The fusion protein was partially purified as inclusion bodies and used to immunize two rabbits. Of these only one gave a strong response. This antiserum, designated anti-71, was used in immunoblotting and immunoprecipitation against EHV-1 Ab4-infected BHK-21 (C13 cells). The epitope recognized by anti-71 was mapped to amino acids 517 to 529 of the putative gene 71 polypeptide sequence with a set of synthetic overlapping peptides (Fig. 1 and data not shown).

Identification of the gene 71 product in EHV-1-infected cells

Anti-71 was used to identify the product of the putative gene 71 of EHV-1 Ab4 by immunoblotting and immunoprecipitation. In immunoblotting experiments, anti-71...
recognized a polypeptide from EHV-1 Ab4-infected cell extracts that migrated at a position corresponding to a Mr of approximately 192K (Fig. 2, lane 1) on 10% SDS-PAGE. The polypeptide was not recognized in mock-infected cell extracts (Lane 2), or by preimmune serum (lane 4). Recognition was also inhibited by the relevant fusion protein (lane 6), but not by an unrelated fusion protein (lane 5). In the extract from mutant ED71-infected BHK-21 (C13) cells, the 192K band was absent. However a band corresponding to a Mr of 116K was observed (lane 3). Since the antiserum was raised against a β-galactosidase fusion protein, this band was presumed to correspond to β-galactosidase (116K) produced by the lacZ insertion in ED71. This was confirmed by Western blotting with a commercial β-galactosidase antiserum (data not shown).

Regulation of expression of the gene 71 protein product

To study gene regulation, a time course analysis of gene 71 expression was carried out. Cells were harvested at various times after infection and polypeptides were separated by 8% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-71 serum. The 192K protein was first detected at 8 h p.i.; the amount increased until about 12 h and remained constant up to 24 h (Fig. 3). This pattern was reproducible in duplicate experiments and suggests that gene 71 is regulated as a late gene. To determine whether gene 71 is regulated as a true late gene, expression of the 192K protein was examined in the presence of 300 μg/ml PAA. At this concentration of PAA, virus DNA replication is reduced to undetectable levels (< 5% of the untreated control) in cells infected with HSV-1 strain 17⁺ (Johnson et al., 1986). Similarly, we have demonstrated that PAA inhibits EHV-1 (Ab4) DNA synthesis to less than 5% of controls.

Infected cells were harvested at 0, 6, 12 and 24 h post-absorption. The 192K Mr polypeptide was first detected at 8 h after adsorption both in the absence and presence
fraction. The 192K protein was detected in the membrane-associated fraction by immunoblotting and was not released by treatment with 1 M NaCl, indicating that it is bound with relatively high affinity to cellular membranes (Fig. 4).

Location of the gene 71 polypeptide within the virus particle

EHV-1 virus preparations consist of virions and L particles, which are essentially envelope–tegument structures (Szilágyi & Cunningham, 1991; McLauchlan & Rixon, 1992). The gene 71-encoded 192K protein was found in both L particles and virions, indicating that it is a component of the envelope or tegument (Fig. 5a). To distinguish between these two possibilities, purified virions were labelled in vitro with UDP-[14C]galactose by enzymatic labelling, to identify exposed carbohydrate residues on the virion envelope surface. Labelled proteins were analysed by SDS-PAGE (Fig. 5b). The position of the gene 71 polypeptide was determined by alignment with the 192K polypeptide recognized by anti-71. From these experiments we concluded that the protein product of gene 71 is a component of EHV-1 virion envelopes.

Post-translational modification of the gene 71-encoded polypeptide

The gene 71-encoded polypeptide contains a single putative N-linked glycosylation site and an extensive region encoding a high proportion of serine and threonine residues, which is likely to be heavily O-glycosylated (Telford et al., 1992). To determine whether the gene 71 polypeptide is glycosylated, extracts from cells infected with EHV-1 Ab4 in the presence of [35S]methionine, [3H]mannose and [14C]glucosamine were immunoprecipitated with anti-71 serum. The gene 71 polypeptide was labelled with [35S]methionine (Fig. 6a) and [14C]glucosamine (Fig. 6b) but not by [3H]mannose (data not shown). To determine whether the gene 71 protein is modified by O-linked or N-linked glycosylation, extracts from cells infected with EHV-1 Ab4 in the presence of tunicamycin or monensin were analysed by immunoblotting with anti-71 serum (Fig. 6c). The apparent M, of the gene 71 polypeptide was reduced by about 9K following treatment with tunicamycin. Treatment with monensin resulted in disappearance of the high 192K M, form of the gene 71 polypeptide and an increase in the amount of an 80K M, protein band, which is the predicted primary product. Purified EHV-1 was also digested with glycanase enzymes and analysed by SDS-PAGE followed by Western blotting (Fig. 6d).
The apparent \( M_r \) of the gene 71 polypeptide was only slightly changed by treatment with \( N \)-glycanase. Treatment with neuraminidase reduced the amount of the 192K form of the gene 71 polypeptide and produced a species with a 80K \( M_r \). The 192K \( M_r \) form also disappeared and the band of 80K appeared following treatment with exoglycanases. These results revealed that the gene 71-encoded polypeptide is a heavily O-linked glycoprotein with only a small amount of N-linked glycosylation.

**Immunofluorescence staining pattern of the gene 71 protein product on the surface of EHV-1-infected cells**

The location of the gene 71 protein in EHV-1-infected BHK-21 (C13) cells was examined at 20 h.p.i. by immunofluorescence using anti-71 (data not shown). The product of gene 71 was detected specifically on the surface of EHV-1-infected BHK-21 (C13) cells.

**The relationship between the gene 71 protein product and gp300 of EHV-1**

It became apparent during the course of this study that the high \( M_r \) glycoprotein encoded by gene 71 of EHV-1 Ab4 was very similar in size and other characteristics to gp300, a polypeptide previously identified as the product of EHV-1 gene 28 (Allen & Yeargan, 1987; Whittaker et al., 1990, 1992).

To resolve this discrepancy we constructed a revertant, designated Re71, in which the deletion of gene 71 in ED71 was restored. The restriction enzyme profile of Re71 was identical to that of wild-type EHV-1 (data not shown). The growth impairment shown by ED71 (Sun & Brown, 1994) had been corrected in Re71, indicating that this was due to the absence of the gene 71 polypeptide.
The protein of EHV-1 gene 71

Fig. 7. The relationship between the gene 71 product and gp300. Extracts from mock-infected cells (lanes 3 and 9) or cells infected with mutant ED71 (lanes 4 and 10), revertant of Re71 (lanes 1 and 7), EHV-1 Ab4 (lanes 2, 6, 8 and 12) or EHV-1 Ab4 in presence of 1 μg/ml tunicamycin (lanes 5 and 11) were analysed by 8% SDS-PAGE cross-linked with bis-acrylamide (a) or DATD (b). The gels were probed with either anti-71 serum or P19 MAb. M, values are shown on the left. The gene 71 polypeptides (g71) and 116K β-galactosidase (β-gal) bands are indicated.

It is therefore clear that the protein designated gp300 recognized by MAB P19 is the same as the product of gene 71 identified by the antiserum we have raised against a gene 71 fusion protein. Thus we conclude that gp300 is the product of EHV-1 gene 71 and not of gene 28 as previously published.

Discussion

EHV-1 gene 71 is located in the short unique region of the genome and is predicted to encode a 797 amino acid polypeptide with a M, of 80.1K (Telford et al., 1992). The putative polypeptide of gene 71 possesses hydrophobic amino- and carboxy-terminals which may serve as a potential signal sequence and trans-membrane domain respectively (Fig. 1). The observations presented in this paper are consistent with the sequence analysis. Similar sequence features have been found in HSV-1 gC and HSV-2 gG (Serafini-Cessi et al., 1985) which are heavily O-linked and have an extended region containing a high proportion of serine and threonine residues. These features suggest that the gene 71 product is a class 1 membrane glycoprotein with heavy O-linked and limited N-linked glycosylation, which is likely to result in very slow electrophoretic mobility in SDS-PAGE.

We have demonstrated that gene 71 encodes a 192K product (data not shown). We then compared the polypeptide product of gene 71 with gp300 using EHV-1, ED71, Re71 and two antibodies, anti-71 and MAB P19, which had previously been used to characterize gp300 (Whittaker et al., 1990; Fig. 7). Extracts from cells infected with EHV-1 (lanes 2, 6, 8 and 12), ED71 (lanes 4 and 10), Re71 (lanes 1 and 7) or mock-infected (lanes 3 and 9), and EHV-1 in the presence of tunicamycin (lanes 5 and 11) were analysed by 8% SDS–PAGE cross-linked with either bis-acrylamide (Fig. 7a) or N,N'-diallyltartardiamide (DATD; Fig. 7b). Western blotting was carried out using either anti-71 (lanes 7 to 12) or MAB P19 (lanes 1 to 6). The polypeptide recognized following EHV-1 infection by both anti-71 and MAB P19 had the same electrophoretic mobility (192K). In addition both antibodies detected the same M, shift following treatment with tunicamycin. Neither antibody detected the 192K polypeptide in cells infected with ED71 (although anti-71 did recognize the 116K β-galactosidase polypeptide). The same polypeptide with a M, of 192K was detected with the two antibodies in the cells infected with Re71. Identical results were obtained under both gel conditions (bis-acrylamide and DATD).

It is therefore clear that the protein designated gp300 recognized by MAB P19 is the same as the product of gene 71 identified by the antiserum we have raised against a gene 71 fusion protein. Thus we conclude that gp300 is the product of EHV-1 gene 71 and not of gene 28 as previously published.
polypeptide in EHV-1 Ab4-infected cells, a $M_r$ much greater than the putative $M_r$ of the primary product predicted from the sequence. Post-translational modification experiments confirmed that the gene 71 polypeptide is heavily $O$-glycosylated and that this is primarily responsible for the large difference from its predicted electrophoretic mobility. Following treatment with monensin, neuraminidase or exoglycanases, the apparent $M_r$ of the gene 71 polypeptide was reduced to 80K, which is the same as that predicted from the amino acid sequence. This 80K species was also found in untreated, infected cell extracts, suggesting that it is the primary product of gene 71 in EHV-1-infected cells. In analysing N-glycosylation modification of the gene 71 product, it was shown that (i) the gene 71 polypeptide was apparently not labelled by $[^{3}H]$_mannose, (ii) the apparent $M_r$ of the gene 71 polypeptide was reduced to 183K when the polypeptide was synthesized in the presence of tunicamycin and (iii) treatment with N-glycosidase only slightly reduced the $M_r$ of the gene 71 polypeptide in virions. These observations indicate that the gene 71 polypeptide is lightly $N$-glycosylated. The cellular fractionation experiments confirmed that the gene 71 polypeptide is strongly associated with membranes, suggesting that it is indeed a membrane-inserted protein. The immunofluorescence and virion surface labelling experiments showed that the gene 71 product is located on the surfaces of virion envelopes and infected cell membranes. It is hoped that, in conjunction with antibodies to specific domains of the protein and by electron microscopy, the precise arrangement of the gene 71 polypeptide on the virion envelope can be revealed. The transcriptional pattern of the genome of EHV-1 is similar to HSV-1 (Cohen et al., 1975; Clements et al., 1977). On comparison with two HSV-1 genes, a true late gene (US11) and an early gene (UL42), we concluded that expression of the gene 71 polypeptide is regulated in a leaky late manner in infected cells.

Among the identified EHV-1 glycoproteins (Abodeely et al., 1971; Kemp et al., 1974; Perdue et al., 1974; Turtinen & Allen, 1982; Meredith et al., 1989), one with a $M_r$ in excess of 200K, designated either gp1, gp2, gp1/2 or gp300, has been identified and subsequently characterized as a heavily $O$-linked glycoprotein with a $M_r$ estimated as 400K by SDS-PAGE cross-linked with DATD (Whittaker et al., 1990). Recently, gp300 was demonstrated to be encoded by EHV-1 gene 28, using an anti-gp300 MAb, P19, and a fusion protein from part of the gene 28 ORF (Whittaker et al., 1992). During the course of our study it became apparent that gp300 has very similar characteristics to the gene 71 polypeptide. The comparative experimental data using anti-71 and P19 presented here demonstrated that the gene 71 polypeptide and gp300 synthesized in EHV-1 Ab4-infected cells have identical electrophoretic mobilities to each other by SDS-PAGE, cross-linked either with bisacrylamide or DATD in the presence or absence of tunicamycin. Also, gp300 was absent in cells infected with the mutant ED71 in which the gene 71 ORF was deleted, but was detected in cells infected with the revertant Re71 in which the gene 71 ORF had been restored. The result with Re71 ruled out the possibility of a secondary mutation in ED71 gene 28, which could have disrupted the gene. We concluded therefore that gp300 is in fact encoded by EHV-1 gene 71 and not by gene 28 as previously determined. The sequence of EHV-1 gene 28, unlike that of gene 71 (Telford et al., 1992), is not typical of a membrane protein; there is no apparent signal sequence (McGeoch, 1985) and there is only one region (amino acids 130 to 148) of sufficient length and hydrophobicity to be membrane spanning (Kyte & Doolittle, 1982). Furthermore, unlike gene 71 there is no region rich in serine or threonine residues to suggest heavy $O$-glycosylation.

The precise stage(s) of the virus life cycle at which the gene 71 polypeptide plays a role is uncertain. Some reports about the possible functions of $O$-linked oligosaccharides suggest that they could play a role in polypeptides acquiring their final conformation (Gibson et al., 1980), in increasing hydration of cells, in protecting virus from degradation or in transport of viral glycoproteins or virions to the cell surface (Johnson & Spear, 1983). It has been suggested that if the serine- and threonine-rich region of gene 71 is heavily $O$-glycosylated, this region will form a highly extended semi-flexible rod of 70 nm in length on the surface of the virus particle (McGeoch et al., 1993). The data using monensin presented in this paper confirms that gene 71 is heavily $O$-glycosylated and would therefore support this prediction. Interestingly, despite being released from infected BHK-21 (C13) cells, mutant ED71 virions could not be banded on 5 to 15% Ficoll gradients (data not shown), suggesting that virions lacking gene 71 may band aberrantly.

We have previously demonstrated that the gene 71 product is dispensable for growth in tissue culture. The polypeptide gp300 has been reported to be involved in cell–cell fusion. A MAb, G12, against gp300 was found to inhibit cell–cell fusion of EHV-1-infected cells (Whittaker et al., 1992). In agreement with this observation, ED71 causes small plaques in infected cells. Observations in vivo have shown that the mutant ED71 grows poorly in the lungs of mice and is less virulent than wild-type virus and other EHV-1 lacZ insertion mutants (K. Marshall and H. J. Field, personal communication).

We thank Professor John H. Subak-Sharpe for his interest in this project, Dr Liz Telford for providing virus and making available...
unpublished data and Dr David M. Meredith for his co-operation and for providing MAb P19. We are also grateful to Dr Christine MacLean, Dr David Stevenson and Mr David Miller for advice and technical help and Drs Andrew Davison, Amina Fu, June Harland and Duncan McGeoch for their collaboration and critical reading of this manuscript. This work was funded by the Equine Virus Research Foundation.

References


COLLE, C. F., III, FLOWERS, C. C. & O’CALLAGHAN, D. J. (1992). Open reading frames encoding a protein kinase, homologue of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the reading frames encoding a protein kinase, homologue of glycoprotein gX of pseudorabies virus, and a novel glycoprotein map within the transcription groups and recombination analysis leading to a linkage map. *Journal of General Virology* **63**, 269–274.


(Received 28 February 1994; Accepted 13 June 1994)