Characterization of the herpes simplex virus type 1 strain 17+ neurovirulence gene RL1 and its expression in a bacterial system

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The DNA sequence of herpes simplex virus type 1 (HSV-1) strain 17+ in the region coding for the polypeptide ICP34.5 predicts a protein of 248 amino acids with a proposed M, of 26 158. The entire RL1 open reading frame was cloned into the expression vector pET8c to enable over-expression of ICP34.5 in Escherichia coli. The expressed protein was partially purified and used as an immunogen to produce a polyclonal antiserum in rabbits. Construction of an ICP34.5 null mutant (1771), demonstrated that the predicted open reading frame for ICP34.5 in strain 17+ is correct and confirmed that HSV-1 strain 17+ ICP34.5 specifically determines neurovirulence. The specificity of the antiserum directed against the E. coli-expressed ICP34.5 was defined by Western blotting of wild-type and RL1-negative infected cell extracts.

Introduction

Herpes simplex virus types 1 (HSV-1) and 2 are important human pathogens which cause a variety of diseases ranging from benign superficial cutaneous lesions to life-threatening encephalitis (Whitley, 1985). Identification of the viral genes that control pathogenic properties and elucidation of their functions are of fundamental importance if we are to understand the basic mechanisms of these diseases.

Any alteration in a virus gene that impairs replication in vitro will also affect the performance of the virus in vivo and therefore all essential genes could be considered as 'virulence' genes (Darby et al., 1984; Larder et al., 1986). In HSV-1 it has been clearly demonstrated that there are several genes whose expression is not required for multiplication in dividing cells in tissue culture, presumably because a cellular homologue can compensate for the HSV gene. However, these genes are required for maximum demonstration of neurovirulence in various animal models and can thus be regarded as neurovirulence genes. These include virus-encoded enzymes involved in DNA metabolism such as thymidine kinase (Tenser et al., 1981; Ben-Hur et al., 1983; Brandt et al., 1991), ribonucleotide reductase (Cameron et al., 1988) and dUTPase (Thompson & Wagner, 1988; Pyles et al., 1992).

Other genes have been identified that play a role in viral neuroinvasiveness [as defined by the ability of the virus to spread to the central nervous system (CNS) following peripheral inoculation]. A single base change in either the glycoprotein gD (Izumi & Stevens, 1990) or glycoprotein gB (Yuhaz & Stevens, 1993) open reading frame (ORF) does not have any effect on virus growth in vitro but is sufficient to produce a non-neuroinvasive phenotype in vivo. gB and gD have roles in virus adsorption and penetration respectively (Fuller & Spear, 1987; Cai et al., 1988), and the decrease in neuroinvasiveness associated with these amino acid substitutions is probably due to a decreased ability of the mutant glycoproteins to bind to their cellular receptors.

The existence of a neurovirulence locus in the long repeat region of the HSV-1 genome is well documented, and this region has been shown to contain a gene, RL1, in HSV-1 strain 17+ (Dolan et al., 1992). HSV-1 strain F (Chou & Roizman, 1986) and HSV-2 strain HG52 (McGeoch et al., 1991). RL1 codes for the protein ICP34.5 (Ackermann et al., 1986; Dolan et al., 1992). In the mouse model, variants with deletions in RL1 are non-neurovirulent being unable to replicate in the CNS or cause encephalitis (Thompson et al., 1983; Taha et al., 1989a, b; Chou et al., 1990; MacLean et al., 1991). However they retain their wild-type phenotype under standard tissue culture conditions.

An HSV-1 strain 17+ RL1 deletion variant (1716) with a 759 bp deletion which removes one 18-bp DR1 element of the 'a' sequence and extends to the 3' end of RL1 has been isolated and characterized (MacLean et al., 1991). The LD50 of 1716 is 7 x 10⁶ p.f.u./mouse compared with an LD50 of < 10 p.f.u./mouse for the wild-type HSV-1 strain 17+ following intracerebral inoculation of mice. The non-neurovirulent phenotype of 1716 is attributed to its inability to replicate in neurons of the CNS. The deletion in 1716 removes most of RL1 including the
incorporated into the 92 bp inserted oligonucleotide was provided by blunt-ended, cloned into M13 and sequenced using a Sequenase version sequence analysis. A 760 bp fragment from the unique BstEII site in the 2.0 kit (Amersham). Following electrophoresis, gels were dried and in the correct position in pEA.10 and that no aberrant bases had been plasmid was designated pEA.10.

Methods

Cells. Baby hamster kidney 21 clone 13 (BHK21/C13) cells were used throughout this study (Macpherson & Stoker, 1962). They were grown in Eagle's medium (Gibco), supplemented with 10% newborn calf serum (Gibco) and 10% (v/v) tryptose phosphate broth.

Virus. The wild-type parental virus used in this study was HSV-1 strain 17+, which was grown and titrated on BHK21/C13 cells as previously described (Brown et al., 1973). The HSV-1 strain 17+ RLI deletion variant 1716 has been described by MacLean et al. (1991).

Construction of the plasmid pEA.10. To produce the recombinant plasmid pGEM34.5, a 1.46 kb Abl/AseI restriction enzyme subfragment of HSV-1 BamHI k, (nucleotides 125074 to 126530; Perry & McGeoch, 1988) which contains the entire RLI ORF was cloned into the Smal site of pGEM-3Zf(-) using standard procedures (Manatis et al., 1982). The RLI gene contains a unique Ncol site at the initiating ATG and approximately 96 bp downstream from this there is a unique BsrEI site. pGEM34.5 was digested with these enzymes and a double-stranded oligonucleotide was inserted. This oligonucleotide was identical to the RLI sequence except that it contained a 6 bp insert which introduced an in-frame stop codon into the ICP34.5 coding sequence and had an Abl restriction enzyme site so that recombinant viruses and plasmids could be easily identified. This recombinant plasmid was designated pEA.10.

Sequence analysis of pEA.10. Confirmation that the stop codon was in the correct position in pEA.10 and that no aberrant bases had been incorporated into the 92 bp inserted oligonucleotide was provided by sequence analysis. A 760 bp fragment from the unique BsrEI site in the RLI gene to an EcoR1 site in the polylinker of pGEM-3Zf(-) was blunt-ended, cloned into M13 and sequenced using a Sequenase version 2.0 kit (Amersham). Following electrophoresis, gels were dried and exposed to Kodak X-1 film.

Construction of an ICP34.5 null mutant. BHK21/C13 cells were cotransfected with HSV-1 strain 17+ DNA and various amounts of the linearized plasmid pEA.10 as previously described (Stow & Wilkie, 1976). When c.p.e. was widespread, the cells were scraped into the medium, sonicated and released virus was plated onto semi-confluent monolayers of BHK21/C13 cells. Following 2 days incubation at 37°C, single plaques were individually isolated and plaque-purified. Stocks were grown and their genomes analysed by Southern blotting using the wild-type strain 17+ BamHI k restriction enzyme fragment as a hybridization probe (Southern, 1975).

A wild-type recombinant was constructed in a similar way. Variant 1771 DNA was cotransfected onto BHK21/C13 cells with the HSV-1 strain 17+ BamHI k restriction enzyme fragment and viruses with a wild-type profile were isolated and purified.

Neurovirulence studies. The neurovirulence of 1771 was determined by estimation of its LD50 value (Reed & Muench, 1938) compared with those of the wild-type parental strain 17+ and the ICP34.5 deletion variant 1716, following intracerebral inoculation of BALB/c mice. Twenty-five μl aliquots of 17+, 1771 and 1716 were separately inoculated into the left cerebral hemisphere of 3-week-old mice. Mice were monitored for up to 21 days post-infection using a scoring system (Morton & Griffiths, 1985) and were then humanely destroyed if the score was above a defined threshold.

In vivo growth curves. At various times post-inoculation with wild-type (105 p.f.u./mouse) or mutant virus (106 p.f.u./mouse), mice were killed and their brains were removed and homogenized in 1 ml PBS/calf serum. The volume of each was recorded before storing at 1 ml at –70°C. When samples at all time points had been collected, the tissue suspension was sonicated and the viruses that were released were applied to monolayers of BHK21/C13 cells. Virus titres were calculated as p.f.u./animal. The virus from each brain was titrated separately and the titre at each time point was calculated as the mean of values from two animals.

Virus growth properties in vitro. Semi-confluent monolayers of BHK21/C13 cells were infected at a multiplicity of 10 p.f.u./cell or 04001 p.f.u./cell for one-cycle and multi-cycle growth experiments, respectively. At various times post-infection (p.i.), the cells were harvested and stored at –70°C. When all samples had been collected, the progeny virus titres were calculated by plaque assay on BHK21/C13 cells at 37°C.

Construction of the expression plasmid pET34.5. The intact ICP34.5 ORF from HSV-1 strain 17+ was cloned into pET8c. This plasmid contains the T7 gene promoter including the Shine-Delgarno sequence and initiating ATG (Studier et al., 1990). The starting plasmid was pGEM34.5. Following digestion with NcoI at the initiating ATG and BamHI from the pGEM-3Zf(-) polylinker, the entire ORF of ICP34.5 was released from the initiation codon to beyond the stop codon of the gene. This fragment was inserted into pET8c, which also contains the T7 RNA polymerase gene, which was under the control of the inducible lac UV5 promoter, and for each experiment single colonies were used to inoculate larger cultures.

Expression of ICP34.5. A 500 ml overnight culture was diluted 20-fold into ten 1 litre cultures which were incubated at 37°C with shaking. On reaching an optical density of 0.5 at 600 nm, the temperature of the cultures was rapidly decreased to 28°C. T7 RNA polymerase expression was induced by the addition of IPTG, followed by incubation of the culture for 1 h at 28°C. Cells from 10 litres of culture were collected by centrifugation at 5000 r.p.m. for 10 min in a GS3 rotor and resuspended in 100 ml of 50 mM HEPES pH 7.6, 50 mM NaCl, 2 mM DTT.

Preparation of crude bacterial extracts. Bacteria were lysed by addition of lysozyme to a final concentration of 500 μg/ml, followed by
incubation on ice for 30 min. Cell debris was pelleted by centrifugation at 15,000 rpm for 30 min at 4 °C and the supernatant containing the expressed protein was stored at −70 °C.

Partial purification of ICP34.5. To determine ICP34.5 solubility over a range of ammonium sulphate concentrations, increasing amounts of a saturated solution of ammonium sulphate were added to 100 μl aliquots of crude extract. After being kept on ice for 10 min., samples were clarified by centrifugation at 13,000 g for 30 min in a microfuge at 4 °C. Pellets were redissolved in 300 μl of 50 mM-HEPES pH 7.6, 50 mM-NaCl and supernatants were standardized to a volume of 300 μl using the same buffer. Pellet and supernatant fractions were analysed by SDS-PAGE. For large-scale purification of ICP34.5, the volume of saturated ammonium sulphate solution was adjusted accordingly.

The salt-fractionated material was resuspended in 50 mM-HEPES pH 7.6, 50 mM-NaCl and applied onto a FPLC Mono-Q HR 5/5 (1 ml) anion-exchange column, which had been equilibrated with the same buffer. At this pH the expressed protein did not bind to the column, whereas the vast majority of the E. coli proteins did, so the flowthrough was collected and stored at −70 °C and the column was thoroughly washed with buffer B (50 mM-HEPES pH 7.6, 1 M-NaCl) to remove the bound E. coli proteins, which were subsequently discarded.

SDS-PAGE. SDS-PAGE of proteins was performed using 10% single-concentration gels cross-linked with 2.5% (w/w) N,N'-methylene-bis-acrylamide.

Production of polyclonal ICP34.5 specific antisera. Rabbit antisera to ICP34.5 were produced using bacterially expressed ICP34.5 as the antigen. New Zealand White rabbits were immunized intramuscularly with 2 mg total protein (approximately 100 μg ICP34.5) in Freund's complete adjuvant followed by four boosts, each at a 14-day interval, using the same amount of antigen but in Freund's incomplete adjuvant.

Western blotting. Extracts from mock- and HSV-1-infected cells were prepared at 16 h p.i. SDS-polyacrylamide gels were prepared as above and the method used was essentially the one described by Towbin et al. (1979). Detection was by Protein A peroxidase and enhanced chemiluminescence (Amersham). A peptide antiserum designated 78, specific to ICP34.5, was produced using bacterially expressed ICP34.5 as the antigen. The neurovirulence of 1771 was examined following intracerebral inoculation of BALB/c mice. Aliquots (25 μl) of 17 +, 1771 and 1716 were inoculated into the left cerebral hemisphere of 3-week-old mice and deaths were scored up to 21 days p.i. HSV-1 strain 17 + was inoculated at doses of 10^4 and 10^5 p.f.u./mouse. At the lower dose of 10^5 p.f.u./mouse, HSV-1 strain 17 + killed half of the mice and thus is likely to have an LD50 value of 7 p.f.u./mouse. The ICP34.5 deletion variant 1716, inoculated at doses of 10^5 and 10^6 p.f.u./mouse, showed no deaths indicating probable LD50 values of > 10^6 p.f.u./mouse. The recombinant virus 1771 was inoculated at doses of 10^5 and 10^6. As with 1716 no animals died at either dose, again giving a probable LD50 value of > 10^6 p.f.u./mouse.

Neurovirulence of 1771 in BALB/c mice

The neurovirulence of 1771 was examined following intracerebral inoculation of BALB/c mice. Aliquots (25 μl) of 17 +, 1771 and 1716 were inoculated into the left cerebral hemisphere of 3-week-old mice and deaths were scored up to 21 days p.i. HSV-1 strain 17 + was inoculated at doses of 10^4 and 10^5 p.f.u./mouse. At the lower dose of 10^5 p.f.u./mouse, HSV-1 strain 17 + killed half of the mice and thus is likely to have an LD50 value of 7 p.f.u./mouse. The ICP34.5 deletion variant 1716, inoculated at doses of 10^5 and 10^6 p.f.u., caused no deaths indicating probable LD50 values of > 10^6 p.f.u./animal. The recombinant virus 1771 was inoculated at doses of 10^5 and 10^6. As with 1716 no animals died at either dose, again giving a probable LD50 value of > 10^6 p.f.u./animal.

Results

Isolation of the variant 1771

A plasmid (pEA.10) was constructed containing an in-frame stop codon in the RL1 ORF 9 bp downstream of the initiating ATG. The insert contained an XbaI site to facilitate identification of recombinant viruses and plasmids. pEA.10 was linearized and cotransfected with intact HSV-1 strain 17 + DNA. The DNA profile of single plaque isolates was analysed by Southern blotting and restriction endonuclease digestion to identify recombinant viruses.

Digestion of the HSV-1 strain 17 + genome with BamHI/XbaI and hybridization with the BamHI L–S junction fragment k leads to detection of the L terminal fragment s, the S terminal fragment q and the L–S junction fragment k.

Since RL1 is a diploid gene, any recombinants that contain the stop codon close to the initiating ATG of RL1 will have two additional XbaI sites, one in the terminal long repeat region of the genome and the other in the internal long repeat region. On digestion of the DNA from such a variant with BamHI/XbaI, BamHI s is cut to give two fragments, one of 511 bp which cannot be detected and one that is equivalent to BamHI s minus 511 bp and runs slightly below where s normally migrates. Similarly BamHI k is cleaved into two fragments, one that is equivalent to BamHI s minus 511 bp and the other that corresponds to BamHI q + 511 bp (Fig. 1). BamHI q is unaltered. A variant (1771) with this genome profile was isolated and plaque-purified a further three times before preparation of a virus stock.

Neurovirulence of 1771 in BALB/c mice

The neurovirulence of 1771 was examined following intracerebral inoculation of BALB/c mice. Aliquots (25 μl) of 17 +, 1771 and 1716 were inoculated into the left cerebral hemisphere of 3-week-old mice and deaths were scored up to 21 days p.i. HSV-1 strain 17 + was inoculated at doses of 10^4 and 10^5 p.f.u./mouse. At the lower dose of 10^5 p.f.u./mouse, HSV-1 strain 17 + killed half of the mice and thus is likely to have an LD50 value of 7 p.f.u./mouse. The ICP34.5 deletion variant 1716, inoculated at doses of 10^5 and 10^6, caused no deaths indicating probable LD50 values of > 10^6 p.f.u./animal. The recombinant virus 1771 was inoculated at doses of 10^5 and 10^6. As with 1716 no animals died at either dose, again giving a probable LD50 value of > 10^6 p.f.u./animal.

Growth of 1771 in vitro and in vivo

To determine the in vitro growth pattern of 1771, single-cycle and multi-cycle growth experiments were carried out on BHK21/C13 cells. Strain 17 + and the variant 1771 grew equally well, giving equivalent final yields (results not shown) indicating that 1771 is not impaired for growth in BHK21/C13 cells.
For *in vivo* studies, aliquots (25 μl) of HSV-1 strains 17+ (10^2 p.f.u./animal) and 1771 (10^5 p.f.u./animal) were injected into the left cerebral hemisphere of 3-week-old BALB/c mice. At various times p.i., (0, 1, 2, 3, 4 and 5 days), two mice per virus were killed, their brains were homogenized and the progeny virus titre was calculated using plaque titration on BHK21/C13 cells. Only one mouse inoculated with HSV-1 strain 17+ survived until day 5, thus this time point is represented by a single mouse. The final titres (Fig. 2) were calculated as p.f.u./animal. The parental strain 17+ titre rose rapidly following intracerebral inoculation, reaching a titre of 1 × 10^6 p.f.u./animal by days 4 to 5. In contrast, no replication was apparent in 1771-inoculated animals and the input virus was rapidly cleared, until by day 3 p.i. there was no detectable virus (< 10 p.f.u./animal).

**Induction of ICP34.5 protein synthesis using IPTG**

The RL1 gene from HSV-1 strain 17+ was cloned into the pET8c expression vector and was expressed under the control of IPTG-induced T7 RNA polymerase. Induction was carried out at 28 °C since at this temperature the expressed protein was produced in a soluble form whereas at higher growth temperatures (31 °C or 37 °C) the protein was insoluble. In an attempt to optimize ICP34.5 production, cultures were induced with various concentrations of IPTG. ICP34.5 production was assayed by running proteins from cells extracted at various times post-induction on SDS-polyacrylamide gels and Western blotting using antiserum 78, at a 1:50 dilution (Fig. 3). The antibody detected a 37K band. The specificity of this antibody reaction was demonstrated by its inhibition, following preincubation of the antiserum with the relevant peptide. Maximum protein production occurred 1 h post-induction with 0.05 to 0.2 mM-IPTG. It appears that the expressed protein is relatively non-toxic to the cells since ICP34.5 is produced even in the absence of IPTG. Several lower Mᵣ bands appeared to be associated with ICP34.5 production and one with an apparent Mᵣ 25K to 28K was reactive with peptide antiserum 78 and appeared to co-purify with the larger protein.
**HSV neurovirulence gene characterization**

![Fig. 3](image_url)

**Fig. 3. ICP34.5 expression with various concentrations of IPTG.** The level of expression of ICP34.5 was examined using various concentrations of IPTG: (a) without IPTG; (b) 0.2 mM-IPTG; (c) 0.4 mM-IPTG. Western blotting with the peptide antiserum at a dilution of 1/50 was used to assay ICP34.5 expression. *M*<sub>r</sub> markers are on the left-hand side. Times p.i. (h) are indicated above each lane.

**Partial purification of extracts containing ICP34.5**

The initial step in purification of bacterially expressed ICP34.5 was ammonium sulphate precipitation. ICP34.5 started to precipitate at an ammonium sulphate concentration of approximately 10%, with increasing amounts precipitating up to 40% salt (results not shown). To ensure that no ICP34.5 was retained in the supernatant fraction, 50% ammonium sulphate was used for routine purification. Following precipitation on ice for 30 min, the protein was collected using centrifugation, resuspended in 100 ml 50 mM-HEPES pH 7.6, 50 mM-NaCl and applied directly to a Mono-Q column which had been equilibrated with the same buffer (results not shown). The flowthrough which contained the *E. coli*-expressed ICP34.5 was collected. Attempts to purify the protein further were unsuccessful and generally resulted in the recovery of negligible amounts of protein. The flowthrough from the Mono-Q was concentrated and used directly for immunization of rabbits. Animals were bled 10 days after each boost and the antisera were tested at different dilutions against HSV-1 strain F- and HSV-1 strain 17+-infected cell extracts for reaction with ICP34.5-specific bands. After five injections only one rabbit had produced an immune response, and the titre appeared to have reached its maximum. This animal was therefore bled and its serum was used as the polyclonal anti-ICP34.5 serum.

**Detection of ICP34.5 using polyclonal antiserum**

Protein extracts were prepared 16 h p.i. from three separate HSV-1 strain 17<sup>+</sup> and 1771 plaque isolate infections, separated by 10% SDS–PAGE and analysed by Western blotting using the rabbit polyclonal antiserum at a 1:20 dilution (Fig. 4). No band corresponding to the expected *M*<sub>r</sub> of ICP34.5 was observed in 1771-infected cell extracts, whereas in HSV-1 strain 17<sup>+</sup>-infected cells extracts, a 37K band corresponding to the expected size of ICP34.5 was detected.

It has previously been shown that strain 17<sup>+</sup> produces a smaller protein than strain F (MacKay et al., 1993) and that this difference is likely to be due to a variation in the number of copies of a PAT repeat sequence found 10 times in the HSV-1 strain F sequence, but only five times in the HSV-1 strain 17<sup>+</sup> sequence. The peptide antiserum, 78, was approximately 30-fold less efficient at detecting the HSV-1 strain 17<sup>+</sup> protein than it was at detecting the HSV-1 strain F protein. This could be because of one (or both) of the following reasons: either in HSV-1 strain 17<sup>+</sup>-infected cells there were lower levels of ICP34.5 compared with those in HSV-1 strain F-infected cells or, because the peptide antiserum was raised against 10 copies of the PAT repeat, the difference could simply be due to antibody affinity differences. By using the rabbit polyclonal antiserum, HSV-1 strain 17<sup>+</sup> ICP34.5 was detected at levels comparable to the HSV-1 strain F.
polypeptide (data not shown) indicating that the previous differences in detection had been due to antibody affinity differences and that the protein accumulated to equivalent levels in cells infected with either strain.

**Immunofluorescence studies**

Ackermann et al. (1986) and MacKay et al. (1993) using cell fractionation studies reported the accumulation of ICP34.5 largely in the cytoplasm of Hep-2 and BHK21/C13 cells, respectively. The availability of the ICP34.5 polyclonal antiserum allowed localization to be studied using immunofluorescence. When cells were infected with wild-type strain 17+ (Fig. 5a), dense punctate patches of fluorescence could be seen in the cell cytoplasm, with minimal signs of perinuclear staining. Cells infected with the mutant 1771 (Fig. 5b), or mock-infected (Fig. 5c) showed a general low level of background fluorescence, with no intense patches of

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![Fig. 4](image_url)  
**Fig. 4.** Western blot analysis of 1771. Detection of ICP34.5 in variant 1771 (a) and strain 17+ (b) by Western blot analysis, using the protein antiserum at a 1/20 dilution. \( M_r \) markers are shown on the right-hand side. ICP34.5-specific bands are indicated by an arrow.

![Fig. 5](image_url)  
**Fig. 5.** Localization of ICP34.5 in the cytoplasm of HSV-1-infected cells using the rabbit protein antiserum. Immunofluorescence of HSV-1 strain 17+ (a), variant 1771 (b) or mock- (c) infected cells incubated with the ICP34.5 rabbit protein antiserum at a 1/50 dilution. Anti-rabbit IgG conjugated to fluorescein isothiocyanate was used as the second antibody.
staining as were observed with the wild-type virus-infected cells. These results are consistent with those obtained in previous cell fractionation studies and suggest localization of the protein in specific cytoplasmic organelles e.g. the Golgi apparatus or ribosomes.

Discussion

The work presented in this paper was conducted (i) to confirm that ICP34.5, the product of the HSV RL1 gene, confers neurovirulence to the virus independently of any other genes or transcripts encoded in the repeat regions of the genome, (ii) to produce a high titre ICP34.5 antiserum and (iii) to determine whether the apparent differences in the levels of ICP34.5 accumulation in HSV-1 strain F- and HSV-1 strain 17+infected cells were real or merely due to antibody affinity differences.

Deletions in the long repeat region of the HSV-1 genome that remove the RL1 gene (Thompson et al., 1983, 1989; Taha et al., 1989a, b; Chou et al., 1990; MacLean et al., 1991) abolish the neurovirulence phenotype of HSV and prevent it from replicating in the CNS of mice. RL1 encodes the polypeptide ICP34.5 (Ackermann et al., 1986; Chou & Roizman, 1990; McGeoch et al., 1991; Dolan et al., 1992). Most of the existing mutants in ICP34.5 have deletions which affect additional sequences in the repeat region of the HSV-1 genome with the exception of one mutant in which a stop codon in all six frames was inserted near the amino terminus of ICP34.5 of HSV-1 strain F (Chou et al., 1990). However, this mutation could also affect overlapping reading frames. To demonstrate conclusively that lack of ICP34.5 expression alone leads to a non-neurovirulent phenotype we have constructed an HSV-1 strain 17+ RL1-negative variant, 1771, which has a stop codon functional only in the assigned ICP34.5 reading frame 9 bp downstream from the initiating ATG. The neurovirulence phenotype was compared with those of wild-type HSV-1 strain 17+ and the previously characterized non-neurovirulent variant 1716, which has a 759 bp deletion removing RL1. Variant 1771 has a probable LD90 value of >106 p.f.u./mouse, a value comparable to that of 1716 and fails to grow in the mouse CNS, confirming that abolition of ICP34.5 expression alone abolishes neurovirulence.

As found with variant 1716 (MacLean et al., 1991), variant 1771 is not impaired in growth in vitro in BHK21/C13 cells and this is a consistent finding in our laboratory for all RL1 mutants. However, in mouse 3T6 cells, RL1 variants mimic the in vivo phenotype and fail to replicate, whereas the wild-type virus is not impaired in replication (S. M. Brown, unpublished results). As both 1716 and 1771 replicate efficiently in undifferentiated F9 cells (a mouse embryo testicular carcinoma cell line) (S. M. Brown, unpublished results) and 1716 can replicate at the periphery following footpad inoculation (Robertson et al., 1992) this would indicate that the replication defect observed in variants that fail to produce ICP34.5 is not host-specific, but is tissue- and/or cell type-specific. Moreover the ability of 1716 to establish a latent infection in dorsal root ganglia demonstrates that it can at least enter neurons.

In a previous attempt to detect ICP34.5 in HSV-1 strain 17+infected cells, antisera were raised against peptides from eight different regions of the HSV-1 strain 17+ RL1 predicted amino acid sequence. Originally none of these peptide antisera successfully detected ICP34.5 in HSV-1 strain 17+infected cells and only one successfully detected ICP34.5 in strain F-infected cell extracts. After optimization of the assay conditions this antiserum also detects ICP34.5 in HSV-1 strain 17+infected cells, although at a level 30 times lower than that for HSV-1 strain F-infected cells (MacKay et al., 1993). To maximize the ease of detection of ICP34.5 in strain 17+infected cells, it was decided to express the RL1 gene in a bacterial expression system to raise polyclonal anti-ICP34.5 sera directed against the expressed protein. The expression and subsequent purification of the bacterial protein was monitored by using the peptide antiserum which weakly recognized the HSV-1 strain 17+ protein in infected cells. The availability of the polyclonal antiserum raised the possibility of obtaining an antibody that would identify the HSV-2-encoded ICP34.5. Additionally the availability of a semi-pure form of ICP34.5 would facilitate the analysis of protein-protein interactions involving ICP34.5. It was anticipated that the polyclonal antiserum would determine whether the differences in the levels of detection between the HSV-1 strain 17+ and the HSV-1 strain F polypeptides were real, or merely due to antibody affinity differences.

The E. coli pET system (Studier et al., 1990) has previously been used for the high-level expression of HSV-1 R1 (Furlong et al., 1991) and R2 (Lankinen et al., 1991) and was chosen for expression of the entire ORF of ICP34.5. Incubation of cultures at 37 °C following induction resulted in an insoluble form of ICP34.5; however, lowering the growth temperature of the cultures to 28 °C following induction allowed production of soluble E. coli-expressed ICP34.5. Because ICP34.5 was not expressed at high levels and previous studies using the peptide antisera indicated that it might not be highly immunogenic, it was thought necessary to remove as many contaminating proteins from the crude bacterial extract as possible before using it in antiserum production. The first stage in purification was achieved by ammonium sulphate fractionation. Several columns were tested for ICP34.5 binding at various pH ranges and salt concentrations, but in almost every case the protein
failed to bind. When it did bind, the protein tended to elute over almost the entire salt gradient indicating that the E. coli-expressed ICP34.5 was aggregating with other proteins in the sample. Although ICP34.5 did not bind with the Mono-Q column most bacterial bands did and hence the flowthrough contained larger and purer yields of ICP34.5 than the eluate from any other column tested. Because of the small amount of protein finally obtained following Mono-Q purification it was not considered feasible to purify the protein further for use in the production of polyclonal antisera.

Unfortunately the polyclonal antiserum produced did not have as high a sensitivity as might have been predicted and it was actually no more sensitive in detecting the HSV-1 strain F ICP34.5 than the available peptide antiserum. However, the polyclonal antiserum recognized HSV-1 strain F and HSV-1 strain 17+ ICP34.5 with equivalent sensitivity, demonstrating that the previous differential in detection level (MacKay et al., 1993) was due to antibody affinity differences and that the level of ICP34.5 accumulation in cells infected with either strain is in fact the same. The polyclonal antiserum failed to detect HSV-2 strain HG52 ICP34.5 by either immunoprecipitation or Western blotting.

To obtain a higher titre and a more sensitive antiserum will probably require the immunogen to be of higher purity. This may be achieved by the use of alternative expression systems e.g. baculovirus or glutathione S-transferase-fusion might give higher expression of ICP34.5 and higher levels of crude protein for purification. Changing the expression system however will not overcome the problems of protein aggregation, thus alternative 5′ deletions are currently being made in the existing construct in the hope that a smaller truncated form of the protein will be more readily purified. For an antiserum to identify the HSV-2 strain HG52 ICP34.5, it will be necessary to conserve the 63 amino acid region of identity between the HSV-1 strain 17+ and HSV-2 strain HG52 proteins. As ICP34.5 is a small protein (248 amino acids in strain 17+) this obviously limits the alterations which can be made and it may be necessary also to express the HG52 polypeptide. It is hoped that eventually ICP34.5 will be purified to homogeneity, for use in monoclonal antibody production. In conjunction with further defined lesions in the RL1 ORF these monoclonal antibodies will be used to determine the functional regions of ICP34.5.

E. McKie is the recipient of an MRC studentship.

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