Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo

Christine A. MacLean,* Lesley M. Robertson† and Fiona E. Jamieson

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

On the basis of predicted amino acid sequence characteristics, herpes simplex virus type 1 gene UL10 is thought likely to encode a membrane protein with eight potential transmembrane regions. Previously, a protein with an apparent Mr 47000 on SDS-PAGE was identified as a product of this gene. Here we have further characterized this protein, and show that it is modified by N-linked glycosylation, associates with membranes from infected cells, and is a component of the virus particle. It is not essential for virus growth in tissue culture. To investigate its role in vivo a deletion mutant lacking the majority of the UL10 open reading frame was constructed (UL10-del). The in vitro growth properties of this virus were consistent with previous studies; it grew to give slightly lower yields than wild-type and revertant viruses, and had no apparent temperature-sensitive or host range phenotype. In vivo, in a mouse model, UL10-del was capable of establishing a latent infection, although it was impaired for growth at the periphery, and for spread to and/or growth within the nervous system relative to wild-type or revertant viruses.

Introduction

Membrane proteins of enveloped viruses have a number of important functions. They are involved in virus entry into and egress from cells, may mediate transport across virion or infected cell membranes and are often key participants in virus interactions with the host immune system. Herpes simplex virus type 1 (HSV-1) encodes a large number of membrane proteins. Of the 73 distinct gene products so far predicted to be encoded by the HSV-1 genome, 20 contain a possible signal sequence and/or one or more potential transmembrane domains (McGeoch et al., 1985, 1988, 1991; Barker & Roizman, 1992). To date, 10 HSV-1 glycoproteins have been identified, and some of these have been extensively studied (for reviews see Spear, 1985, 1993; Marsden, 1987). However, little is known about the other potential membrane proteins.

The products of four virus genes (UL10, UL20, UL43 and UL53) are predicted to be membrane-inserted proteins with several transmembrane segments (McGeoch et al., 1988). Both the UL20 and UL53 gene products contain four potential transmembrane regions, and the UL53 product also contains a signal sequence which is probably cleaved (Ramaswamy & Holland, 1992). UL53 encodes a glycosylated polypeptide, gK, thought to be essential for virus replication (Hutchinson et al., 1992; Ramaswamy & Holland, 1992). A single amino acid substitution within gK results in extensive cell-to-cell fusion (Debroy et al., 1985; Pogue-Geile & Spear, 1987), suggesting a role in virus penetration, an event involving fusion between the virus and cell membranes. UL20 encodes a membrane-associated protein essential for virus growth in some, but not all, cell types, where it is required for the intracellular transport of the newly enveloped virus particles (Baines et al., 1991).

In contrast, no role has been described for the UL10 or UL43 gene products. Neither of these genes is essential for virus growth in tissue culture, although inactivation of UL10 does impair growth of the virus (Baines & Roizman, 1991; MacLean et al., 1991). We were interested in investigating further the role of UL10. UL10 encodes a polypeptide which is predicted to contain eight potential transmembrane segments, including a signal sequence if the second in-frame initiation codon is used, and two potential sites for N-linked glycosylation. A protein synthesized predominantly late in infection, with an apparent Mr 47000 following SDS-PAGE, has been identified as a product of this gene (MacLean et al., 1991). The existence of corresponding open reading frames (ORFs) in other herpesvirus genomes, including varicella-zoster virus (VZV),

* Present address: Department of Pharmaceutical Science, Royal College, University of Strathclyde, 204 George Street, Glasgow G1 1XW, U.K.

† Present address: Department of Pharmaceutical Science, Royal College, University of Strathclyde, 204 George Street, Glasgow G1 1XW, U.K.
Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), equine herpesvirus type 1 (EHV-1) and herpesvirus saimiri (HVS) (Albrecht et al., 1992; Baer et al., 1984; Davison & Scott, 1986; Lehner et al., 1989; Telford et al., 1992), suggests an important role for UL10 in the natural life cycle of the virus. In this communication, we have further characterized the UL10 polypeptide and investigated the effect of deleting the majority of the UL10 ORF on virus growth in vivo.

Methods

Cells. BHK-21 clone 13 (C13) cells (Macpherson & Stoker, 1962) were grown in Eagle's MEM supplemented with 10% newborn calf serum.

Virus. HSV-1 strain 17syn-7 (Brown et al., 1973) was the wild-type virus strain used in this study. UL10-lacZ contains the Escherichia coli lacZ gene, under the control of the simian virus 40 early promoter, within the UL10 ORF (MacLean et al., 1991). The thymidine kinase-negative virus X4 has been described previously (MacLean & Brown, 1987).

Plasmids. Plasmid construction and propagation were carried out by standard procedures (Maniatis et al., 1982). In the following descriptions HSV-1 DNA sequences are numbered according to McGeoch et al. (1988). Plasmid pC73.1 contains a 3.5 kb BamHI-BglII fragment (residues 21655 to 25153), encompassing the UL10 ORF (residues 23206 to 24627, including the termination codon), within the BamHI site of pUC19. PpuMI-digested pC73.1 was blunt-ended with the Klenow fragment of DNA polymerase, digested with BalI and religated, resulting in a plasmid lacking the HSV DNA sequences from residues 23606 to 24594 inclusive (pc122). To introduce a novel XbaI site within the intragenic region between genes UL10 and UL11, a SalI subfragment from pC73.1, containing HSV-1 sequences from residues 22872 to 25153, was cloned into the SalI site of pGem-1, generating plasmid pB3.1. MluI-digested pB3.1 was blunt-ended with the Klenow fragment and religated in the presence of an 8 bp XbaI linker. This effectively introduced a 12 bp insertion after HSV-1 residue 24771 (plasmid pC343).

Antiserum. The anti-UL10 serum was raised against the carboxy-terminal region of UL10 (amino acids 458 to 467; MacLean et al., 1991).

Construction of deletion and revertant viruses. Transfections were carried out according to the procedure of Graham & van der Eb (1973), as modified by Stow & Wilkie (1976). C13 cells were cotransfected with 1 to 2 μg of virus DNA and various amounts of linearized plasmid DNA, and the progeny virus were harvested and titrated as previously (MacLean & Brown, 1987).

Virus growth in vivo. Four week old female BALB/c mice were inoculated in the right rear footpad with 25 μl of 10-fold serial dilutions of virus (four mice per virus dose) and the virus inoculum was confirmed by titration. Six to 8 weeks post-infection (p.i.) the mice were killed, and nine dorsal root ganglia (DRG; the two lowest thoracic, six lumbar and the upper sacral) were removed from the right-hand side of the spinal cord and placed separately in culture medium within microtitre wells. Ganglia were screened for release of infectious virus for 3 weeks, by transferring the culture medium every 2 to 3 days onto a C13 cell monolayer, and scoring for plaque formation or c.p.e.

Preparation of infected cell extracts. Confluent cell monolayers in 50 mm Petri dishes were infected at a multiplicity of 20 in Eagle's MEM containing 5% newborn calf serum. The virus was adsorbed for 1 h at 37 °C and the infected cells then washed twice with, and subsequently maintained in, Eagle's MEM containing one-fifth the normal concentration of methionine and 2% newborn calf serum. [35S]Methionine (Amersham; specific activity > 1000 Ci/mmol) was added directly to the cells at a final concentration of 50 μCi/ml at 3 h p.i. Infected cell polypeptides were harvested 24 h.p.i. by washing the cells twice with PBS and then adding 0.5 ml extraction buffer containing 100 mM-Tris-HCl pH 8.0, 10% glycerol, 0.1% NP40, 0.5% sodium deoxycholate and 0.2 mM-PMSF. Cell debris was removed by centrifugation at 110000 g for 10 min in a Beckman microfuge and the supernatant was stored at −70 °C until use.

For glycosylation studies, infected cells were labelled either with [3H]mannose (Amersham; specific activity 30 to 60 Ci/mmol) at 100 μCi/ml in PBS from 6 to 14 h.p.i., or with [3H]glucosamine (Amersham; specific activity 50 to 60 Ci/mmol) at 10 to 20 μCi/ml in Eagle's MEM containing 2% newborn calf serum, from 3 to 20 h.p.i., and harvested as described above. To inhibit glycosylation events, cells were infected and maintained continually in either 2 μg/ml tunicamycin (which inhibits N-linked glycosylation), 10 μg/ml monensin (which inhibits Golgi apparatus function, i.e. both O-linked glycosylation and the processing of the N-linked moieties), or both.

Subcellular fractionation. Subcellular fractionation was based on the method described by Bryant & Ratner (1990), with minor modifications. Cell monolayers, rinsed twice with PBS and once with distilled water, were scraped into homogenization buffer (20 mM-Tris-HCl pH 8.0, 10 mM-MgCl2, 0.5 mM-EDTA, 7 mM-2-mercaptoethanol) and swollen on ice for 20 min. After disruption of the cells by Dounce homogenization (30 strokes), intact cells and nuclei were removed by centrifugation at 2000 r.p.m, for 3 min at 4 °C in a Fisons Coolspin centrifuge, and the supernatant was then adjusted to 150 mM-NaCl and centrifuged in a Sorvall Ti50 rotor at 45000 r.p.m. for 30 min at 4 °C. The supernatant (cytosol) was removed, and the pellet (total membranes) was then resuspended in homogenization buffer containing 1 M-NaCl and this was then centrifuged as before, to elute those proteins which bind to membranes at only low ionic strength. The final dose. Mice were observed daily for 10 days after inoculation and the LD50 was calculated. Brains were removed from dead animals, homogenized and sonicated, and virus was then grown by plating approximately 10% of the resultant suspension onto a C13 cell monolayer in a Linbro well. The resultant virus stocks were analysed by restriction enzyme digestion.

Latency studies. Five week old female BALB/c mice were inoculated in the right rear footpad with 25 μl of 10-fold serial dilutions of virus (four mice per virus dose) and the virus inoculum was confirmed by titration. Six to 8 weeks post-infection (p.i.) the mice were killed, and nine dorsal root ganglia (DRG; the two lowest thoracic, six lumbar and the upper sacral) were removed from the right-hand side of the spinal cord and placed separately in culture medium within microtitre wells. Ganglia were screened for release of infectious virus for 3 weeks, by transferring the culture medium every 2 to 3 days onto a C13 cell monolayer, and scoring for plaque formation or c.p.e.
pellet was resuspended in homogenization buffer containing 150 mM-
NaCl. Samples were diluted 1:1 with twofold concentrated extraction
buffer for immunoprecipitation.

**Purification of virus particles.** The procedure was essentially that
described by Szilágyi & Cunningham (1991), with minor modifications.
Cell monolayers in 80 oz roller bottles were infected at a multiplicity of
0.001, incubated at 31°C until c.p.e. was evident, and then labelled with
[35S]methionine as described above. Once c.p.e. was complete, the
culture supernatants were centrifuged in a Fisons Coolspin centrifuge
(2000 r.p.m. for 30 min at 4°C) to remove cell debris. Virions were
centrifuged in a Sorvall GSA rotor (12000 r.p.m. for 2 h at 4°C),
resuspended gently in a small volume of Eagle's MEM without phenol
red and layered on a 35 ml continuous 5 to 15 % Ficoll gradient which
was centrifuged in a Sorvall AH629 rotor at 12000 r.p.m. for 2 h at
4°C. Both the lower virion band and the upper, more diffuse band
containing the light particles (nucleocapsid-free, tegument-envelope
structures) were collected by side puncture, diluted and centrifuged in
a Sorvall AH629 rotor at 21000 r.p.m. for 16 h at 4°C. Purified virions
and light particles were solubilized in extraction buffer prior to
immunoprecipitation.

**Immunoprecipitation.** Immunoprecipitation reactions were carried
out as described previously (MacLean et al., 1991), and the samples
were analysed on 5 to 12.5 % SDS-polyacrylamide gels, cross-linked
with bisacrylamide (Marsden et al., 1978), or on 10 % SDS-
polyacrylamide gels cross-linked with diallyltartardiamide (DATD;

**Fluorography.** SDS-polyacrylamide gels were treated with EnHance
(New England Nuclear), dried and exposed to Kodak X-Omat XS-1
film at -70°C.

**Results**

**The UL10 protein associates with membranes from infected cells**

UL10 is predicted to encode a membrane protein (McGeoch et al., 1986). To determine whether the UL10 polypeptide is membrane-associated, cytosol and mem-
brane fractions were isolated from infected cells labelled with [35S]methionine, and immunoprecipitated with the anti-UL10 serum (see Methods). From Fig. 1 it can be seen that the UL10 encoded polypeptide associated with the membrane fraction, and that this interaction was stable in the presence of 1 M-NaCl. At this concentration NaCl elutes proteins that associate only weakly with membranes, but does not affect the more strongly associated or integral membrane proteins. In most ex-
periments, the anti-UL10 serum specifically precipitated only a 47K polypeptide from infected cell extracts. Two additional higher M, bands were precipitated from membrane fractions (these can also be seen in the experiment in Fig. 2a). Since the pattern of these bands was reminiscent of gD, immunoprecipitations were also carried out using an anti-gD monoclonal antibody; these bands did not comigrate with gD (Fig. 1). It is likely that these represent alternative processed forms of the UL10 encoded polypeptide, not normally apparent in precip-
itations from total cell extracts due to comigrating bands in this area of the gel. Increasing the amount of competing peptide did not prevent precipitation of these comigrating bands (results not shown).

**The UL10 protein is modified by N-linked glycosylation**

The UL10 encoded polypeptide contains two potential N-linked glycosylation sites. To determine whether this protein is glycosylated, extracts from cells infected in the presence of [35S]methionine, [3H]mannose or [3H]glucos-amine were immunoprecipitated with anti-UL10 serum (Fig. 2a). The UL10 polypeptide was labelled weakly by both mannose and glucosamine, suggesting that it is glycosylated, at least to a minor extent. To determine whether the UL10 protein is modified by N-linked or O-
linked glycosylation, cells were infected in the presence of tunicamycin, monensin or both, and labelled with [35S]methionine. Cell extracts were then immunoprecipitated with anti-UL10 serum. The apparent M, of the UL10 polypeptide was reduced by about 3K following treatment with tunicamycin, but was not appreciably affected by monensin (Fig. 2b; for clarity a thymidine kinase-negative virus was used in this experiment, as the UL10 protein migrates between the thymidine kinase and actin bands following tunicamycin treatment). Analysis on DATD cross-linked gels showed the mobility shift more clearly (Fig. 2c).

**The UL10 protein is a virion component**

The HCMV counterpart of UL10 protein is a virion component (Lehner et al., 1989), and it seemed reason-
able to expect that the HSV-1 UL10 protein would also be present in purified virus particles. Cells were infected with HSV-1 strain 17syn + in the presence of [35S]methionine, and both virions and light particles (virus-like structures which lack a nucleocapsid, but contain the tegument and envelope components) were purified on Ficoll gradients, solubilized and then immunoprecipitated with anti-UL10 serum. The UL10 protein was detected in both virions and light particles, but only on long exposures of the gels (results not shown). In an attempt to improve detection, similar experiments were carried out with HSV-1 strain F, which grows to higher titres than strain 17syn +. The UL10 polypeptide could be precipitated from both the strain F virions and light particles (Fig. 3).

**Construction of UL10 deletion and revertant viruses**

We previously constructed a UL10-lacZ insertion mu-
tant; this virus could potentially encode a truncated
UL10 protein containing all the transmembrane regions of the intact product (MacLean et al., 1991). Therefore,
to investigate the role of UL10 \textit{in vivo}, we first deleted the majority of the UL10 ORF.

Plasmid pC122 contains HSV DNA sequences encompassing UL10, but lacking 989 bp from within the UL10 ORF (see Methods, and Fig. 4). This construct potentially encodes a polypeptide possessing the N-terminal 133 amino acids of the full-length (473 amino acid) UL10 product, containing only the first two transmembrane domains, and a novel 92 amino acid C terminus. This mutation was recombined into the virus genome by cotransfecting C13 cells with \textit{ULIO-lacZ} virus DNA and plasmid pC122 (see Methods). Recombinant viruses were detected by their clear plaque morphology in the presence of X-gal. Purified virus was analysed by restriction enzyme digestion of \textit{\textsuperscript{32}P}-labelled viral DNA (results not shown), and one isolate, designated \textit{ULIO-del}, was chosen for further study.

To ensure that any observed growth alterations resulted from the introduced mutation, revertant viruses were generated. C13 cells were cotransfected with \textit{ULIO-del} virus DNA and either a plasmid containing the wild-type UL10 ORF (pC73.1), or a plasmid with a 12 bp insertion within the intragenic region between UL10 and UL11, introducing an \textit{XbaI} site (pC343). The latter plasmid was included as a control, to ensure that any wild-type isolates arose through recombination, and not through wild-type contamination. Recombinant viruses were readily detected by virtue of their larger plaque morphology, and their genome structures were analysed by restriction enzyme digestion. None of 22 large plaque isolates from control transfections lacking plasmid DNA had a wild-type genotype, while 11/11 wild-type isolates from the transfections with pC343 contained the novel \textit{XbaI} site (results not shown). We were therefore confident that all the wild-type isolates represented true revertant virus. Two 'wild-type' and two '\textit{XbaI}+' revertants were purified, and working stocks grown. These viruses grew indistinguishably following infection.
at low multiplicity (Fig. 5), and one 'wild-type' revertant (rev-2) was chosen as the prototype UL10-rev virus for subsequent studies.

Fig. 2. The UL10 polypeptide is modified by N-linked glycosylation. (a) Extracts from cells infected in the presence of [35S]methionine (Met), [3H]mannose (Mann) or [14C]glucosamine (Gluc) were immunoprecipitated with anti-UL10 serum, in the presence (+) or absence (-) of the relevant peptide, and analysed on 5 to 12.5% SDS–polyacrylamide gels. Only the relevant portion of the gel is shown. E, Total cell extract. Mr values are shown on the left. The UL10 47K polypeptide is indicated (▲). (b, c) Extracts from cells untreated or treated with tunicamycin (T), monensin (M) or both (T/M) were immunoprecipitated with anti-UL10 serum, in the presence (+) or absence (-) of the relevant peptide, and analysed on either a 5 to 12.5% SDS–polyacrylamide gel cross-linked with bisacrylamide (b), or on a 10% DATD cross-linked SDS–polyacrylamide gel (c). Only the relevant portions of the gels are shown. E, Total cell extract from untreated cells. Mr values are shown on the left. The UL10 polypeptide is indicated (▲). The thymidine kinase-negative virus X4 was used in (b); wild-type strain 17syn+ was used in all other experiments.

Fig. 3. The UL10 polypeptide is a virion component. Virions (V) and light particles (L), labelled with [35S]methionine and purified as described in Methods, were immunoprecipitated with anti-UL10 serum in the absence (−) or presence (+) of the relevant peptide, and analysed on 5 to 12.5% SDS–polyacrylamide gels. A total infected cell extract (E) was included as a marker lane. Mr values are shown on the left. The UL10 47K protein is indicated (▲).

Fig. 4. UL10 deletion and revertant constructs. The gene arrangement in the region of UL10 is shown, with the DNA sequence numbering of McGeoch et al. (1988) given above. The sequences deleted from the UL10 ORF (■■) and the site of insertion of an XbaI linker (X) are indicated.

In vitro growth properties of UL10-del

UL10-del, like UL10-lacZ, consistently showed a smaller plaque morphology than the wild-type or revertant...
viruses. A pattern of growth similar to that of the wild-type viruses was seen following infection of C13 cells at low multiplicity, but UL10-del reached yields 10- to 20-fold lower than the controls (Fig. 5), and about twofold lower than its parent virus, UL10-lacZ (not shown). Following infection at high multiplicity, UL10-del reached a final yield one- to sevenfold lower than that of the revertant virus in a variety of cell types tested (C13, MDCK, MA104, Vero, CV-1, BSC-1, HeLa and Flow 2002), and about 18-fold lower in FG cells. UL10-del was no more impaired for growth in resting C13 cells (three- to sevenfold reduced yields), nor was the plaquing efficiency of UL10-del notably different at 31 °C, 37 °C or 39.5 °C (not shown).

In vivo growth properties of UL10-del

The ability of the UL10 deletion mutant to replicate in vivo at the periphery and to spread to and replicate within the nervous system was compared with that of the wild-type and revertant viruses. Mice were inoculated in the footpad as described (Methods) and virus titres in the footpad, DRG and brainstem measured at various times p.i. Initial experiments were carried out using an input dose of 1 × 10^6 p.f.u./mouse, the peripheral LD_{50} of our wild-type stock (both UL10-del and UL10-rev had a peripheral LD_{50} > 10^7 p.f.u.). Although both wild-type and UL10-rev viruses could be detected in the DRG and brainstem, UL10-del was detectable only at the periphery, and at a significantly lower level than UL10-rev (P < 0.02). Even at the highest possible input dose (5 × 10^7 p.f.u.), UL10-del was only barely detectable in the DRG, and did not reach detectable levels in the brainstem (Fig. 6). At this dose, the amount of UL10-del virus present at the periphery was similar to that found when using 50-fold less UL10-rev. These results suggest that UL10-del is impaired in its ability to grow at the periphery, and to spread to and/or grow within the nervous system, in the mouse footpad model.

To determine whether UL10-del could establish and reactivate from a latent infection, mice were inoculated in the right rear footpad with 10-fold serial dilutions of virus (four mice per group), and nine ipsilateral ganglia per mouse were explanted 6 to 8 weeks p.i. The ganglia were screened individually for release of infectious virus over a 3 week period. Results are shown in Table 1. At each dose tested, similar numbers of mice infected with UL10-del and UL10-rev developed a detectable latent infection. Thus, UL10-del is capable of establishing and reactivating from a latent infection. However, UL10-del was slower to reactivate following explantation, generally taking 2 to 4 days longer than UL10-rev to score positive (not shown), and did not appear to spread as efficiently through the nervous system as the wild-type or revertant viruses; only one or two ganglia per mouse scored positive, even with the highest input dose (Table 1).

The impaired spread of virus through the nervous system could be due to the lower titres of virus reaching the ganglia, or to an inability of the virus to grow in nervous tissue. To determine whether UL10-del could replicate within the nervous system, the neurovirulence of the UL10 deletion mutant was compared to wild-type and revertant viruses. Mice were inoculated intracerebrally with 10-fold serial dilutions of virus, and deaths from encephalitis by 10 days p.i. scored (Table 2). UL10-del appeared to be slightly less pathogenic than its revertant virus, with a LD_{50} of 3.5 × 10^5 compared to 1 × 10^6 p.f.u./mouse. Restriction enzyme analysis of virus recovered from the brains of dead animals confirmed that these deaths were caused by UL10-del, and were not the result of wild-type contamination.

Limitations of the in vivo model

Although UL10-del appeared to be impaired for growth in vivo compared to UL10-rev, it was nevertheless capable of replication both at the periphery and within the
3.0 "2, ~0 0 3 2 1 1 1 I I I

(a) [ ' I ' I ' 4 6 8 2.5 2.0 1.5 1.0 0.5 0 2 10 2 4 6 8 10 10

Fig. 6. Growth of UL10-del in vivo. Mice were inoculated in the right rear footpad, and the right rear footpad (a), nine ipsilateral DRG (b) and the brainstem (c) were removed at the times indicated. Each point is the mean virus yield from four animals. In one set of experiments UL10-rev (□) and UL10-del (■) were each used at 10^6 p.f.u./mouse; in another the dose of UL10-rev (○), 1.7 x 10^8 p.f.u./mouse, was threefold higher than that of UL10-del (●), 5.4 x 10^7 p.f.u./mouse.

Table 1. Determination of the capacity of UL10-del to establish and reactivate from a latent infection

<table>
<thead>
<tr>
<th>Input dose (p.f.u./mouse)</th>
<th>17syn^+</th>
<th>UL10-rev</th>
<th>UL10-del</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7</td>
<td>−</td>
<td>1,1,1,2*</td>
<td>1,1,1,2*</td>
</tr>
<tr>
<td>10^6</td>
<td>6.8†</td>
<td>4.8,4.7</td>
<td>0.1,2.1</td>
</tr>
<tr>
<td>10^5</td>
<td>1,3,1,1</td>
<td>1.1,0.0</td>
<td>1.1,0.1</td>
</tr>
<tr>
<td>10^4</td>
<td>1.1,0,1</td>
<td>0.0,0,1</td>
<td>0.0,0,1</td>
</tr>
</tbody>
</table>

* Number of ganglia/mouse yielding infectious virus. † Two mice from this group died following the acute infection.

Table 2. Comparison of neurovirulence of UL10-del, UL10-rev and wild-type

<table>
<thead>
<tr>
<th>Virus</th>
<th>Input dose (p.f.u./mouse)</th>
<th>10^4</th>
<th>10^3</th>
<th>10^2</th>
<th>LD_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>17syn^+</td>
<td>−</td>
<td>−</td>
<td>4/4*</td>
<td>&lt; 10^3</td>
<td></td>
</tr>
<tr>
<td>UL10-rev</td>
<td>−</td>
<td>3/4</td>
<td>2/4</td>
<td>1 x 10^2</td>
<td></td>
</tr>
<tr>
<td>UL10-del</td>
<td>4/4</td>
<td>1/4</td>
<td>0/4</td>
<td>3.5 x 10^2</td>
<td></td>
</tr>
</tbody>
</table>

* Number of animals dead/number of animals tested.

nervous system, in this mouse model. It is difficult to assess the significance of the impairment in terms of a specific role for UL10 in vivo (i.e. is the effect greater than might be expected from the in vitro studies). The footpad replication model is limited in its usefulness for virus strains, such as 17syn^+, that have a high LD_{50} following infection by this route. The assay was relatively insensitive, requiring an input virus dose near the peripheral LD_{50} for efficient virus growth and spread. Thus, virus was detected over a longer period of time, and reached significantly higher titres in animals infected with 1 x 10^6 p.f.u. wild-type 17syn^+ than 1 x 10^5 p.f.u. Peak titres (log_{10} p.f.u.) were: footpad 4.92 ± 0.08, 4.17 ± 0.35 (P < 0.05); DRG 4.52 ± 0.5, 2.29 ± 0.19 (P < 0.01); brainstem 4.15 ± 0.52, 2.05 ± 0.95 (P < 0.05), respectively. In addition, inoculation of UL10-rev and UL10-del at high input dose (but still less than the LD_{50}) caused severe leg swelling and lesions in almost all animals. Thus, the dose of UL10-del required for efficient spread to the nervous system could not be established.

Furthermore, UL10-rev appeared to be impaired in its ability to spread to and/or replicate within the nervous system, compared to the wild-type virus. Infection with 1 x 10^6 p.f.u. wild-type 17syn^+ produced significantly higher peak titres in the DRG (P < 0.05) and brainstem (P < 0.02) than infection with 1.7 x 10^8 p.f.u. UL10-rev, although UL10-rev reached significantly higher titres in the footpad (P < 0.01) (compare results above with Fig. 6). Whether this impairment is due to a secondary mutation which arose during the generation of UL10-del or UL10-rev, or whether it simply reflects individual plaque variation pre-existing within the wild-type stock used to generate UL10-del, is unclear. Interestingly, 10 individual plaque isolates of wild-type 17syn^+ were found to vary in intracranial LD_{50}, from < 10 to 100 p.f.u. (L. Robertson, unpublished). The intracranial LD_{50} of UL10-rev is within this range (100 p.f.u.), but at the less
virulent extreme. Analysis of individual plaque variation in peripheral LD<sub>50</sub> has not been carried out.

**Discussion**

HSV-1 gene UL10 is predicted to encode a very hydrophobic protein with eight potential transmembrane segments (McGeoch et al., 1986). Two potential N-linked glycosylation sites are present within the predicted amino acid sequence of the UL10 protein, and one of these is conserved in all other known corresponding herpesvirus ORFs (VZV gene 50, Davison & Scott, 1986; EHV-1 gene 52, Telford et al., 1992; EBV gene BBRF3, Baer et al., 1984; HCMV gene UL100, Lehner et al., 1989; Chee et al., 1990; HSV gene 39, Albrecht et al., 1992). These features would suggest that the UL10 product is a glycosylated membrane protein. The results presented here support this prediction. In cell fractionation experiments, the UL10 polypeptide was found to be associated with infected cell membranes, it also was labelled weakly by both [3H]mannose and [14C]glucosamine and had a higher electrophoretic mobility when synthesized in the presence of tunicamycin, an inhibitor of N-linked glycosylation. The poor incorporation of mannose and glucosamine, and the relatively small change in M<sub>r</sub> with tunicamycin treatment, would suggest that the 47K UL10 protein is not heavily glycosylated. The two additional higher M<sub>r</sub> species specifically precipitated from membrane fractions by the anti-UL10 serum probably represent alternatively processed forms of the UL10 protein, and would appear to be at least as abundant as the 47K species. We are currently trying to determine whether they represent more highly glycosylated forms of the UL10 product.

The UL10 product is dispensable for growth in tissue culture (Baines & Roizman, 1991 ; MacLean et al., 1991). Although we had previously constructed a UL10-lacZ insertion mutant, we could not exclude the possibility that this virus synthesized a partially functional UL10 protein (MacLean et al., 1991). Therefore, to investigate the role of UL10 in vivo, we first deleted the majority of the UL10 ORF (since UL10 overlaps UL9, an essential gene, we could not simply delete the entire UL10 ORF). The deleted gene retains only the first two transmembrane segments, and is therefore unlikely to encode a functional UL10 protein.

In agreement with the previous studies, UL10-del is impaired for growth in tissue culture, and does not have an obvious temperature-sensitive or host range phenotype. In addition, it is no more impaired for growth in resting as compared to exponentially growing cells (results not shown). In vivo, UL10-del is impaired for growth at the periphery, and for spread to and/or growth within the nervous system, in a mouse model: following footpad inoculation, UL10-del grew less well at the periphery than control viruses did, and was barely detectable in the DRG. It was capable of establishing a latent infection and reactivating following explantation, but, unlike UL10-rev, only those ganglia directly innervating the site of inoculation appeared to be involved; and the intracranial LD<sub>50</sub> of UL10-del was approximately 35-fold higher than that of the revertant virus. The level of impairment in growth in vivo is difficult to quantify, but is compatible with the in vitro growth properties of this virus: i.e. there is no evidence that UL10 has a specific in vivo role not detectable in tissue culture assays.

In this communication we show that HSV-1 gene UL10 encodes a glycosylated membrane protein required for optimal virus growth both in vitro and in vivo. Like its HCMV counterpart (Lehner et al., 1989), the UL10 protein is a virion component, presumably located within the virus envelope. However, the precise function of the UL10 protein is still unclear. In contrast to the other multiply inserted membrane proteins encoded by HSV-1, the highest similarity between the UL10 protein and its counterparts in the other herpesviruses lies within the transmembrane regions (it should be noted that overall amino acid similarity is low). This would not be expected if the transmembrane regions simply serve a structural role, and may favour a role for this protein in transmembrane transport events. Elucidation of the structure of the UL10 protein within the membrane, and the development of functional assays, will be important to determine whether this is indeed the case.

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**References**


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