Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins

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The herpes simplex virus type 1 genome contains four open reading frames (ORFs) which are predicted to encode hydrophobic proteins with the potential to cross a membrane several times. The products of these genes (genes UL10, UL20, UL43 and UL53) have not previously been identified. To investigate the role of these proteins in the virus life cycle, we attempted to inactivate the genes individually by inserting the lacZ gene from Escherichia coli within the ORFs. Using this approach we have isolated insertion mutants for UL10 and UL43, as well as a deletion mutant lacking the majority of the UL43 ORF. The growth of the UL10-lacZ virus was slightly impaired in tissue culture compared to that of the wild-type virus parent, whereas the growth of the UL43 mutants was indistinguishable from that of wild-type virus. Furthermore, deletion of the majority of the UL43 ORF did not impair the ability of the virus to replicate in vivo at the periphery, or to spread to and replicate within the nervous system, in a mouse ear model. Repeated attempts to isolate lacZ insertion mutants for UL20 and UL53 were unsuccessful, suggesting that these genes may be essential for virus growth, at least in tissue culture. Using antipeptide sera, the products of genes UL10 and UL20 have been detected.

Introduction

Herpes simplex virus (HSV) is a large dsDNA virus, with a genome of approximately 152000 bp. The complete DNA sequence of one HSV-1 strain (strain 17 syn +) has been determined and it suggests that this virus encodes at least 70 distinct gene products (McGeoch et al., 1985, 1986a, 1988a; Perry & McGeoch, 1988). Although many of these proteins are known to play roles in various aspects of virus replication and virion structure, almost one-third of the identified genes have no attributed function (McGeoch et al., 1988a). We are therefore interested in investigating some of these genes with a view to identifying new virus-encoded polypeptides and elucidating their roles in the virus life cycle.

Four of these ‘unknown’ genes encode proteins which are notably hydrophobic, containing several stretches of amino acids which may be membrane-inserted; these genes are designated UL10, UL20, UL43 and UL53 (McGeoch et al., 1988a). The hydropathicity profiles of the predicted proteins are shown in Fig. 1.

There are two potential in-frame translation initiation codons for UL10 (McGeoch et al., 1986b). Thus, UL10 may encode a polypeptide of 473 or 455 amino acids, possessing eight potential transmembrane regions in either case and a potential N-terminal signal sequence (McGeoch, 1985) if the second initiation codon is used. Although no protein product has been identified for UL10, the product of the corresponding gene of human cytomegalovirus (HCMV) has recently been shown to be a virion component (Lehner et al., 1989). UL20 encodes a polypeptide of 222 amino acids, with two to four potential transmembrane segments. It does not have a potential N-terminal signal sequence and nothing is known about this product. The UL43 polypeptide is 434 amino acids in length, possessing at least six potential transmembrane segments. It lacks an N-terminal signal sequence and again nothing is known about this product. UL53 encodes a 338 amino acid polypeptide, with two to four possible transmembrane regions, as well as a potential N-terminal signal sequence. Although no protein product has been identified for UL53, the UL53 open reading frame (ORF) has been shown to be the locus of a mutation causing syncytial plaque morphology (Debroy et al., 1985), suggesting that the gene product may indeed be a membrane protein.

In order to investigate the role of these gene products in the virus life cycle, we attempted to construct virus strains in which each gene is inactivated by the insertion of the Escherichia coli lacZ gene into the ORF (Chakra-
barti et al., 1985; Panicali et al., 1986; Goldstein & Weller, 1988). Viral recombinants containing the lacZ insert are detectable by their blue plaque morphology in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a chromogenic substrate for β-galactosidase. Using this approach, we generated lacZ insertion mutants in UL10 and UL43 but were unable to obtain such mutants in UL20 or UL53, suggesting that these latter genes may be essential for virus growth in tissue culture. In addition, using antipeptide sera we have identified polypeptides encoded by the UL10 and UL20 ORFs.

Methods

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

Virus. HSV-1 strain 17 syn* (Brown et al., 1973) was the wild-type virus strain used in this study.

Plasmids. Plasmids pFJ3 and pFJ5ACAT were kindly provided by Drs Frazer Rixon and John McLauchlan. pFJ3 contains the lacZ gene from E. coli, under the control of the simian virus 40 early promoter; pFJ5ACAT is similar but also contains an HSV polyadenylation signal downstream from, and in the opposite orientation to, the lacZ polyadenylation signal (Rixon & McLauchlan, 1990). The lacZ gene is contained within a 4.1 kb (pFJ3) or 4.2 kb (pFJ5ACAT) Xbal fragment. Plasmid construction and propagation were carried out by standard procedures (Maniatis et al., 1982). In describing the construction of plasmids containing HSV-1 DNA sequences, we have used the numbering system of McGeoch et al. (1988). The numbers given represent either the first base of the restriction enzyme site or the first and last bases of the ORF, including the termination codon.

To construct plasmid pC78, a 3.5 kb BamHI (21655)-BglII (25149) fragment containing the UL10 ORF (23206 to 24627) was cloned into the BamHI site of pUC19. The 4.1 kb Xbal fragment from pFJ3 was blunt-ended using the Klenow fragment of DNA polymerase and ligated, using T4 DNA ligase, into the unique NsiI site within the UL10 ORF (24345).

To construct plasmid pC75.1, a 5.1 kb BamHI (91610)-EcoRI (96751) fragment containing the UL43 ORF (94748 to 96052) was cloned into the BamHI and EcoRI sites of pUC19 to generate plasmid p35. The 4.1 kb Xbal fragment from pFJ3 was blunt-ended using the Klenow fragment of DNA polymerase and ligated, using T4 DNA ligase, into the unique NsiI site within the UL10 ORF (24345).

To construct plasmid pC71, a 3.2 kb EcoRI (110095)-BamHI (113322) fragment containing the UL53 ORF (112179 to 113195) was cloned into the EcoRI and BamHI sites of pUC19. An Xbal site present within the pUC19 mult cloning site was then removed by sequential Xbal digestion, blunt-ending with the Klenow fragment of DNA polymerase and religation. A unique Hpal site was present within the UL53 ORF at residue 112512. Hpal digestion and religation in the presence of an Xbal linker then generated a unique Xbal site within the UL53 ORF; the 4.2 kb Xbal fragment from pFJ5ACAT was ligated into this Xbal site. This fragment was used in order to provide an alternative polyadenylation signal for UL52, which is thought to be 3’ coterminal with UL53 (see Discussion).

All plasmids were propagated in E. coli strains DH5 or DH5x (Gibco-BRL). The positions of the lacZ inserts within the four ORFs are shown in Fig. 1.

Generation of virus mutants. Cells were transfected with DNA according to the procedure of Graham & van der Eb (1973), as modified by Stow & Wilkie (1976). HSV DNA (200 to 1000 p.f.u.; 1 to 2 μg) was cotransfected with varying amounts of linearized plasmid DNA (0.4 to 3.2 μg; an approximately threefold to 39-fold molar excess) in the presence of carrier calf thymus DNA, to a total of 6 μg DNA per transfection. The cells were incubated at 37 °C in Eagle’s medium containing 5% newborn calf serum until the c.p.e. was relatively widespread. The cells were then scraped into the medium, sonicated and the virus yield was titrated under methylcellulose.

Cells were infected with virus at approximately 20 p.f.u. per 50 mm Petri dish and overlaid with Eagle’s medium containing 0.6% Noble agar and 1 μg/ml X-gal. Individual blue plaques were isolated and subjected to several further rounds of plaque purification. The genome structure of the virus mutants was determined by restriction enzyme analysis before virus stocks were grown.

Restriction enzyme analysis of in vivo 32P-labelled virus DNA. Restriction enzyme analysis of single plaque isolates was based on the method of Lonsdale (1979), as described by MacLean & Brown (1987). Briefly, cells were infected at 10 p.f.u./cell in phosphate-free Eagle’s medium containing 1% newborn calf serum and 10 μCi/ml 32P for 48 h at 31 °C. Viral DNA was isolated by extraction with SDS and phenol, and ethanol precipitation. The DNA was resuspended in water containing pre-boiled RNase A (1.5 μg/ml) and RNase T1 (0.0625 μg/ml) for 2 h at 37 °C, before being digested with 1 to 2 units of the relevant restriction enzyme under the manufacturer’s recommended conditions. The digests were analysed by electrophoresis on 0.6% or 0.8% agarose gels in E buffer (36 μM-Tris, 30 mM NaOH, 0.5 mM EDTA). The dried gels were exposed to X-Omat X-S films at room temperature, or at −70 °C against an image intensifying screen.

Pathogenicity studies. Virus suspension (20 μl) containing 5 × 106 p.f.u. of either HSV-1 strain 17 syn* or UL43–lacZ or UL43–Del virus, was inoculated subcutaneously into the left ears of anaesthetized 4 to 5 week old female BALB/c mice (Hill et al., 1975). The left ear, pooled CII, CIII and CIV cervical dorsal root ganglia, and brainstem were removed from animals killed at various times after infection, and stored at −70 °C. The virus titre present in these tissues was determined by homogenizing the tissues in 0.2 ml Eagle’s medium containing 10% newborn calf serum and 10% tryptose phosphate broth and assaying for infectious virus on C13 cells.

Oligopeptide antiserum. Oligopeptides were synthesized representing amino acids 1 to 12 and 458 to 467 of the UL10 ORF, 1 to 12 and 458 to 467 of the UL10 ORF, 20 to 30 and 126 to 137 of the UL43 ORF, and 270 to 281 and 327 to 338 of the UL53 ORF. Antiserum against synthetic peptides representing amino acids 80 to 90 and 118 to 129 of the UL10 ORF, and 423 to 434 of the UL43 ORF, were raised by Dr Margaret Frame.

To construct plasmid pC79, a 3.2 kb EcoRI (110095)-BamHI (113322) fragment containing the UL53 ORF (112179 to 113195) was cloned into the EcoRI and BamHI sites of pUC19. An Xbal site present within the pUC19 mult cloning site was then removed by sequential Xbal digestion, blunt-ending with the Klenow fragment of DNA polymerase and religation. A unique Hpal site was present within the UL53 ORF at residue 112512. Hpal digestion and religation in the presence of an Xbal linker then generated a unique Xbal site within the UL53 ORF; the 4-2 kb Xbal fragment from pFJ5ACAT was ligated into this Xbal site. This fragment was used in order to provide an alternative polyadenylation signal for UL52, which is thought to be 3’ coterminal with UL53 (see Discussion).
HSV-1 membrane proteins

Fig. 1. Hydropathicity profiles of the putative membrane proteins. Hydropathicity profiles (Kyte & Doolittle, 1982) are shown for the predicted products of genes UL10 (a), UL20 (b), UL43 (c) and UL53 (d). N and C represent the amino and carboxy termini of the predicted proteins, respectively. Hydrophobic regions are shown above and hydrophilic regions below the dotted lines. Those regions of the polypeptides which are predicted by both the method of Kyte & Doolittle (1982) and that of Eisenberg et al. (1984) to be membrane-spanning, and which also contain at least 16 sequential uncharged residues, are indicated by the short solid lines. Regions of the polypeptides that fulfil two of these three criteria, and are thus also considered likely to be membrane-spanning, are indicated by the short dashed lines. The locations within the ORFs of the inserted lacZ gene are indicated (J), as are those sequences deleted from the UL43 ORF in the UL43-del mutant (→). Oligopeptides representing the regions indicated by the black boxes (■) were synthesized and used to raise antisera.

Oligopeptides were either synthesized using an LKB Biolynx peptide synthesizer and continuous flow Fmoc chemistry (reviewed by Atherton et al., 1979; Sheppard, 1983), or were obtained from Peptide and Protein Research. All chemicals were purchased from LKB Biochrom with the exception of dimethylformamide (Rathburn Chemicals), trifluoroacetic acid (Aldrich) and resin to which the first amino acid had been coupled via an acid-labile handle (Peptide and Protein Research). Following synthesis the peptides were cleaved from the resin and side-chain protecting groups were removed with either 95% trifluoroacetic acid in water, or with 95% trifluoroacetic acid, 4% phenol in water. In the latter case, the peptide was then extracted with ether prior to freeze-drying. The peptides were analysed by mass spectrometry (M-Scan) to check that the product's Mr was identical to that predicted from the amino acid sequence.

Two Sandy Lop rabbits were immunized intramuscularly with 100 µg bovine serum albumin (BSA)-conjugated peptide emulsified in Freund's complete adjuvant for the first injection, and Freund's incomplete adjuvant for subsequent injections. The animals were bled 10 days after each injection. The sera were raised by Serotec. Anti-BSA antibodies were subsequently removed by absorption with BSA immobilized on Sepharose 4B.

Preparation of infected cell extracts. Confluent cell monolayers in 50 mm Petri dishes were infected at 20 p.f.u. virus/cell in Eagle's medium containing 5% newborn calf serum. The virus was absorbed for 1 h at 37 °C and the infected cells were then washed twice with, and subsequently maintained in, Eagle's medium 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 post-infection (p.i.). Infected cell polypeptides were harvested 24 h post-infection with PBS and then adding 0.5 ml extraction buffer containing 10 mM-Tris–HCl pH 8.0, 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate and 0.2 mM-PMSF (Zweig et al., 1980). Cell debris was pelleted by centrifugation at 11000 g for 10 min in a Beckman microfuge and the supernatant was stored at −70 °C until use.

Immunoprecipitation. Antiserum (50 µl) was incubated at 37 °C for 30 min in the presence or absence of peptide. Infected cell extract (100 µl), diluted to 200 µl with extraction buffer, was then added and the samples were incubated either overnight at 4 °C, or for 1 h at 37 °C. The samples were 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. Immunoprecipitates were washed several times with extraction buffer, and bound proteins were then eluted by boiling in denaturation buffer (50 mM-Tris–HCl pH 6.7, 2% SDS, 700 mM-2-mercaptoethanol, 10% glycerol and 1% bromophenol blue) and analysed by SDS-PAGE (Marsden et al., 1978).

Fluorography. SDS-PAGE gels were treated with Enhance (New England Nuclear), dried and exposed to X-Omat XS-1 film at −70 °C.

Results

Generation of a UL10–lacZ insertion mutant

A plasmid was constructed, pC78.1, which contains the lacZ gene from E. coli inserted into a unique NruI site towards the 3' end of the UL10 ORF (see Fig. 3a); the UL10 ORF is thus interrupted at codon 381. An in-frame termination codon occurs 57 codons downstream within the promoter region of the lacZ insert. Therefore, should
an altered UL10 polypeptide be synthesized, it should contain the first 380 amino acids of the wild-type UL10 protein, followed by a novel 56 amino acid carboxy terminus. Such a protein would contain all the hydrophobic regions of the wild-type product (see Fig. 1a). This mutation was recombined into the wild-type virus genome by cotransfecting C13 cells with HSV DNA and plasmid pC78.1. Recombinant viruses were detected by their blue plaque morphology in the presence of X-gal and a number of individual plaques were isolated and purified. One of these was subsequently chosen for further study and was designated UL10-lacZ.

To confirm that the UL10-lacZ virus had the expected genome structure, restriction enzyme analysis of 32P-labelled DNA was carried out. An example is shown in Fig. 2(a). UL10 lies within the BglII fragment k (10·6 kb). Insertion of the lacZ gene into UL10 introduces an additional BglII site (see Fig. 3a) which should lead to loss of the k fragment and the generation of two novel fragments: one of approximately 13·8 kb, which should run just below the j fragment (14·6 kb), and a small fragment of 0·8 kb. The loss of the wild-type k fragment and the appearance of a novel fragment running below j are clearly seen in Fig. 2(a); the small 0·8 kb fragment would have run off the bottom of the gel. No obvious additional differences between the UL10-lacZ and wild-type virus profiles are seen, with the exception of fragments h and l. These represent end fragments from the short region of the genome which are known to vary between different plaque isolates (Davison & Wilkie, 1981). Results using a number of other restriction enzymes (not shown) were also consistent with UL10-lacZ having the expected genome structure, with no additional gross differences from the wild-type virus genome.

**Generation of a UL43-lacZ insertion mutant**

A plasmid was constructed, pC75.1, which contains the lacZ gene inserted into a unique NsiI site towards the 5' end of UL43 (Fig. 3b). The inserted DNA interrupts the UL43 ORF at codon 54 and introduces an in-frame termination codon a further 35 codons downstream. Should an altered UL43 protein be synthesized, it would contain only the first 53 amino acids of the wild-type
UL43 protein followed by a novel 34 amino acid carboxy terminus. Such a protein would lack the hydrophobic regions of the intact gene product (see Fig. 1c). However, a number of methionine codons occur immediately downstream from the insertion site (amino acids 54, 71 and 94) and re-initiation from these could result in a polypeptide retaining the majority of the hydrophobic domains. The mutation was recombined into the wild-type virus genome by cotransfection of C13 cells with HSV DNA and plasmid pC75.1. Several individual blue plaques were isolated and purified, and one was chosen for further study. This was designated UL43-lacZ.

Restriction enzyme analysis of UL43-lacZ DNA was carried out and an example is shown in Fig. 2(a). UL43 lies within BglII fragment g (17-4 kb). Insertion of the lacZ gene into UL43 introduces an additional BglII site (see Fig. 3b) which should result in loss of the g fragment and the generation of two novel fragments: one of approximately 15-9 kb, which should run at or above the i fragment (15-6 kb), and one of approximately 5-6 kb, which should run between the n (5-7 kb) and o (5-3 kb) fragments. The loss of the wild-type g fragment and the appearance of novel fragments running just above fragment i and just below fragment n can be seen in Fig. 2(a). The results from this and other restriction enzyme digestions were consistent with UL43-lacZ having the expected genome structure and no additional visible differences from the wild-type virus genome.

Generation of a UL43 deletion mutant

Deletion of a 1118 bp region of DNA from within the UL43 ORF generated plasmid pC180.1 (see Methods and Fig. 1c). This construct could theoretically encode a polypeptide possessing the amino-terminal 53 amino acids of the UL43 protein and a novel 31 amino acid carboxy terminus. The mutation was recombined into the virus genome by cotransfecting C13 cells with UL43-lacZ virus DNA and plasmid pC180.1. Recombinant viruses were detected in this case by their non-blue, or clear, plaque morphology in the presence of X-gal. A number of clear plaques were isolated and purified and one, designated UL43-del, was chosen for further study.

Restriction enzyme analysis confirmed the structure of the UL43-del virus genome. An example is shown in Fig. 2(b). UL43 lies within the BamHI i fragment (6-6 kb). Insertion of the lacZ gene into UL43 introduces a new BamHI site (Fig. 3b), resulting in loss of the i fragment and the generation of two novel fragments: one of approximately 7-4 kb, migrating at or below fragment h (7-5 kb), and one of approximately 3-3 kb, running near fragment r (3-4 kb). In contrast, UL43-del should show a loss of the i fragment and the generation of a deleted i fragment of approximately 5-5 kb running just above fragment l (5-4 kb). Fig. 2(b) shows the BamHI profiles of wild-type virus, UL43-lacZ and UL43-del DNA. Wild-type virus DNA (lane 1) showed partial digestion products and therefore a second experiment aligning the wild-type virus and UL43-lacZ DNA profiles is shown (lanes 5 and 4, respectively). Both mutants show the expected loss of the i band and UL43-lacZ DNA has a novel band just below fragment h, but the novel band near fragment r is not readily apparent. Interpretation of bands in this region is difficult due to the variable migration of the q fragment, the end fragment from the short region of the genome. UL43-del DNA has a novel band running just above fragment l but has lost the band below fragment h which was present in the UL43-lacZ parent. Variation in the k fragment between the isolates is also observed; this represents the L-S joint fragment. From this and other experiments it was concluded that UL43-del has the expected genome structure and does not obviously differ in any other way from wild-type virus.

Attempts to generate lacZ insertion mutants in UL20 and UL53

Plasmids pC94 and pC179 were constructed as described in Methods, interrupting the UL20 and UL53 ORFs, respectively, at the positions shown in Fig. 1(b and d). Several attempts were made to recombine these mutations into the virus genome. Blue virus plaques were detectable at a frequency similar to that found in the previous experiments (approximately 0.3 to 3% depending on the experiment) but only when there was sufficient wild-type virus for complementation. Although restriction enzyme analysis of mixed plaques suggested that a minor population of virus with the lacZ genotype was present, the blue plaque isolates could not be purified from contaminating wild-type virus. Therefore, it would appear that the introduction of these mutations into the virus genome has a profound effect on the ability of the virus to grow in tissue culture.

Growth properties of the lacZ insertion and deletion mutants

The behaviour of the mutant viruses in tissue culture was compared with that of wild-type virus. The plaque morphology of the UL43-lacZ and UL43-del isolates was very similar to wild-type virus, whereas UL10-lacZ consistently formed smaller plaques (results not shown). The pattern of virus growth following infection at low multiplicity (0.001 p.f.u./cell) is shown in Fig. 4. The UL43 mutants grew to the same titre as the wild-type virus, following a similar pattern of growth (Fig. 4b). In contrast, the UL10-lacZ mutant grew more slowly and...
reached a final yield about 10-fold less than that of the wild-type virus (Fig. 4a). Similar results were found following infection at high multiplicity (not shown), although the reduction in yield of UL10-lacZ was less marked (two to 10-fold), varying slightly from experiment to experiment.

In order to determine whether the mutant viruses exhibited a temperature-sensitive or host-range phenotype, virus was grown at high multiplicity either in C13 cells at different temperatures (31 °C, 37 °C, 38.5 °C or 39.5 °C), or at 37 °C in various different cell types (C13, HFL, Vero, Bsc-1, MDCK or 3T6 cells). Under all conditions tested, the UL43 mutants again grew to levels very similar to those of the wild-type virus whereas UL10-lacZ gave slightly reduced yields (two- to 10-fold) (results not shown).

The ability of the UL43 mutants to grow in vivo was tested using a mouse ear model (Hill et al., 1975). The results are shown in Fig. 5. Neither mutant showed any gross impairment in its ability to grow at the site of inoculation (Fig. 5a). The mutants also followed a similar pattern of growth to that of the wild-type virus in both the ganglia and the brainstem (Fig. 5b and c), although slightly lower peak titres were reached. The UL43 deletion virus reached a mean peak titre in the brainstem which was threefold less than that of the wild-type virus; the results for the individual mice were 6.21, 4.72 and 5.14 compared to 6.16, 5.21 and 6.17 log_{10} p.f.u., respectively. From these results, it would appear that the lack of UL43 does not significantly impair the ability of the virus to spread to and grow within the nervous system. The apparently greater impairment observed for the UL43-lacZ virus cannot be due only to the lack of the wild-type UL43 product but has not been investigated further. The UL43-del virus was also found to reactivate from latently infected ganglia following explantation at the same frequency as wild-type virus (S. Efstathiou, unpublished observations).

Identification of the putative integral membrane proteins

We attempted to identify the polypeptide products of the four ORFs using antipeptide sera. Oligopeptides were synthesized which represent four regions from within UL10, two from UL20, three from UL43 and two from UL53 (Fig. 1). Antisera raised against the peptides were tested in immunoblotting and immunoprecipitation experiments. Only two sera specifically recognized virus-induced polypeptides: one raised against the carboxy-terminal region of UL10, amino acids 458 to 467, and one raised against an internal region of UL20, amino acids 168 to 179.

The anti-UL10 serum immunoprecipitated a protein from infected cell extracts of approximate Mr 47000
HSV-1 membrane proteins

Fig. 5. Detection of HSV-1 in homogenates of (a) ear, (b) CII, CIII and CIV cervical dorsal root ganglia and (c) brainstem removed from animals at various times after infection with $5 \times 10^6$ p.f.u. of either HSV-1 strain 17 syn* (●), UL43-lacZ (□) or UL43-del (○). Each point represents the mean titre of virus obtained from three animals.

Discussion

Among the genes encoded by the HSV-1 genome is a group of ORFs whose predicted products are hydrophobic proteins with the potential to cross a membrane several times. The function of these genes is not known although one, UL53, is known to be a syn locus (Debroy et al., 1985). To investigate the role of these putative gene products in the virus life cycle, we attempted to disrupt the four ORFs separately. For this we chose to use the lacZ gene from E. coli for two reasons: (i) it serves as a detectable marker, allowing the success of the recombination event to be monitored even if the mutation proves lethal and the recombinant virus cannot be subsequently purified; (ii) a viable lacZ insertion mutant could be used as the parent virus in future experiments to generate additional mutations, for example point mutations or deletions of individual hydrophobic domains. Such virus mutants would be detectable by their non-blue (clear) plaque morphology in the presence of X-gal.

Both a UL43 lacZ insertion mutant and a UL43 deletion mutant, lacking the majority of the ORF, were constructed. The lack of UL43 appears to have little or no effect on the virus phenotype in vitro or in vivo; it does not impair the ability of the virus to grow in tissue culture in different cell types or at different temperatures, nor to replicate in vivo at the periphery, or to spread to and replicate within the nervous system in a mouse ear model. The UL43 polypeptide has not yet been identified. Synthetic oligopeptides representing three different
regions of the UL43 ORF were used to raise antisera, but none of these sera specifically recognized a virus-induced protein. However, it is possible that the UL43 product is present in low amounts and perhaps only at certain times p.i.; a minor transcript, detected only at early times, has been mapped to the corresponding region of the HSV-2 genome (Jenkins & Howett, 1984). It will now be important to identify the corresponding HSV-1 transcript. This, together with the use of higher affinity antisera, for example raised against fusion proteins containing larger regions of the UL43 ORF, should help to identify the UL43 gene products.

A UL10-lacZ insertion mutant, containing the lacZ gene at the carboxy-terminal end of the ORF, was constructed. The growth of this virus is slightly impaired in tissue culture compared to the wild-type parent, suggesting that the UL10 polypeptide may play at least a minor role in virus growth in tissue culture. Since the insertion mutant could potentially synthesize an altered UL10 protein containing the first 380 amino acids and thus all the hydrophobic sequences of the wild-type product, it cannot be concluded that the gene is non-essential. However, we have recently isolated a deletion mutant lacking the majority of the UL10 ORF. Preliminary experiments suggest that the growth of this mutant is also only slightly impaired in tissue culture. It will now be important to generate a revertant virus to determine whether this impairment in growth is due to the UL10 mutation.

By using an antiserum raised against the carboxy-terminal region of the UL10 ORF, a 47K protein product has been identified. The observed Mr is in reasonable agreement with that predicted: either 51K for the intact protein (McGeoch et al., 1986b), or 46 to 47K if cleavage occurs after the potential signal sequence. The UL10 polypeptide labels poorly with both [3H]mannose and [14C]glucosamine (preliminary observations), and further work is in progress to determine whether it is indeed glycosylated. Two potential N-glycosylation sites are present within the predicted amino acid sequence (Hubbard & Ivatt, 1981) and one of these sites is conserved in the predicted protein products of three other corresponding herpesvirus genes: varicella-zoster virus (VZV) gene 50 (Davison & Scott, 1986a), Epstein-Barr virus gene BBRF3 (Baer et al., 1984) and human cytomegalovirus (HCMV) gene UL100 (Lehner et al., 1989; Chee et al., 1990). The corresponding gene product of HCMV has recently been shown to be a structural component, possibly present within the virion envelope (Lehner et al., 1989). We have so far been unable to detect the UL10 polypeptide in purified HSV-1 virions, but the antipeptide serum used may not be of sufficiently high affinity to detect low levels of protein.

Attempts to isolate lacZ insertion mutants in genes UL20 and UL53 were unsuccessful. Recombination of the lacZ constructs into the wild-type genome evidently did occur because blue plaques were detected in the presence of complementing wild-type virus. However, these blue plaques could not be purified, suggesting that the introduction of the mutations caused a significant impairment in the ability of the virus to grow.

The gene to the left of UL20 encodes the major capsid protein, an essential gene. Although the two ORFs do not
overlap, the UL19 promoter region extends 30 to 80 bp into the 3' end of the leftward oriented UL20 ORF (Costa et al., 1985). The lacZ gene was inserted towards the middle of UL20, approximately 356 bp from the 3' end of the ORF, and therefore should not interfere with the function of the UL19 promoter. Little is known about UL21 which is to the right of UL20. The UL21 ORF is rightward oriented and begins about 586 bp from the 5' end of the UL20 ORF. Therefore, it would seem unlikely that the lacZ insert approximately 940 bp upstream from the UL21 ORF would interfere with UL21 transcription. However, Costa et al. (1981, 1984) mapped a rightward transcribed 3'1 kb mRNA overlapping UL20 which could encompass UL21. The lacZ insertion would interrupt this mRNA. A 7 kb RNA of undetermined orientation has also been mapped to a region within and to the right of UL20 (Costa et al., 1981). Thus, it remains possible that the effect of the lacZ insertion is not due to interruption of the UL20 ORF per se but rather to interruption of overlapping transcripts. Use of complementing cell lines expressing UL20 should help to clarify this point.

The lacZ insertion within UL53 should not interfere with neighbouring genes. Although the UL52 ORF overlaps with the UL53 ORF by 15 codons and their transcripts are thought to be 3' coterminal (McGeoch et al., 1988b), the lacZ insert was placed outwith the region of overlap (Fig. 1) and an HSV polyadenylation signal was present in the inserted DNA fragment oriented such that it should provide a functional signal for the UL52 RNA. In addition, the inserted DNA lies over 1 kb from the RNA start site of UL54 and thus should be well outwith the controlling region of this gene (Whitton et al., 1983).

A polypeptide product has not been identified for UL53. However, for UL20, a 22K protein product has been identified. The observed Mr is again close to that predicted (24K; Davison & Scott, 1986b). The UL20 protein does not appear to be glycosylated but preliminary evidence suggests that it is a virion component (results not shown).

This paper reports the initial investigation of four putative integral membrane proteins of HSV-1. The functions of these proteins are unclear. Their potential ability to cross a membrane several times may suggest a role in signal transduction or as ion channels. Interestingly, VZV gene 15, which corresponds to HSV-1 gene UL43, contains a periodic charge pattern similar to that found in voltage-gated ion channels (Karlin et al., 1989). However, this charge pattern is not conserved in UL43. Alternatively, by interacting with tegument proteins via their more hydrophilic regions, these proteins could have a role in tegument–envelope interactions within the virus particle. As already mentioned, the HCMV gene equivalent to UL10 has been shown to be a virion component (Lehner et al., 1989). HCMV appears to encode a large number of multiply hydrophobic proteins, several of which show homology with G-coupled receptors (Chee et al., 1990). However, the function of these hydrophobic proteins is also not known.

To begin to elucidate the function of the HSV-1 proteins, it will be useful to express the ORFs transiently, alone or in combination, and to construct transformed cell lines carrying integrated copies of these genes. Such cell lines should also allow the isolation of viruses carrying mutations in the apparently essential genes UL20 and UL53.

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