The product of the US10 gene of herpes simplex virus type 1 is a capsid/tegument-associated phosphoprotein which copurifies with the nuclear matrix

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We have identified the herpes simplex virus type 1 (HSV-1) US10 gene product using rabbit polyclonal antisera raised against a recombinant 6xHis-US10 fusion protein expressed in Escherichia coli. The antiserum reacted specifically with 34 and 36 kDa proteins in HSV-1 KOS-infected cells as shown by Western blotting and immunoprecipitation experiments. The 36 kDa protein was immunoprecipitated with the US10 antiserum from 32P-labelled lysates of Vero cells infected with HSV-1 KOS, demonstrating that the US10 protein was phosphorylated. Indirect immunofluorescence studies localized the US10 protein mainly to nuclei as large discrete particles at later times post-infection (p.i.), and nuclear fractionation studies revealed that the protein was tightly associated with the nuclear matrix. Moreover, analysis of isolated intracellular capsids showed that both phosphorylated and unphosphorylated forms of the US10 protein were also associated with the capsid/tegument. These results indicate that the US10 gene of HSV-1 encodes a capsid/tegument-associated phosphoprotein which copurifies with the nuclear matrix.

Introduction

The genome of herpes simplex virus type 1 (HSV-1) comprises about 152,000 bp of double-stranded DNA and encodes at least 84 genes (Roizman & Sears, 1996). Recent studies have shown that approximately half the genes are not essential for replication in cell culture. These dispensable gene products, however, are thought to be important for virus growth and spread in the natural host (Ward & Roizman, 1994).

A cluster of dispensable genes has been found in the unique short (US) region of the HSV genome (McGeoch et al., 1985). To date, 14 genes have been identified in the US region (Georgopoulou et al., 1993; McGeoch et al., 1985; Roizman & Sears, 1996), and all but one gene (US6, glycoprotein D) have been reported to be non-essential in cell culture. These US gene products include regulatory protein (US1), protein kinase (US3), glycoproteins G (US4), J (US5), I (US7) and E (US8), a phosphoprotein localized in the nucleoli (US8.5), a virion phosphoprotein (US9), a site-specific RNA-binding protein (US11) and a peptide transporter-binding cytoplasmic protein ICPI74 (US12), as well as US2 and US10 gene products whose functions have not yet been identified (Frame et al., 1986; Georgopoulou et al., 1995; Roizman & Sears, 1996).

The US10 gene of HSV-1 encodes a polypeptide of 313 amino acids predicted to have a molecular mass of 33 kDa (McGeoch et al., 1985). Homologues of the US10 protein are encoded by herpes simplex virus type 2 (HSV-2) (Brown & Harland, 1987), varicella-zoster virus (Davison & Scott, 1986), equine herpesvirus type 1 (EHV-1) (Telford et al., 1992), and Marek’s disease virus (Sakaguchi et al., 1992). US10 homologues of EHV-1 and HSV-1 are known to possess a sequence of 13 amino acids (C-X3-C-X3-H-X3-C) that is a perfect match to the consensus zinc-finger motif (Holden et al., 1992). To our knowledge, however, the product of the US10 gene has not been characterized.

In this study, rabbit polyclonal antisera specific for the US10 protein were made and the expression and properties of the US10 protein were characterized.

Methods

- Cells and viruses. Vero cells, a stable line of African green monkey kidney cells, were grown in Eagle’s minimal essential medium (MEM) supplemented with 5% calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, and were used throughout this study. Wild-type HSV-1 strain KOS and HSV-2 strain 186, an HSV-1 variant N38 which has a
Fig. 1. (a) Schematic representation of the HSV-1 genome showing the US10 gene. The HSV-1 genome is shown with the unique long (UL) and unique short (US) regions, the terminal repeats (TR, and TRs), and interminal repeats (IRs and IRS). The genome is not shown to scale. Boxes and arrows indicate ORFs and mRNAs, respectively. Vertical dotted lines represent the 3' termini of respective mRNA. (b) Plasmid pET28-US10 was transformed into bacteria. The bacteria were grown in the absence (lane 1) or presence (lane 2) of IPTG, and then solubilized and separated by SDS–PAGE. The lysates of bacteria grown in the presence of IPTG were treated with 1% NP40, separated into soluble (lane 3) and insoluble (lane 4) fractions and separated by SDS–PAGE. The mobilities of molecular mass markers are shown in lane M. The arrowhead indicates the induced US10 fusion protein. (c) Detection of the US10 gene product in infected cells. Mock- (lane 1), HSV-1 KOS- (lane 2), SP23- (lane 3), N38- (lane 4), and HSV-2 186- (lane 5) infected cells were harvested at 20 h p.i., separated by SDS–PAGE, and analysed by Western blotting using the US10 antiserum. The mobilities of molecular mass markers are shown on the left. The arrowhead indicates the position of the US10 protein. (d) Identification of the US10 gene product by immunoprecipitation. KOS- (lanes 2, 4) and N38- (lanes 1, 3) infected cells were labelled with [35S]methionine from 15–18 h p.i. The cells were detergent...
deletion in the US9, US10, US11 and US12 genes, and its parental isolate SP23 of strain Patton were used in this study (Nishiya et al., 1993; Jiang et al., 1995). Viruses were propagated and titrated on Vero cells. Subconfluent Vero cells were infected with a multiplicity of 3 p.f.u. per cell.

**DNA manipulation.** The US10 open reading frame (ORF) is located between nucleotide positions 144156 and 145094 of the HSV-1 genome (McGeoch et al., 1985). The US10 coding sequence was cloned by PCR amplification from HSV-1 KOS genomic DNA, using the synthetic oligonucleotide US10f (GTGCA TATGA TCAA CGCGG GGGC) as the forward primer and the synthetic oligonucleotide US10r (CCCAC TCGAG TCGCG ACCTG TTAG) as the reverse primer. *NdeI* and *XhoI* sites were incorporated into the forward and reverse primers, respectively, to facilitate cloning. The PCR consisted of an initial 3 min denaturation step at 94 °C, followed by 30 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 2 min). The 30th cycle concluded with a 30 min extension step at 72 °C. The PCR product was digested with *NdeI* and *XhoI*, and cloned in frame downstream of the region encoding the initiating ATG plus six histidine residues in the *E. coli* expression vector pET28a (Novagen) to give plasmid pET28-US10. The expression of 6xHis-tagged US10 protein is regulated by an IPTG-inducible lac operator sequence and a phage T7 promoter. Translation is expected to terminate at the US10 stop codon. Plasmid pET28-US10 was transformed into *E. coli* strain BL21(DE3) (Novagen) and, following induction with IPTG, expressed large quantities of 6xHis–US10 fusion protein. The fusion protein was not soluble in 0-1% SDS, and so an insoluble fraction was used as an antigen.

**Generation of polyclonal antiserum in rabbits.** Antiserum were produced in two rabbits by immunization with an emulsion containing approximately 0.7 mg of *E. coli*-expressed 6xHis–US10 fusion protein in the form of insoluble inclusion body preparation in Freund’s complete adjuvant. Inoculations were subcutaneous injections on the shaven back. Subsequent boosts used Freund’s incomplete adjuvant and 0.7 mg of the inclusion body preparation. A total of three booster injections were given, each at 3-week intervals after the primary injection. Ten days after the last boost, antiserum were collected from the ear. Both the preimmune and immune antiserum were extensively adsorbed against acetone powder of *E. coli* strain BL21(DE3) and uninfected Vero cells prior to use, as described by Harlow & Lane (1988).

**Metabolic labelling and immunoprecipitation.** Metabolic labelling was performed as described previously (Yamashita et al., 1990). Infected Vero cells were labelled for 3 h with 200 µCi/ml [35S]methionine for 3 h from 15–18 h post-infection (p.i.). Labelling with [32P]orthophosphate was similar to labelling with [35S]methionine. Immunoprecipitation was performed under denaturing conditions.

**Western blotting.** Proteins were electrophoretically transferred from 12% SDS–PAGE gels to Hybond-PVDF membranes (Amersham) as described by Towbin et al. (1979), and probed with the polyclonal US10 antiserum. Bound antibodies were detected using horseradish peroxidase-linked anti-rabbit IgG (Amersham) and the ECL Western blotting detection system (Amersham).

**Indirect immunofluorescence.** An indirect immunofluorescence assay was performed essentially as described by Ward et al. (1990). Vero cells were infected with KOS or N38, fixed with cold acetone at the indicated times, reacted with PBS containing 1% BSA and 20% normal human plasma to block nonspecific reactivity, reacted with the US10 antiserum, and reacted with fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin G (Dako).

**Preparation of nuclear matrix.** Nuclei and nuclear matrix were isolated from cells as described previously (Tsutsui et al., 1983).

**Fractionation of intracellular viral capsids.** Intracellular HSV capsids were prepared as described by Thomsen et al. (1994) and Newcomb et al. (1993). Infected cells were harvested 24 h p.i. by centrifugation and were washed three times with PBS. The cell pellets were resuspended in 1 ml of 2× capsid lysis buffer (40 mM Tris–HCl, 1 M NaCl, 2 mM EDTA, 2% Triton X-100 pH 7.5), freeze-thawed three times, disrupted by sonication and the debris pelleted at 5000 r.p.m. for 10 min. The cleared lysates were layered onto a 12 ml gradient of 20–60% (w/v) sucrose in PBS and centrifuged at 22,000 r.p.m. for 60 min in a Hitachi RPS40 rotor. A 500 µl sample of each fraction was collected by dripping the gradient through an 18-gauge needle, and the position of virus capsids was determined by silver staining following SDS–PAGE.

**Results**

**Preparation and specificity of anti-US10 protein antiserum**

As a first step toward the study of the US10 protein, rabbit polyclonal antiserum specific to this protein were raised using an *E. coli*-produced recombinant US10 fusion protein as antigen. For this purpose the plasmid pET28-US10 was constructed. This plasmid contained all amino acids of the US10 ORF fused in frame in the expression vector pET28a. High levels of the resulting 39 kDa fusion protein were expressed in *E. coli* following induction by IPTG (Fig. 1b). The other two intense bands below the arrowhead, 34 and 32 kDa, probably represent breakdown products of the 6xHis–US10 fusion protein (lane 4).

To examine the reactivity and specificity of the anti-US10 antiserum, Western blotting experiments were performed. Vero cells were mock-infected or infected with HSV-1 KOS, SP23, N38 or HSV-2 186 at 3 p.f.u. per cell. HSV-1 N38 has a deletion of four genes, US9, US10, US11 and US12, and serves as control for serum specificity. Fig. 1(c) shows that the US10 antiserum reacted with two bands in the HSV-1 KOS-infected cell lysates with apparent molecular masses of 36 and 34 kDa. This antiserum also reacted with three bands in the HSV-1 SP23-infected cell lysates with apparent molecular masses of 39, 37 and 35 kDa. However, none of these proteins was detected in mock-infected HSV-1 N38- and HSV-2 186-infected cells. Inasmuch as these proteins exhibited electrophoretic mobilities similar to the predicted molecular mass of the US10 protein and were absent from lysates of N38-infected cells, it is reasonable to conclude that they were the products of the HSV-1 KOS US10 gene.

To analyse further the specificity and reactivity of the US10 antiserum, immunoprecipitation experiments were performed. The results showed that the US10 antiserum immunoprecipi-
proteins in HSV-1 KOS-infected cells. US10 antiserum specifically reacted with 36 and 34 kDa antiserum. These experiments thus demonstrated that the product would be co-immunoprecipitated with the US10 precipitation, proteins tightly associated with the US10 intracellular capsids. Under the conditions used for immunoprecipitation, the proteins reacted with prominent protein bands with molecular masses of 34 and 36 kDa, which were present predominantly in the nuclear matrix. The distinctive electrophoretic profile shown in Fig. 4 was reproducible. The US10 antiserum did not react with lysates of mock-infected cells (data not shown). The abundant species between 50 and 80 kDa observed in lanes 3 and 4 appeared to be gE and gI, which form a hetero-oligomer and act as an Fc receptor (Johnson et al., 1988). The band larger than 100 kDa detected in lane 4 was VP5. As described later, the US10 product was highly insoluble, and associated with the nuclear matrix and intracellular capsids. Under the conditions used for immunoprecipitation, proteins tightly associated with the US10 product would be co-immunoprecipitated with the US10 antiserum. These experiments thus demonstrated that the US10 antiserum specifically reacted with 36 and 34 kDa proteins in HSV-1 KOS-infected cells.

Expression of US10 protein in infected cells

The expression of the US10 protein during a lytic HSV-1 replication cycle in infected cells was analysed by determining the kinetics of appearance of this protein. The temporal class of HSV-1 genes to which the US10 gene belongs was determined from kinetic data obtained by using the DNA synthesis inhibitor phosphonoacetic acid (PAA). To assess the kinetics of appearance of the UL10 products, Vero cells were infected with HSV-1 KOS and extracts of cellular proteins were prepared at the indicated times p.i. As shown in Fig. 2, the US10 protein was detected at 10 h p.i., increased in amount and reached its highest level at 15 h p.i. No appreciable change in the accumulated amount of the protein was detected until 25 h p.i. Since the US10 protein appeared late in infection, it was reasoned that the gene may belong to either the γ1 or γ2 class of HSV genes. To define more precisely the temporal class to which the gene belongs, infected cells were maintained in the presence of 300 µg/ml PAA to inhibit viral DNA synthesis. As a control, the protein extracts were also probed with monoclonal antibody against ICP35, which has been shown to belong to class γ1. ICP35 was detected despite the presence of PAA (data not shown). In contrast, the US10 protein was detected only in the absence of PAA, suggesting that US10 protein is synthesized as a γ2 gene.

Intracellular localization of US10 protein

The intracellular distribution of the US10 protein in HSV-1-infected cells was analysed by indirect immunofluorescence experiments. As shown in Fig. 3, specific fluorescence was observed in KOS-infected cells, whereas no fluorescence was observed in N38-infected cells (Fig. 3d). At 8 and 12 h p.i., cells showed perinuclear staining while the nucleoplasm appeared negative (Fig. 3a, b). At 20 h p.i. the staining was seen in the nucleus as relatively large, discrete particles (Fig. 3c). At earlier times after infection (3 and 5 h p.i.), no specific fluorescence was detectable (data not shown).

Association of US10 protein with the nuclear matrix of infected cells

Preliminary studies showed that US10 protein was highly insoluble in non-ionic detergent extracts of sonicated cells. To examine the solubility, we separated HSV-1 KOS-infected cells into detergent-soluble and insoluble fractions with 1% Triton X-100 plus 150 mM NaCl, and determined the localization of the US10 protein by Western blotting. As shown in Fig. 4, the US10 protein was detected only in the detergent-insoluble fraction. The distribution of US10 protein in the nuclei of infected cells raised the possibility that US10 protein is associated with the nuclear matrix fraction. To test this hypothesis, KOS-infected cells were fractionated as described previously (Tsutsui et al., 1983). As shown in Fig. 4, ICP8 was detected in the supernatant obtained after DNase treatment. In contrast, VP5 was markedly concentrated in the nuclear matrix (Ben-Ze’ev et al., 1983; Quinlan & Knipe, 1983). There were several additional proteins which were associated with the nuclear matrix, some of which could not be distinguished clearly from cellular proteins. The distinctive electrophoretic profile shown in Fig. 4 was reproducible. The US10 antiserum reacted with prominent protein bands with molecular masses of 34 and 36 kDa, which were present predominantly in the nuclear matrix.

Phosphorylation of US10 protein

In an attempt to explain the two bands associated with the US10 protein, we investigated whether US10 protein was
**Product of US10 gene of HSV-1**

**Fig. 3.** Intracellular localization of the US10 protein determined by indirect immunofluorescence. HSV-1 KOS- and N38-infected cells were acetone-fixed at the indicated times and processed for indirect immunofluorescence as described in Methods. KOS-infected cells at (a) 8, (b) 12 and (c) 20 h p.i.; (d) N38-infected cells at 20 h p.i.

**Fig. 4.** Association of the US10 protein with the nuclear matrix. HSV-1 KOS-infected cells were harvested at 20 h p.i. and separated into detergent-soluble (lane 1) and insoluble (lane 2) fractions. Equivalent amounts of each fraction were separated by SDS–PAGE and analysed by Western blotting using US10-specific polyclonal rabbit antiserum. HSV-1 KOS-infected cells were harvested at 20 h p.i. and separated into subnuclear fractions as described in Methods. Three fractions – the supernatant after DNase I treatment (lane 3), pellet after DNase I treatment (lane 4) and pellet after high salt treatment (nuclear matrix) (lane 5) – were separated by SDS–PAGE and the gel was stained with silver. The identities of VP5 and ICP8 are shown. The same blot was probed with US10 antiserum (lanes 6, 7, 8). The mobilities of molecular mass markers are shown. The arrowheads indicates the position of the US10 protein.
US10 is associated with intracellular virus capsids

To determine if the US10 protein is a component of the virus capsid, cell lysates of KOS-infected cells were subjected to sucrose density-gradient centrifugation and fractions were collected as described. The silver-stained protein profiles of these fractions corresponded to those described previously (Tatman et al., 1994; Patel & Maclean, 1995). The identity of the capsid-containing fractions was confirmed on silver-stained SDS–PAGE gels, which revealed the presence of VP22a, indicative of B capsids, in fractions 14 and 15 (Fig. 6a). However, there was no significant change in the abundance of polypeptides indicative of C capsids (VP5, VP19 and VP23) in fractions 1–12. Western blotting of fractions using the US10 antiserum revealed that the US10 protein was predominantly present in fractions 4–8 (probably representing C capsids) and 13–16 (B and A capsids) (Fig. 6b). The US10 proteins were not detected beyond fraction 16. These data suggest that the US10 protein was a capsid/ tegument component and that both 36 kDa phosphorylated and 34 kDa unphosphorylated forms of the US10 product were associated with the capsid/ tegument.

Discussion

In the present study, we produced 6xHis-tagged US10 protein in E. coli and used it as an antigen to produce a rabbit polyclonal antiserum specific for the US10 protein. The
antiserum was found to react specifically with 34 and 36 kDa proteins in HSV-1 KOS-infected cells in both Western blotting and immunoprecipitation experiments. Nucleotide sequence analysis of the coding sequence of US10 predicts a 33.5 kDa basic protein; thus the molecular sizes of the proteins which reacted with the US10 antiserum were consistent with the predicted molecular mass. HSV genes consist of several kinetic groups, designated α, β and γ, whose expression is coordinated, regulated and sequentially ordered in a cascade fashion (Roizman & Sears, 1996). γ gene expression is linked to the onset of viral DNA synthesis. Whereas γ1 polypeptides are made in the absence of viral DNA synthesis, γ2 polypeptides stringently require amplification of viral DNA for their synthesis. Our data showed that the US10 protein was maintained at relatively high levels during the late period of infection, and that the expression of the US10 gene was inhibited by addition of PAA, an inhibitor of viral DNA synthesis. Expression of ICP35, a well-known gene product of HSV, was a component of intracellular capsids. Three types of capsids are seen in HSV-infected cells (Gibson & Roizman, 1972; Heilman et al., 1993). Type B capsids are tightly associated with the nuclear matrix in KOS-infected cells (Capco et al., 1982), and is also defined as the nuclear skeleton which maintains the physical shape and integrity of the nucleus. It has been suggested that the nuclear matrix plays an active role in many molecular processes, such as chromatin organization, DNA replication, gene transcription, RNA splicing and attachment of supercoiled DNA loops (reviewed by Berezney, 1991). In addition, previous studies have shown by electron microscopy that empty HSV capsids are tightly bound to the fibrous structure of the nuclear matrix (Tsutsui et al., 1983), and the UL31 product of HSV-1 has been shown to be associated with the nuclear matrix (Chang & Roizman, 1993). These observations suggest that the nuclear matrix may be involved in HSV replication.

Analysis of purified capsids showed that the US10 protein is a component of intracellular capsids. Three types of capsids are seen in HSV-infected cells (Gibson & Roizman, 1972). Type A or empty capsids lack viral DNA and internal structure; type B or intermediate capsids also lack viral DNA but possess an internal proteinaceous scaffold; and type C or full capsids contain viral genomic DNA (reviewed by Rixon, 1993). Type B capsids are known to be composed of seven proteins designated VP5, VP19C, VP21, VP22a, VP23, VP24 and VP26 (Gibson & Roizman, 1972; Heilman et al., 1979; Cohen et al., 1980), and the genes to which these capsid proteins have been assigned are UL19, UL38, UL26, UL26.5, UL18, UL26 and UL35, respectively (Preston et al., 1983, 1992; Weller et al., 1987; Perduiset et al., 1989; Rixon et al., 1990; Liu & Roizman, 1991; Davison et al., 1992; McNabb & Courtney, 1992). With the exception of the internal scaffolding proteins VP21 and VP22a, the protein composition of type A and C capsids is identical to that of B capsids (see Rixon, 1993). We demonstrated that the US10 protein was cofractionated with the viral capsid/tegument in sucrose gradient sedimentation, and this pattern is very similar to that of UL6 protein, which is a minor component of HSV-1 capsids (Patel & Maclean, 1995). Although the exact location of the US10 protein in the virion remains uncertain, it appears that the protein associates with the capsid rather than the tegument.

Zinc-finger proteins are characterized by the zinc-finger domain, a conserved sequence motif that mediates DNA binding. This sequence motif was first discovered in TFIIB, a 5S gene-specific transcription factor from Xenopus, where it is present nine times (Miller et al., 1985). The motif contains conserved pairs of cysteine and histidine residues that coordinate a zinc atom, thereby folding the domain into a defined three-dimensional structure (Parraga et al., 1988; Lee et al., 1989). In addition, it has been shown that there are several classes of finger proteins according to the number and position of the cysteine and histidine residues available for zinc coordination (Evans & Hollenberg, 1988). The US10 protein contains a typical zinc-finger motif, designated C-X3-C-X3-H-X3-C (C and H indicate cysteine and histidine residues, respectively; X represents any amino acid), and this pattern of motif resembles those of the retroviral nucleocapsid proteins. For example, Rauscher murine leukaemia virus contains a single sequence of the form C-X2-C-X4-H-X4-C, and human immunodeficiency virus contains two such sequences (Darlix et al., 1995). Proteins which contain the CCHC motif have been shown to have specific interactions with single-stranded nucleic acids (Berg, 1990). We have not yet succeeded in showing that either US10 fusion protein or US10 protein itself has the ability to interact with single-stranded RNA or DNA.

Previous studies have shown that US10 is not essential for growth of the virus in tissue culture, but the role of the US10 protein in the virus life-cycle remains uncertain (Nishiyama et al., 1993; Jiang et al., 1995). As regards the function of the US10 protein, it is interesting that US10, US11 and US12 genes specify three mRNAs with distinct 5’ termini but a common 3’ terminus, and that the ORF of the US10 gene has a 110 codon out-of-frame overlap with that of the US11 gene (Rixon & McGeoch, 1984). It has been reported that the US11 protein is an RNA-binding regulatory protein which specifically and stably associates with 60S ribosomal subunits and nucleoli, and is incorporated into virions (Roller & Roizman, 1992; Diaz et al., 1993). The function of the US10 protein and the nature of its association with capsid proteins and the nuclear matrix remain to be determined.
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References


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