

Identification and characterization of the UL14 gene product of herpes simplex virus type 2

K. Wada, F. Goshima, H. Takakuwa, H. Yamada, T. Daikoku and Y. Nishiyama

Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466-8550, Japan

The UL14 gene of herpes simplex virus type 2 (HSV-2) is predicted to encode a 219 amino acid protein with a molecular mass of 23 kDa. In this study, the HSV-2 UL14 gene product has been identified by using a rabbit polyclonal antiserum raised against a recombinant 6 × His–UL14 fusion protein expressed in *E. coli*. The antiserum reacted specifically with 34, 33 and 28 kDa proteins in HSV-2-infected cell lysates and also with a 34 kDa protein produced by *in vitro* transcription and translation reactions, suggesting that the 34 kDa protein is the primary translation product of the UL14 gene. The protein was synthesized at late times post-infection (p.i.) and was not detectable in the presence of the viral DNA synthesis inhibitor acycloguanosine. Indirect immunofluorescence studies localized the UL14 protein both to the nucleus and to perinuclear regions of the cytoplasm, and the nuclear UL14 protein was found to co-localize with the scaffolding protein ICP35 at 9 h p.i. However, the protein accumulated in a perinuclear region of the cytoplasm at 12 h p.i., while most of the ICP35 protein localized within assemblons in the nucleus. Although no detectable UL14 protein was associated with intracellular capsids isolated in the presence of 0.5 M NaCl, it was detected in purified virions. Furthermore, the UL14 protein expressed alone was detected both in the nucleus and in the cytoplasm at 24 h after transfection, but was mainly localized to the cytoplasm at later times.

Introduction

Herpes simplex virus (HSV) is a large, enveloped DNA virus and the genome encodes at least 74 different genes (Roizman & Sears, 1996; Dolan *et al.*, 1998). Of these, approximately half are essential for virus replication in cell cultures (Roizman, 1996). These essential genes encode proteins involved in regulation of gene expression, viral DNA replication and packaging of viral DNA into virions, as well as structural proteins involved in entry of virus into cells and protection of viral DNA. Although recent studies have revealed the properties and functions of many essential genes, the functions of several genes are still unknown (Roizman & Sears, 1996; Dolan *et al.*, 1998).

The UL14 genes of HSV types 1 (HSV-1) and 2 (HSV-2) are located in a region of the HSV genome that is conserved in the whole herpesvirus family, and homologues are detected in alpha-, beta- and gammaherpesviruses including varicella

zoster virus (Davison & Scott, 1986), human cytomegalovirus (HCMV) (Chee *et al.*, 1990), human herpesvirus-6 (Gompels *et al.*, 1995) and Epstein–Barr virus (Baer *et al.*, 1984). In other words, the UL14 gene belongs to the core genes of herpesviruses (McGeoch, 1992). The UL14 genes of both HSV-1 and HSV-2 are predicted to encode 219 amino acid proteins with molecular masses of 23 kDa (McGeoch *et al.*, 1988; Dolan *et al.*, 1998) and their coding regions overlap with the coding region of the UL13 gene, which encodes a protein kinase (Dolan *et al.*, 1998; Daikoku *et al.*, 1997). Although the HSV-1 UL14 gene has been reported not to be dispensable for replication (Roizman, 1996), no information on its properties or function is available at present. This study was thus undertaken to characterize the UL14 product of HSV-2.

Methods

■ **Cell and virus.** 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 U/ml penicillin and 100 µg/ml streptomycin and were used throughout this study. HSV-1 strain KOS and HSV-2 strain 186, derived from single plaques, were propagated in

Author for correspondence: Yukihiro Nishiyama.

Fax +81 52 744 2452. e-mail ynishiya@tsuru.med.nagoya-u.ac.jp

Vero cells by infecting at a low multiplicity (0.01 p.f.u. per cell). Infected cells were harvested when almost all cells exhibited cytopathic effects. After freezing and thawing three times and eliminating cell debris at 3000 r.p.m. for 10 min, virus was stored at -80°C .

DNA manipulations. The UL14 ORF is located between nucleotide positions 28 229 and 28 888 of the HSV-2 genome (Dolan *et al.*, 1998). The UL14 coding sequences were cloned by PCR amplification from plasmid DNA containing the HSV-2 *Hind*III b fragment (Tsurumi *et al.*, 1986), using UL14f (5' GGGCGAATTCATGAGCCGAGACGCC) as the forward primer and UL14r (5' GAGCCTCGAGTCACTCGCCATCGGG) as the reverse primer. *Eco*RI and *Xho*I sites were incorporated into the forward and reverse primers, respectively, to facilitate cloning. The PCR consisted of an initial 5 min denaturation step at 94°C followed by 30 cycles of denaturation (94°C , 30 s), annealing (60°C , 30 s) and extension (72°C , 2 min) and a final extension at 72°C for 7 min. The PCR product was digested with *Eco*RI and *Xho*I and cloned in-frame and downstream of the region encoding the initiating ATG plus six histidine residues ($6 \times \text{His}$) in the *E. coli* expression vector pET-28a (Novagen) to give plasmid pET28-UL14. The expression of $6 \times \text{His}$ -tagged UL14 protein is regulated by the IPTG-inducible *lac* operator sequence and a phage T7 promoter. Translation is expected to terminate at the UL14 stop codon. Plasmid pET28-UL14 was transformed into *E. coli* strain BL21(DE3), which, following induction with IPTG, expressed large quantities of $6 \times \text{His}$ -tagged UL14 fusion protein.

The expression plasmid pcDNA3-UL14 was constructed for expression of the UL14 gene in cultured cells. Cleavage of pET28-UL14 with *Eco*RI and *Xho*I released the UL14 ORF and this DNA fragment was ligated into the multicloning site of pcDNA3.1 (+) (Invitrogen) to give pcDNA3-UL14, which expressed the UL14 gene under the control of the HCMV immediate early promoter. Plasmid pcDNA3-UL14 was also used for *in vitro* transcription and translation, since it contains T7 promoter upstream of the multicloning site.

Plasmid transfection. Cells were transfected by using a lipofection reagent according to the protocols recommended by the supplier (Gibco BRL).

Preparation of polyclonal antisera. Antisera were produced in two rabbits by immunization with an emulsion containing approximately 0.6 mg *E. coli*-expressed, $6 \times \text{His}$ -tagged UL14 protein in the MPL+TDM+CWS emulsion adjuvant system (RIBI ImmunoChem Research). Inoculations were by subcutaneous injection on the shaven back. The same adjuvant and 0.6 mg of the inclusion body preparation were used for subsequent boosts. A total of three booster injections were given at 3 week intervals after the primary injection. One week after the last immunization, we collected blood from the heart. Anti-UL42 polyclonal antisera were also produced in rabbits by immunization with *E. coli*-expressed, $6 \times \text{His}$ -tagged HSV-2 UL42 protein as described above.

Western blotting. At the times indicated, the denatured, solubilized polypeptides from mock-infected and HSV-infected cell lysates were electrophoretically separated on SDS-polyacrylamide gels and electrically transferred to Hybond PVDF membranes (Amersham Japan). Nonspecific protein binding was blocked by treating membranes at 4°C overnight with Tris-buffered saline (TBS; 25 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% skim milk and 0.05% Tween 20. The membranes were washed once with TBS and incubated at 37°C for 1 h with a 1:5000 dilution of the UL14 antiserum in TBS containing 0.1% BSA and 0.05% Tween 20. After washing three times with TBS containing 0.05% Tween 20, the membranes were incubated at 37°C for 1 h with a 1:7000 dilution of goat anti-rabbit peroxidase-labelled second

antibody (BIO SOURCE). The membranes were then washed three times with TBS containing 0.05% Tween 20, treated with the ECL Western blotting detection system (Amersham Japan) and exposed to Hyperfilm-ECL (Amersham Japan).

Immunofluorescence microscopy. Vero cells were grown on coverslips and were either mock-infected or infected with HSV-2 at a multiplicity of 3 p.f.u. per cell. At various times after infection, the cells were fixed in cold acetone. Coverslips were then incubated for 1 h at room temperature with a solution of 20% human serum in PBS in order to reduce levels of background produced as a consequence of the affinity-binding of rabbit immunoglobulin to the Fc receptor formed by glycoproteins E and I. The cells were reacted for 1 h at 37°C with anti-UL14 serum diluted 1:1000 in PBS containing 0.1% BSA, washed in excess PBS and then reacted for 1 h at 37°C with a 1:60 dilution of FITC-conjugated goat anti-rabbit immunoglobulin in blocking solution. Fluorescent images were viewed with a Zeiss laser scanning microscope LSM510.

***In vitro* transcription and translation.** For *in vitro* transcription and translation, the Single Tube protein system 2, T7 (Novagen) was used. In the standard reaction, the DNA template (0.5 μg pcDNA3-UL14 DNA) was transcribed in 10 μl at 30°C for 15 min, followed by the addition of 40 μl translation mixture and continued incubation at 30°C for 60 min. *In vitro* translation products were analysed by Western blotting after SDS-PAGE.

Preparation of nuclear fractions. Infected cells were washed three times with PBS. The cell were suspended in RSB buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl_2 , pH 7.4) and left on ice for 5 min. After adding NP-40 alone or with deoxycholic acid (DOC) to final concentrations of 0.5%, the cells were homogenized by ten strokes with a glass homogenizer. The homogenate was layered over 0.5 M sucrose in RSB buffer and centrifuged at 1500 r.p.m. for 5 min. The nuclear pellet was then washed again with TM sucrose buffer (0.25 M sucrose, 5 mM MgCl_2 , 50 mM Tris-HCl, pH 7.4). After sedimentation, the nuclei were resuspended in TM sucrose buffer and the purity and morphology of isolated nuclei were examined with a light microscope after staining with 0.1% toluidine blue.

Fractionation of intracellular viral capsids. Vero cells were infected with HSV-2 at a multiplicity of 3 p.f.u. per cell and incubated at 37°C for 15 h. Infected cells were harvested by centrifugation and washed three times with PBS. Cell lysates were resuspended in 1 ml lysis buffer I (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5) or lysis buffer II (20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.5) and disrupted by sonication and the debris was pelleted at 3000 r.p.m. for 10 min. The supernatant was layered onto a 12 ml linear gradient of 10–50% (w/v) sucrose in buffer I or buffer II and centrifuged at 24000 r.p.m. for 40 min in a Hitachi RPS 40 rotor. Five hundred μl fractions were collected from the bottom to the top and the position of virus capsids was determined by SDS-PAGE followed by silver staining.

Virion purification. Monolayers of Vero cells cultured in roller bottles (850 cm^2) were infected with HSV-2 at a multiplicity of 3 p.f.u. per cell. After 1 h adsorption at 37°C , maintenance medium containing 5% serum was added. HSV-2 virions were harvested from the extracellular medium at 36 h post-infection (p.i.). After removal of cell debris by low-speed centrifugation, virions were pelleted from the supernatant by centrifugation at 87000 g for 1 h. The virus suspension was layered onto a continuous 10–50% sucrose gradient, followed by centrifugation at 20000 r.p.m. for 1 h at 4°C . The peak virion-containing fractions were collected as described above, diluted in PBS and pelleted

again by centrifugation at 87000 *g* for 1 h. The virions were further purified by a second cycle of sucrose-gradient centrifugation.

Results

Preparation and specificity of anti-UL14 rabbit antiserum

To characterize the UL14 gene product, we first generated anti-UL14 rabbit antisera by using a recombinant HSV-2 UL14 fusion protein as antigen. The plasmid pET28-UL14 was constructed for this purpose as described in Methods and the UL14 fusion protein was expressed in *E. coli* by treatment with IPTG (Fig. 1*a*, lane 2). The UL14 fusion protein, with an apparent molecular mass of 38 kDa, was then purified by using the Prep Cell system (Bio-Rad) (Fig. 1*a*, lane 3) and the purified protein was used to immunize two rabbits. Western blotting analysis was done to examine the reactivity and specificity of the antisera (Fig. 1*b*). One of the antisera reacted strongly to proteins with apparent molecular masses of 34, 33 and 28 kDa in the lysate of HSV-2-infected Vero cells (Fig. 1*b*, lane 3), but did not react strongly with any proteins in the lysates of mock-infected (Fig. 1*b*, lane 1) or HSV-1-infected (Fig. 1*b*, lane 2) cells. Although there was no significant change in the reactivity of the antiserum to these 34, 33 and 28 kDa bands after pre-adsorption with a lysate of control *E. coli* (Fig. 1*b*, lane 6), reactivity was clearly eliminated by pre-adsorption with a lysate of *E. coli* expressing the UL14 fusion protein (Fig. 1*b*, lane 9). Preimmune rabbit serum did not react with any specific proteins in HSV-1- or HSV-2-infected cells (data not shown). These results indicate that the antiserum could detect the UL14 proteins specifically in HSV-2-infected cells, and therefore we used this polyclonal antiserum for further experiments to characterize the UL14 gene product of HSV-2.

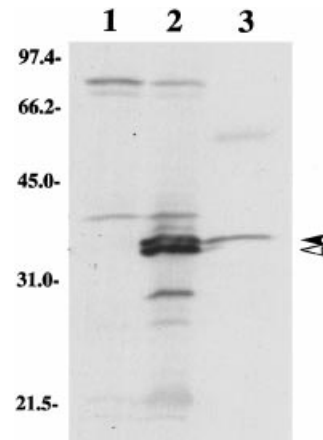


Fig. 2. *In vitro* transcription and translation of the UL14 gene. The UL14 gene under the control of the T7 promoter was transcribed by T7 RNA polymerase followed by translation in an optimized rabbit reticulocyte lysate. The *in vitro* translation products (lane 3) and lysates of mock-infected (lane 1) and HSV-2-infected cells (lane 2) were subjected to SDS-PAGE and analysed by Western blotting using the UL14 antiserum.

In vitro transcription and translation of the UL14 gene

There was a significant difference between the predicted molecular mass of the UL14 protein and the apparent molecular masses on SDS-polyacrylamide gels. We next performed *in vitro* transcription and translation experiments using the Single Tube protein system (Novagen). The system is based on transcription with T7 RNA polymerase followed by translation in an optimized rabbit reticulocyte lysate. The protein products were detected by the UL14 antiserum. The results are shown in Fig. 2, demonstrating that the primary translation product of the UL14 gene had a molecular mass of 34 kDa.

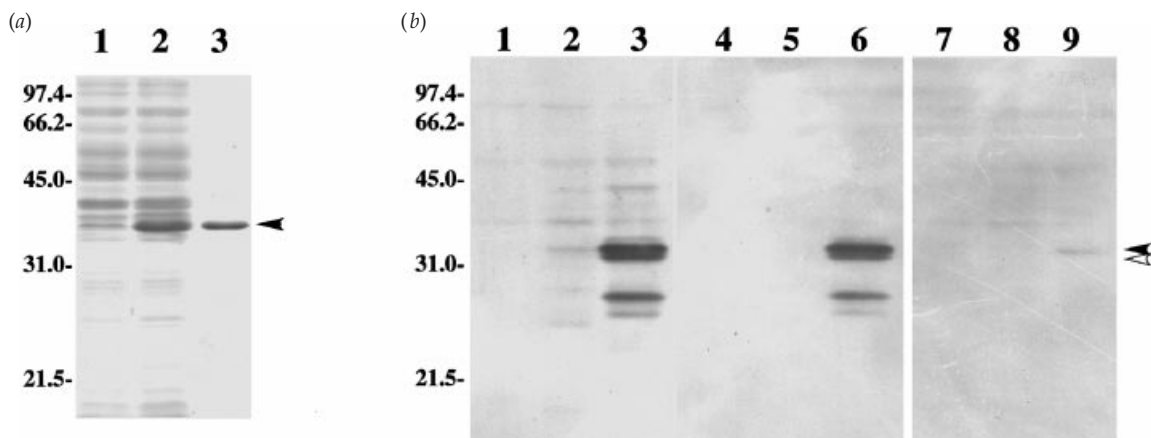


Fig. 1. (*a*) Induction of the His-tagged UL14 fusion protein in *E. coli*. *E. coli* cells harbouring pET28-UL14 were grown in the absence (lane 1) or presence (lane 2) of IPTG and the UL14 fusion protein was purified by using the Prep Cell system (Bio-Rad) (lane 3). Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. Sizes of molecular mass markers (in kDa) are shown on the left. (*b*) Reactivity and specificity of rabbit polyclonal antiserum against the His-tagged UL14 fusion protein. Vero cells were mock-infected (lanes 1, 4 and 7) or infected with HSV-1 (lanes 2, 5 and 8) or HSV-2 (lanes 3, 6 and 9) and harvested at 15 h p.i. Proteins were separated by SDS-PAGE and electrically transferred to Hybond PVDf membranes (Amersham Japan). The membranes were incubated with the UL14 antiserum (lanes 1–3), the UL14 antiserum pre-adsorbed with an *E. coli* lysate (lanes 4–6) or the UL14 antiserum pre-adsorbed with a lysate of *E. coli* expressing the UL14 fusion protein (lanes 7–9). The positions of molecular mass markers (in kDa) are shown on the left.

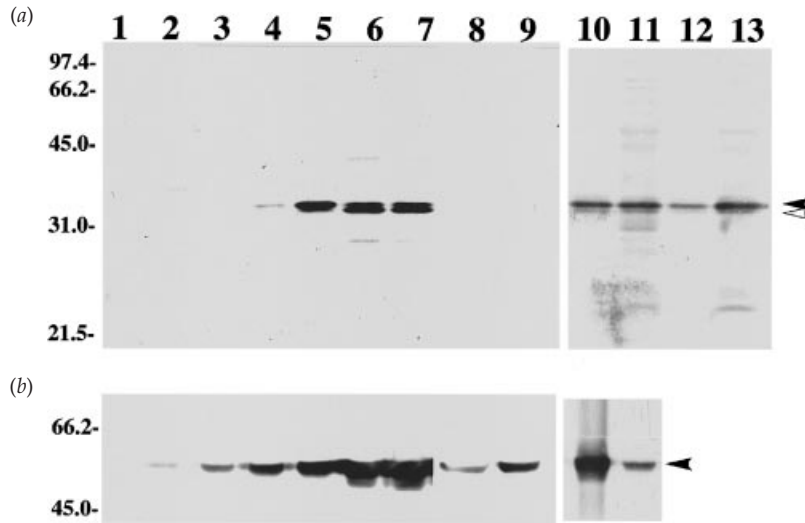


Fig. 3. Production of the UL14 protein in HSV-2-infected cells. Vero cells were mock-infected (lanes 1) or infected with HSV-2 (lanes 2–9) at a multiplicity of 3 p.f.u. per cell. The cells were cultured in the absence (lanes 1–7) or presence of 300 µg/ml ACV (lanes 8 and 9) and harvested at 3 (lanes 2), 6 (3), 9 (4 and 8), 12 (5), 15 (6 and 9) or 18 (7) h p.i. HSV-2-infected Vero cells 12 h p.i. were separated into nuclear (lanes 10 and 12) and cytoplasmic (11 and 13) fractions using either NP-40 (10 and 11) or NP-40 and DOC (12 and 13). Proteins were separated by SDS-PAGE and analysed by Western blotting using the UL14 antiserum (a) or the UL42 antiserum (b). The positions of molecular mass markers (in kDa) are shown on the left.

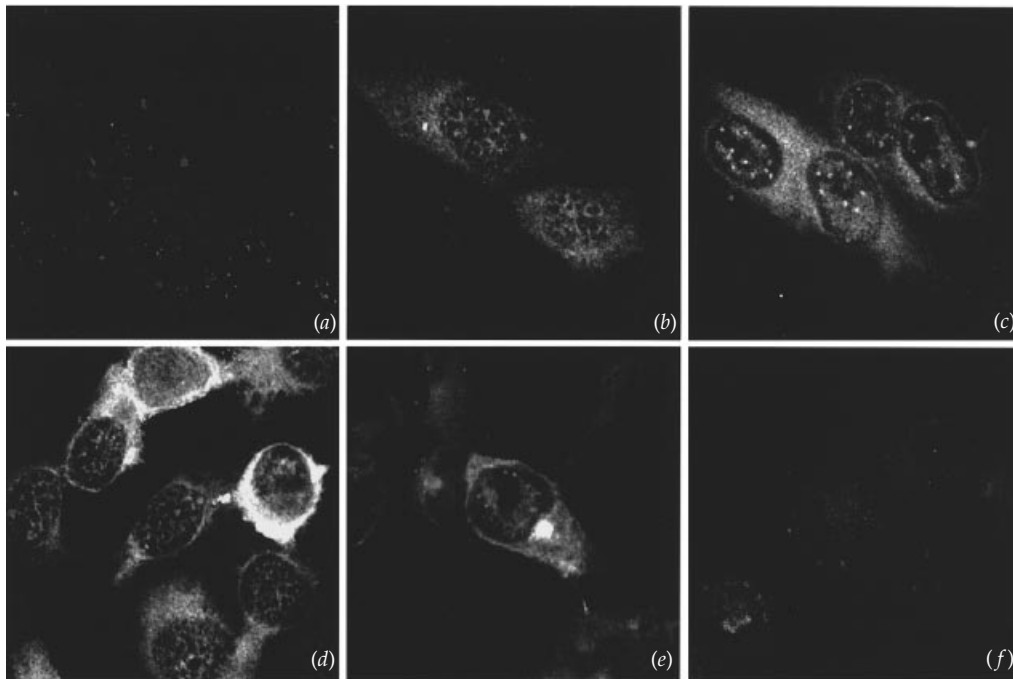


Fig. 4. Localization of the UL14 protein in HSV-2-infected cells. Mock-infected (a) and HSV-2-infected (b–f) Vero cells were fixed with cold acetone at 6 (b, c), 9 (d) and 12 h (e, f) p.i. and stained with the UL14 antiserum (a–e) or with preimmune serum (f).

Expression of the UL14 product in HSV-2-infected cells

The kinetics of UL14 protein expression in HSV-2-infected Vero cells were analysed by Western blotting. At various times after infection, cell lysates were subjected to electrophoresis, transferred to PVDF membranes and reacted with the UL14 antiserum. As shown in Fig. 3(a), the UL14 gene product was detectable at 9 h p.i. as a protein band with a molecular mass of 34 kDa and increased in amount until 12 h p.i. However, the 33 kDa protein species was not detected until

12 h p.i. In contrast, a well-characterized HSV early protein, the DNA polymerase accessory protein (the UL42 gene product), which was used as a control, was detected from 3 h p.i. (Fig. 3b).

To determine the dependence of production of the UL14 gene product on viral DNA synthesis, infected cells were maintained for various times after a 1 h adsorption period in the presence or absence of acycloguanosine (ACV) (300 µg/ml). No detectable UL14 protein was produced in the presence of ACV at 15 h p.i. (Fig. 3a, lane 9), while a significant amount of the UL42 protein was detected in the presence of

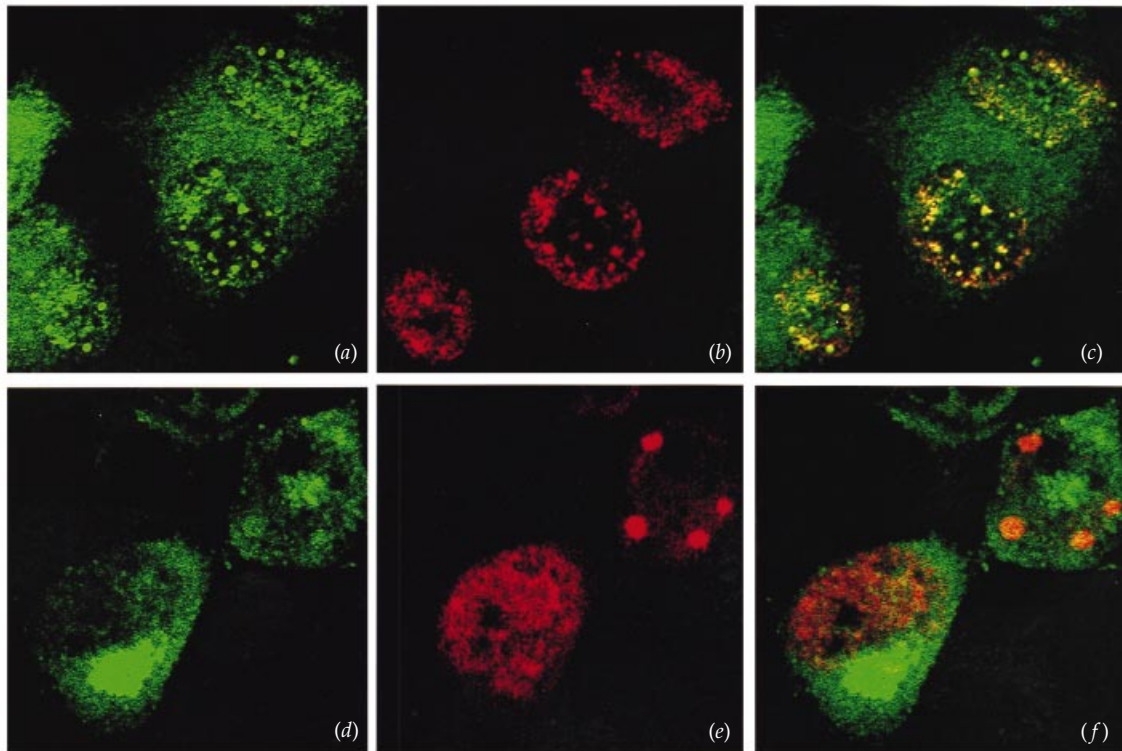


Fig. 5. Indirect double immunofluorescence of HSV-2-infected cells. HSV-2-infected Vero cells were fixed with cold acetone at 9 (*a–c*) and 12 (*d–f*) h p.i. and double-stained with the anti-UL14 rabbit serum (*a, d*) and anti-ICP35 mouse monoclonal antibody (*b, e*). The cells were then reacted with FITC-conjugated goat anti-rabbit immunoglobulin and rhodamine-conjugated goat anti-mouse immunoglobulin and examined with a Zeiss laser scanning microscope. Fluorescent images were obtained with 488 and 568 nm band-pass filters for excitation of FITC and rhodamine, respectively. The merged images are shown on the right (*c, f*). Co-localization of the proteins is visualized by yellow fluorescence.

ACV (Fig. 3*b*, lane 9). The results indicate that synthesis of the UL14 protein was highly dependent on viral DNA synthesis, suggesting that the UL14 protein is a late gene product whose expression is regulated as a $\gamma 2$ gene.

In order to examine the subcellular localization of the UL14 protein in infected cells, the cells were next fractionated into nuclear and cytoplasmic fractions by NP-40 lysis. Western blot analysis showed that approximately equal amounts of the UL14 product were distributed in the crude nuclear and cytoplasmic/membrane fractions of infected cells at 12 h p.i. (Fig. 3*a*, lanes 10 and 11). Since the nuclear fraction obtained by NP-40 lysis contains perinuclear structures, the ionic detergent DOC was also used to remove perinuclear structures from the crude nuclear fractions. The addition of DOC reduced the amount of UL14 protein detected in the nuclear fraction (Fig. 3*a*, lanes 12 and 13), suggesting that a significant amount of the UL14 protein was present in a perinuclear region. However, it would be very difficult to estimate accurately the relative amounts of nuclear and cytoplasmic UL14 protein in infected cells, as the UL42 protein, which is a typical nuclear protein, was easily detected in the cytoplasmic fraction even after NP-40 lysis, a very mild fractionation procedure (Fig. 3*b*, lanes 10 and 11).

Intracellular localization of the UL14 gene product in HSV-2-infected cells

The intracellular distribution of UL14 protein was also examined by indirect immunofluorescence staining. At various times after infection, Vero cells infected with HSV-2 were fixed with cold acetone, treated with human serum to block nonspecific binding and reacted with the UL14 antiserum. No specific staining was observed in mock-infected cells that were reacted with the UL14 antiserum (Fig. 4*a*) or in HSV-2-infected cells reacted with preimmune serum (Fig. 4*f*). Specific fluorescence became detectable both in the cytoplasm and in the nucleus of infected cells at 6 h p.i.: in the nucleus, the UL14 protein was detected in a net-like structure (Fig. 4*b*) or as fine, discrete particles (Fig. 4*c*). Similar patterns of immunofluorescence were observed at 9 h p.i., but in some cells the UL14 protein localized predominantly to the perinuclear region of the cytoplasm (Fig. 4*d*). The UL14 protein thereafter formed a mass in the perinuclear region by 12 h p.i. (Fig. 4*e*).

The distribution of the UL14 protein in the nucleus was reminiscent of that of the major capsid protein VP5 and the scaffolding protein ICP35, and HSV-2-infected cells were therefore double-stained with the UL14 antiserum and with a

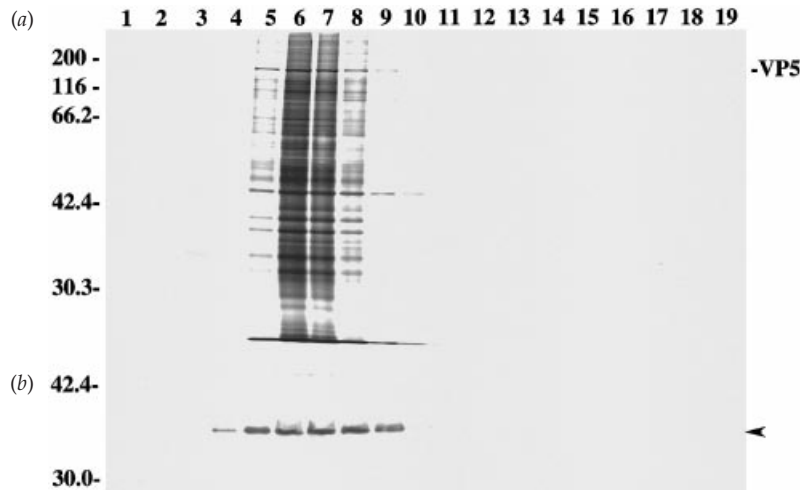


Fig. 6. Association of the UL14 protein with extracellular virions. Virus particles were collected from the culture medium harvested at 36 h p.i. and purified by two cycles of 10–50% (w/v) sucrose-gradient centrifugation as described in Methods. Fractions of the second sucrose-gradient centrifugation were collected from the bottom to the top. Proteins in each fraction were separated by SDS-PAGE and stained with silver (a) or analysed by Western blotting using the UL14 antiserum (b). The positions of molecular mass markers (in kDa) are shown on the left.

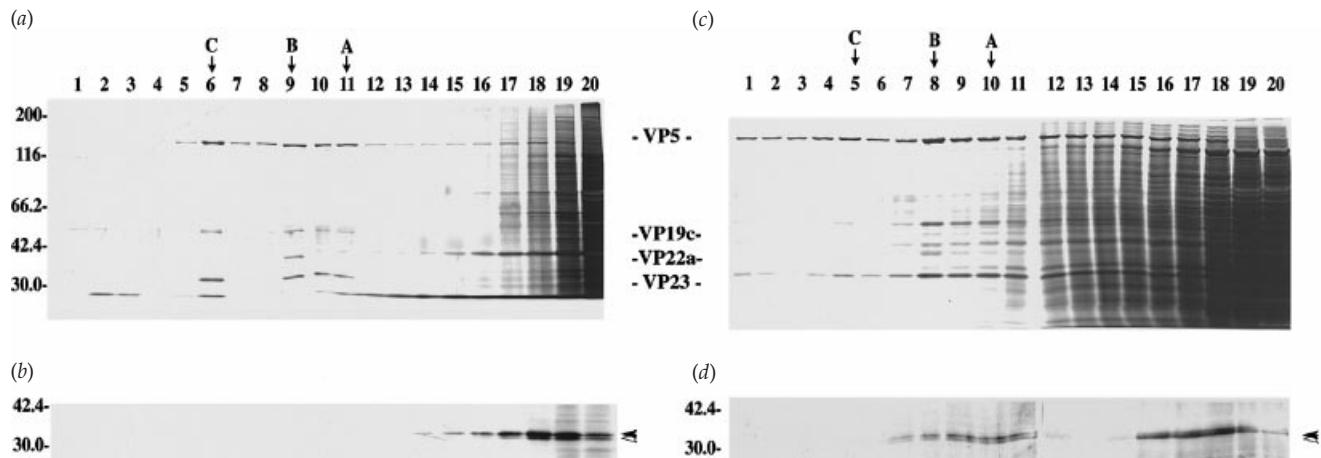


Fig. 7. Association of the UL14 protein with intracellular virus capsids. HSV-2-infected cells were harvested at 15 h p.i. Cell lysates were prepared in lysis buffer II containing 0.5 M NaCl (a, b) or lysis buffer I containing 0.1 M NaCl (c, d), as described in Methods, and were subjected to 10–50% (w/v) sucrose-gradient centrifugation. Fractions were collected from the bottom to the top. Proteins in each fraction were separated by SDS-PAGE and stained with silver (a, c) or analysed by Western blotting (b, d). Arrowheads indicate the position of the UL14 protein. Positions of molecular mass markers (in kDa) are indicated on the left.

mouse monoclonal antibody against ICP35. As shown in Fig. 5(c), the UL14 protein was found to co-localize with ICP35 in the nucleus at 9 h p.i. However, at later times, when ICP35 formed the assemblon structure (Ward *et al.*, 1996), the UL14 protein was mainly detected in the perinuclear region and did not co-localize with ICP35 (Fig. 5f).

Association of the UL14 product with intracellular capsids and virions

To determine whether the UL14 protein is a component of HSV-2 virions, extracellular virions were purified from culture media harvested at 36 h p.i. Virus particles were pelleted by centrifugation at 87 000 *g* for 1 h and purified by two cycles of 10–50% sucrose-gradient centrifugation as described in Methods. Fractions of the second sucrose-gradient centrifugation were collected from the bottom to the top and the

peak virion-containing fractions were identified by SDS-PAGE analysis followed by silver-staining (Fig. 6a). Each fraction was also subjected to Western blot analysis with the UL14 antiserum, and the UL14 protein was detected in fractions corresponding to the peak virion-containing fractions (Fig. 6b).

To determine whether the UL14 protein is associated with intracellular capsids, cell lysates were prepared from HSV-2-infected cells at 15 h p.i. and subjected to sucrose density-gradient centrifugation, after which fractions were collected from the bottom to the top. Each fraction was then applied to SDS-PAGE and stained with silver to identify the position of A, B and C capsids. It is known that A and B capsids lack viral DNA, while C capsids contain viral DNA. Moreover, A and C capsids differ in protein composition from B capsids in that they lack the scaffolding protein VP22a (Rixon, 1993). As shown in Fig. 7(a), the peak fraction of B capsids was readily

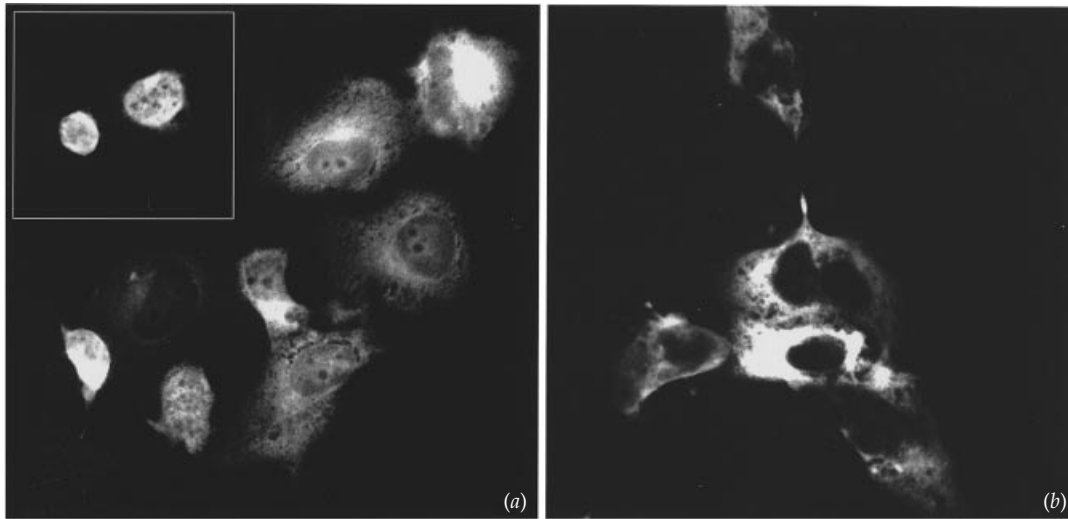


Fig. 8. Indirect immunofluorescence of COS-7 cells expressing the UL14 protein alone. Semiconfluent monolayers of COS-7 cells on glass coverslips in 35 mm plastic dishes were washed with PBS and overlaid with 1 ml Dulbecco's modified Eagle's MEM containing 10 μ l lipofectin reagent (Gibco BRL) and 1 μ g pcDNA3-UL14, the UL14 protein expression vector. The cells were incubated at 37 °C for 5 h and the medium was replaced with 2 ml Eagle's MEM containing 10% foetal calf serum. After incubation at 37 °C for 24 (a) and 48 h (b), cells were fixed and treated with the anti-UL14 serum for indirect immunofluorescence staining.

identified by the presence of VP22a (40 kDa). Judging from the abundance of the major capsid protein VP5 (155 kDa) and the presence of VP22a, the peak positions of A, B and C capsids appeared to be fractions 11, 9 and 6, respectively. Each fraction of the same preparation was also examined by Western blotting with the UL14 antiserum. No detectable UL14 protein was associated with any capsids, but the UL14 protein was detected in the top fractions of the sucrose density gradient (Fig. 7b). In these experiments, the intracellular capsids were isolated in the presence of 0.5 M NaCl. We then examined the association of the UL14 protein with capsids in the presence of 0.1 M NaCl, a relatively low concentration of salt for capsid isolation (Fig. 7c). Under these conditions, the peaks of A, B and C capsids appeared to be fractions 10, 8 and 5, respectively, and some UL14 protein was detectable in the fractions containing B and A capsids (Fig. 7d). Taken together, these results suggest that the UL14 protein may be a minor tegument component that is associated weakly with the capsids.

Intracellular distribution of the UL14 protein in singly expressing cells

To determine the intracellular distribution of the UL14 protein when expressed alone, we constructed the expression plasmid pcDNA3-UL14, which expressed the UL14 gene under the control of the HCMV promoter. COS-7 cells were transfected with the plasmid DNA and examined by immunofluorescence staining using the UL14 antiserum. At 24 h after transfection, the UL14 protein was detected both in the cytoplasm and in the nucleus (Fig. 8a). In some cells, specific immunofluorescence was only detectable in the nucleus (inset). At 48 h after transfection, however, the UL14 protein mainly

localized to the cytoplasm (Fig. 8b). These results indicate that no other virus proteins were required for nuclear and cytoplasmic localization of the UL14 protein.

Discussion

In this study, we have generated a polyclonal antiserum specific for the UL14 protein by using 6 \times His-tagged HSV-2 UL14 protein as an antigen. The antiserum was found to react strongly with 34, 33 and 28 kDa proteins produced at late times of infection in HSV-2-infected cells, and its reactivity for these proteins was clearly eliminated by pre-adsorption with the recombinant HSV-2 UL14 protein, suggesting that all of them are UL14 gene products. However, nucleotide sequence analysis of the coding sequence of UL14 predicts an acidic protein with a molecular mass of 23 869 Da (Dolan *et al.*, 1998), so the apparent molecular masses of these three proteins are considerably larger than the predicted molecular mass. Such a discrepancy between the predicted molecular mass and the apparent molecular masses on SDS-polyacrylamide gels could be due either to post-translational modification or to an unusual amino acid composition. The UL14 protein has a relatively high content of hydrophobic amino acids such as alanine (17.4%), leucine (9.1%) and proline (8.7%). This could account for the discrepancy between the apparent and predicted molecular masses of the UL14 protein. To test this possibility, we performed *in vitro* transcription and translation experiments using the cloned UL14 coding sequence of HSV-2 and found that the primary translation product had an apparent molecular mass of 34 kDa. It is thus suggested that the discrepancy in molecular mass may be due to the unusual amino acid composition of the UL14 protein. Moreover, the

time-course experiments showed that the 34 kDa protein appeared first in HSV-2-infected cells and then the 33 kDa species became detectable. The 28 kDa species was usually detected at later times of infection and the amount of this protein species was much less than that of the 34 and 33 kDa proteins. These observations support the idea that the 34 kDa protein species is the primary translation product of the UL14 gene in HSV-2-infected cells. The 28 kDa species may be a degradation product of the UL14 protein, since detection of this species was not constant. In contrast, it seems that processing to the 33 kDa species may be important for some function of the UL14 protein.

The UL14 protein localized both to the nucleus and to the perinuclear region of the cytoplasm. At 9 h p.i., the UL14 protein within the nucleus co-localized with the scaffolding protein ICP35, suggesting a possible role either in capsid assembly or capsid maturation, including viral DNA cleavage/packaging. While the UL14 gene of HSV-1 is not dispensable for replication (Roizman, 1996), it is known that only six genes (UL18, UL19, UL35, UL38, UL26 and UL26·5) encoding capsid proteins and scaffolding proteins are necessary for the formation of capsids. In fact, co-expression of these six genes enables cells to produce capsids that are indistinguishable in appearance and protein composition from those made during HSV infection (Tatman *et al.*, 1994; Thomsen *et al.*, 1994). It thus seems unlikely that the UL14 protein plays a critical role in the formation of capsids. On the other hand, it has been reported that at least six genes (UL6, UL15, UL25, UL28, UL32 and UL33) are essential for the cleavage and packaging of viral DNA (Steven & Spear, 1996). Temperature-sensitive mutants and genetically engineered mutants with mutations in these genes have been isolated and characterized, showing that these mutants have similar phenotypes, in that viral DNA can be replicated but not packaged (Addison *et al.*, 1990; al-Kobaisi *et al.*, 1991; Poon & Roizman, 1993; Schaffer *et al.*, 1973; Sherman & Bachenheimer, 1987; Weller *et al.*, 1987). A recent study has also shown that the UL17 gene product plays an essential role in cleavage and packaging of viral DNA (Salmon *et al.*, 1998). Among the proteins involved in this process, some, such as the UL6 and UL25 proteins, are tightly associated with B or C capsids (Ali *et al.*, 1996; Patel & MacLean, 1995) and some are not (Lamberti & Weller, 1998; Yu & Weller, 1998). Although the UL14 protein was detectable in the mature virion, the interaction with capsids in the nucleus appeared to be weak. The properties of the UL14 protein seem consistent with its putative role in capsid maturation, but further experiments using deletion mutants will be required to test this possibility.

In cells transfected with the UL14 gene, both nuclear and cytoplasmic staining were observed. At early times of transfection, the UL14 protein was detected in the nucleus or in both the nucleus and the cytoplasm, but at later times cytoplasmic staining was predominant. Western blot analysis revealed that only the 34 kDa species was detected in

transfected cells (not shown), suggesting that the modification to the 33 kDa species may not be involved in the change in localization. Although the distribution patterns of proteins overexpressed in transfected cells may not always reflect the normal behaviour of the proteins, the marked change in the UL14 staining pattern in transfected cells seems very interesting. We are planning to study the time-course of distribution after transfection and the mechanism of the change by using various kinds of UL14–green fluorescent protein fusion.

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