Arch Virol (1998) 143: 863–880

Archives of Virology

Characterization of the UL16 gene product of herpes simplex virus type 2

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Accepted January 12, 1998

Summary. We have raised rabbit polyclonal antisera against a His-tagged herpes simplex virus type 1 (HSV-1) UL16 fusion protein, one of which very specifically reacted with 40 kDa and 41 kDa proteins in the lysates of HSV-1 and HSV-2-infected cells, respectively. Since its reactivity to the 41 kDa protein was clearly eliminated by pre-adsorption with *E. coli* lysates expressing the UL16 fusion protein, the antiserum was used to characterize the UL16 products of HSV-2. The HSV-2 UL16 protein was produced at the late phase of infection in a manner highly dependent on viral DNA synthesis and was distributed in both the nuclei and the cytoplasma of infected cells. In immunofluorescence studies, the UL16-specific fluorescence in the nuclei was shown to be detected as small discrete granules. On the other hand, the cytoplasmic fluorescence was diffusely distributed around the nucleus at 8 h postinfection but, at later times of infection, it was mainly detected as a mass at a perinuclear region. The analysis on its association with capsids has revealed that the UL16 protein copurified with C capsids but not B and A capsids, and that the association with C capsids was not tight. Moreover, our experiments have shown that a detectable level of the UL16 protein was not associated with extracellular virions, and that the partially purified UL16 proteins had a DNA-binding activity. These observations are consistent with the hypothesis that the UL16 protein plays a role in capsid maturation including DNA packaging/cleavage. We have also determined the complete nucleotide sequence of the HSV-2 UL16 gene and found that a nonstandard initiation codon may be used for its translation.

Introduction

Herpes simplex virus (HSV) is a large enveloped DNA virus and the genome encodes more than 80 genes. Approximately half of the viral genes have been

shown to be nonessential for viral replication in cell cultures [26]. However, these dispensable genes play important roles in the viral growth and spread in the host [4, 18, 21, 33]. Although recent efforts have identified most of the dispensable genes of HSV [27], the precise function of the products, except a few, remains obscure.

The UL16 gene of HSV-1 is one of the dispensable genes [3] and located within the intron of an essential gene, UL15, in a region of the HSV genome which is conserved in all other herpesviruses [1, 8, 11, 17]. The UL16 homologs are thus detected in alpha, beta and gamma herpesviruses including human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV). The UL16 gene of HSV-1 encodes a protein with the predicted molecular mass of 40 kDa and other homologs have also similar molecular weight [9, 32]. Moreover, the pileup analysis of the UL16 homologs have revealed that they contain seven conserved cystein residues as well as two conserved histidine residues in the middle of the ORFs, suggesting the presence of a possible zinc and/or nucleic acid binding function for these homologs [32].

This study was initially undertaken to characterize the UL16 product of HSV types 1. In the course of this study, however, Nalwanga et al. [19] reported a primary characterization of the UL16 protein of HSV-1; they showed that the HSV-1 UL16 product is a virion protein with an apparent molecular mass of 40 kDa that colocalizes with intranuclear capsid proteins. We therefore focused on the HSV-2 counterpart. Although most of our results support those of Nalwanga et al. (1996), there were some differences. Additionally we demonstrate that the UL16 protein was associated with C capsids and had a DNA-binding activity. Furthermore, we determined the nucleotide sequences of the region including the complete open reading frame of the HSV-2 UL16, demonstrating that the HSV-2 UL16 gene, unlike the HSV-1 counterpart, did not have the ATG codon at the putative initiation site for translation.

Materials and 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 units/ml of penicillin, and 100 μg/ml streptomycin and were used throughout this study. HSV-1 strain KOS and HSV-2 strain 186 derived from a single plaque were propagated in Vero cells by infecting at a low multiplicity (0.01 PFU/cell). Infected cells were harvested when almost all cells exhibited cytopathic effects (CPE). After freezing and thawing three times and eliminating cell debris at 3 000 rpm for 10 minutes, virus was stored at $-80\,^{\circ}$C.

DNA manipulations

The UL16 ORF is located between nucleotide positions 30 174 to 31 295 of the HSV-1 genome [17]. The UL16 coding sequences were cloned by PCR amplification from HSV1 KOS genomic DNA, using UL16f (TAG TGC GGC CGC ATG GCG CAG CTG GGA CCC CGG CGG) as the forward primer and UL16r (TAG TCT CGA GTT ATT CGG GAT CGC TTG AGG AGG) as the reverse primer. NotI and XhoI sites were incorporated into the forward

and reverse primers, respectively, to facilitate cloning. The PCR consisted of an initial 10 minute denaturation step at 94 °C, followed by 29 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 3 min). The 30th cycle concluded with a 30 min extension step. The PCR product was digested with NotI and XhoI, and cloned in frame and downstream of the region encoding the initiating ATG plus six histidine (6 × His) residues in *E. coli* expression vector pET-28a (Novagen) to give plasmid pET28-UL16. The expression of 6 × His-tagged UL16 protein is regulated by isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lac operator sequence and a phage T7 promoter. The translation is expected to terminate at the UL16 stop codon. Plasmid pET28-UL16 was transformed into *E. coli* strain BL21 (DE3) (Novagen, New York) which, following induction with IPTG, expressed large quantities of 6 × His-tagged UL16 fusion protein. Because this recombinant protein was highly insoluble, it was purified by a conventional inclusion body purification procedure [34].

Preparation of polyclonal antisera

Antisera were produced in three rabbits by immunization with an emulsion containing approximately 0.6 mg of *E. coli*-expressed 6 × His-tagged UL16 protein in the form of insoluble inclusion body preparation in MPL + TDM + CWS emulsion adjuvant system (RIBI ImmunoChem Research, Inc., Montana). Inoculations were subcutaneous injections 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 each at 2-week intervals after primary injection. Two weeks after the last immunization, we collected blood from heart.

Western blotting

We performed Western blotting as described previously [10]. Briefly, at the indicated times, the denatured solubilized polypeptides from mock-infected and HSV-2 186-infected cell lysates were electrophoretically separated on 4% stacking- 10% SDS-polyacrylamide gels and electrically transferred to Hybound-PVDF membranes (Amersham Japan, Tokyo). Nonspecific protein binding was blocked by treating membranes with Tris-buffer saline (TBS: 25 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% bovine serum albumin (BSA) and 0.05% Tween20 at 4 °C overnight. The membranes were washed once with TBS and incubated with a 1: 350 dilution of the UL16 antiserum in TBS containing 1% BSA and 0.05% Tween20 at 37 °C for 1 h. After washing three times with TBS containing 0.05% Tween20, the membranes were incubated with a 1: 7 000 dilution of goat anti-rabbit peroxidase-labeled second antibody (BIO SOURCE) at 37 °C for 1 h. The membranes were then washed three times with TBS containing 0.05% Tween20, treated with ECL Western blotting detection system (Amersham Japan), and exposed to Hyperfilm-ECL (Amersham Japan).

Immunoprecipitation and electrophoresis

HSV-2-infected Vero cells were labeled for 20 min with 60 μ Ci/ml [^{35}S]methionine at 9 h or 12 h postinfection. At the end of the labeling, cells were quickly chilled by placing them on ice and treated with ice-cold phosphate-buffered saline (PBS). The radio labeled cells were lysed in ice-cold 50 mM Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl, 0.1% SDS, 0.5% DOC, 0.5% NP-40, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin and 1 mg/ml BSA. The lysate was precleared twice with normal rabbit serum and Protein A Sepharose 4 FF (Pharmacia LKB Biotechnology, Uppsala, Sweden) and immunoprecipitated with either a polyclonal rabbit antiserum against UL16 and Protein G Sepharose 4 FF (Pharmacia LKB Biotechnology, Uppsala, Sweden). The sepharose beads were washed in 50 mM Tris-HCl,

pH 7.5, 150 mM NaCl, 0.5% NP-40 5 times to remove nonspecifically adsorbed proteins. Immunoprecipitates were eluted from the beads by boiling in 50 μl of × 2 SDS sample buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 20% glycerol and 4% SDS) for reducing conditions. Then antigens were separated by SDS-PAGE. After the gels were fixed with 10% acetic acid and 10% methanol and dried, protein bands were visualized with the Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Confocal immunofluorescence microscopy

Vero cells were grown in coverslips and were either mock-infected or infected with HSV-2 at a multiplicity of 3 PFU/cell. At various times after infection, the cells were fixed in cold acetone. Coverslips were then incubated with a solution of 20% human serum in PBS for 1 h at room temperature to reduce background levels produced as a consequence of the affinity binding of rabbit immunoglobulin to the Fc receptor formed by glycoprotein E and I. The cells were reacted with anti-UL16 serum diluted 1: 400 in PBS containing 0.1% BSA for 1 h at 37 °C, washed in excess PBS and then reacted with 1: 60 dilution of FITC-conjugated goat anti-rabbit immunoglobulin in blocking solution for 1 h at 37 °C. Fluorescent images were viewed and recorded with the Bio-Rad MRC-series confocal imaging system.

Preparation of nuclear fractions

Nuclei were isolated from cells as described previously [30]. Briefly, infected cells were washed with PBS three times. The cells were suspended in RSB buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.4), and left for 5 min on ice. After adding Nonidet P-40 (NP-40) alone or NP-40 and deoxycholic acid (DOC) to a final concentration of 0.5%, respectively, the cells were homogenized by 10 strokes with a glass homogenizer. The homogenate was layered over 0.5 M sucrose in RSB buffer, and centrifuged at 1 500 rpm 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 [30]. The nuclear fraction was further incubated with 20 μg/ml DNase I (Takara Shuzo Co. Ltd., Ohtsu, Japan) for 30 min at room temperature under continuous shaking and washed once in LM buffer (0.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The final LM pellet was washed with cold LM buffer three times and then pelleted at 3 000 rpm for 15 min.

Fractionation of intracellular viral capsids

Vero cells were infected with HSV-2 at a multiplicity of 3 PFU/cell and incubated at 37 °C for 15 h. Infected cells were harvested by centrifugation and washed with PBS three times. Cell lysates were resuspended in 1 ml of lysis buffer I (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 1% Triton-X 100, pH 7.5), or buffer II (20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 1% Triton-X 100, pH7.5), disrupted by sonication and the debris was pelleted at 3 000 rpm for 10 minutes. 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 24 000 rpm for 40 min in a Hitachi RPS 40 rotor. Five hundred μl of 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 PFU/cell. After 1 h for adsorption at 37 °C, maintenance medium containing

5% serum was added. HSV-2 virions were harvested from the extracellular media at 36 h postinfection. After removal of cell debris by low-speed centrifugation, virions were pelleted from the supernatant by centrifugation at 87 000 **g** for 1 h. For purification of virions on Ficoll gradients, the virus pellet was resuspended in MEM without serum and phenol red. The virus suspension was layered onto a continuous 5–15% Ficoll gradient, followed by centrifugation at 26 000 **g** for 2 h at 4 °C [28]. The virion band was collected as described above and diluted in MEM.

Partial purification of the UL16 protein and DNA binding assay

HSV-2-infected cells (approximately 0.5 g) were harvested at 24 h postinfection, suspended in extraction buffer (5 ml) containing 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 10% glycerol, 10 mM 2-ME, 1 mM PMSF and sonicated in ice using a Microson ultrasonic cell disruptor. The sonicated cell suspension was centrifuged at 60 000 **g** for 60 min. The supernatant was dialyzed against buffer A (50 mM Tris-HCl pH 7.5, 10% glycerol, 10 mM 2-ME, 1 mM PMSF) and then applied to a phosphocellulose column (P11 cellulose, Whatman) equilibrated with buffer A. The column was washed with buffer A and proteins were eluted with a linear gradient (5 ml) from 0.0 to 0.5 M NaCl in buffer A. Fractions containing the UL16 protein from the phosphocellulose column were pooled, dialyzed against two changes of buffer A, and applied to the single stranded (ss) DNA agarose column. The column was washed with buffer A and proteins were eluted with a gradient from 0.0 to 0.5 M NaCl in buffer A.

Sequencing of the HSV-2 UL16 open reading frame

The UL16 gene of HSV-2 was sequenced by the primer extension/dideoxy chain termination method using ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, New Jersey). A 20.7-kb Hind III B fragment of HSV-2 strain 186 DNA has been cloned into the Hind III site of plasmid pACYC 184 [29]. The 6.0 kb fragment, which contains the complete open reading frames of the UL13, UL14, UL15 and UL16 genes of HSV-2, was obtained by digesting the isolated Hind III B fragment with Bam HI and Eco RI, and was inserted into the multi-cloning site of plasmid pUC19. Primers specific for the plasmid were used to initiate sequencing. Subsequent primer sequences were selected from the newly determined sequences. Using a total of 11 primers, 18 to 21 nucleotides in length, nucleotide sequences spanning the entire UL16 gene were obtained. Sequences were determined at least from three clones on both DNA strands. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and Genbank nucleotide sequence databases with the following accession number AB007142.

Results

Preparation and specificity of anti-UL16 antiserum

To characterize the UL16 product, we first generated anti-UL16 rabbit antisera by using a recombinant HSV-1 UL16 fusion protein as antigen. For this purpose the plasmid pET28-UL16 was constructed as described under Materials and methods, and the UL16 fusion protein was expressed in *E. coli* by treatment with IPTG (Fig. 1). Two of three rabbits which were immunized produced antisera specifically reacting with the UL16 fusion protein. To examine the reactivity and specificity of the antisera, Western blotting and immunoprecipitation analysis were done (Fig. 2). One of the antisera strongly reacted with a protein with an apparent

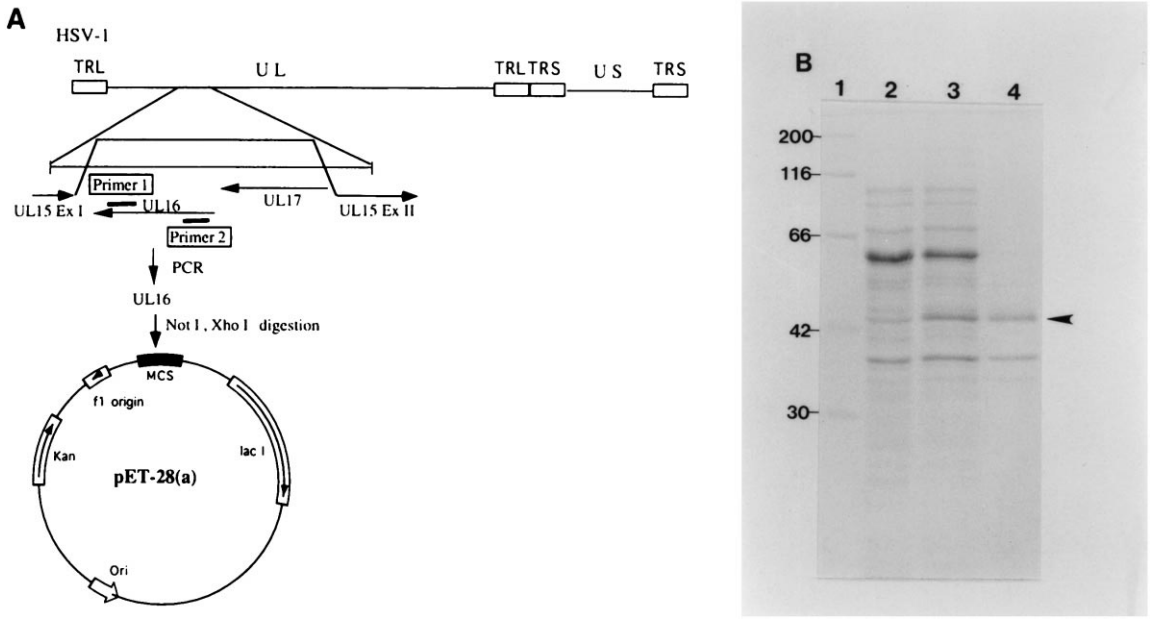

Fig. 1. A Schematic representation of the HSV genome containing the UL16 gene and strategy for construction of the expression plasmid pET 28-UL16. **B** Induction of the His-tagged UL16 fusion protein in *E. coli. E. coli* harboring pET 28-UL16 were grown in the absence (*2*) or presence (*3*) of IPTG, and the UL16 fusion protein was partially purified by a conventional inclusion body purification procedure (*4*). Proteins were separated by SDS-PAGE and stained with Coomasie brilliant blue. Molecular weight markers are shown in *1*. The arrow indicates the position of the UL16 protein

molecular mass of 40 kDa in the lysate of HSV-1-infected Vero cells but did not react with any proteins in the lysates of mock-infected and HSV-2-infected cells. Whereas the other antiserum could specifically detect 40 kDa and 41 kDa band in the lysates of HSV-1- and HSV-2-infected cells, respectively (Fig. 2A). The reactivity of the latter antiserum with the 41 kDa band was clearly eliminated by pre-adsorption of the antiserum with the lysate of *E. coli* expressing the UL16 fusion protein, but there was no significant change in the reactivity after pre-adsorption with the lysate of control *E. coli* (Fig. 2B). The latter antiserum was also found to immunoprecipitate the 41 kDa protein from the lysate of HSV-2-infected cells labeled with [^{35}S]methionine (Fig. 2C). Preimmune rabbit serum did not react any specific proteins in HSV-1- and HSV-2-infected cells (data not shown), and therefore we used this polyclonal antiserum for further experiments to characterize the UL16 product of HSV-2.

Expression of the UL16 product in HSV-2-infected cells

The kinetics of UL16 protein expression was analyzed by Western blotting in HSV-2-infected Vero cells. At various times after infection cell lysates were subjected to electrophoresis, transferred to PVDF membranes and reacted with the

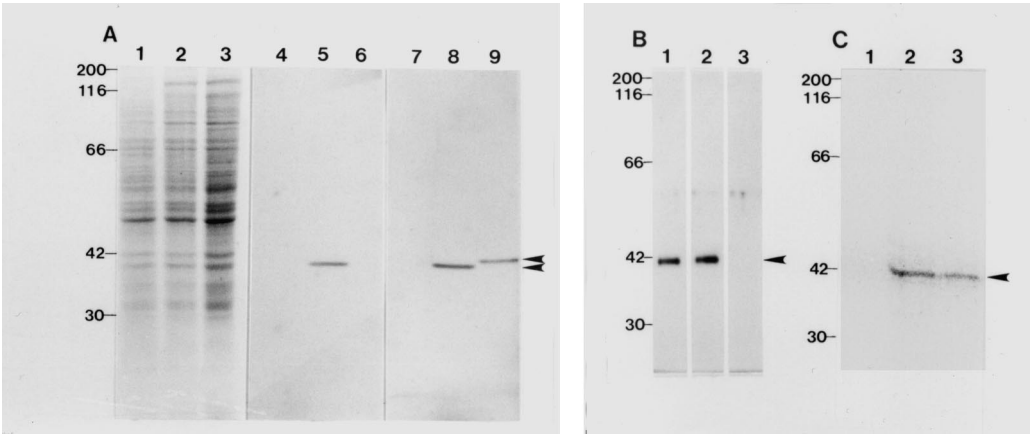

Fig. 2. Reactivity and specificity of rabbit polyclonal antisera against the His-tagged UL16 fusion protein. **A** Detection of the UL16 protein in infected cells. Vero cells were mock-infected (*1*, *4*, *7*) or infected with HSV-1 (*2*, *5*, *8*) and HSV-2 (*3*, *6*, *9*), and harvested at 15 h postinfection. Proteins were separated by SDS-PAGE and stained with Coomasie brilliant blue (*1–3*). Antisera from two rabbits were used for Western blotting (*4–6*, *7–9*). **B** Specificity of the anti-UL16 serum. The antiserum which reacted with a 41 kDa protein in HSV-2-infected cells was pre-adsorbed with lysates of non-treated *E. coli* (*1*), IPTG-treated control *E. coli* (*2*) and IPTG-treated, UL16 fusion protein-expressing *E. coli* (*3*), and was used for Western blotting. **C** Detection of the UL16 protein by immunoprecipitation. Vero cells were mock-infected (*1*) or infected with HSV-2 (*2* and *3*), and were labeled with [^{35}S]methionine for 20 min at 9 h (*2*) and 12 h (*3*) postinfection. Immunoprecipitation using the anti-UL16 serum was performed as described under Materials and methods

UL16 antiserum. As shown in Fig. 3A, the UL16 product was detectable at 9 h postinfection, and thereafter gradually increased in amount between 12 and 24 h postinfection, indicating that the UL16 protein is a late gene product.

To determine the dependence of its production on viral DNA synthesis, infected cells were maintained for various times after a 1 h adsorption period in the presence or absence of ACV (300 μg/ml). No detectable amount of the UL16 protein was produced in the presence of ACV even at 24 h postinfection (Fig. 3B), indicating that the UL16 protein synthesis was highly dependent on viral DNA synthesis. The results suggest that the UL16 is regulated as γ2 gene.

Subcellular localization of the UL16 product in HSV-2-infected cells

Intracellular distribution of the UL16 protein was examined by indirect immunofluorescence staining. At various times after infection, Vero cells infected with HSV-2 were fixed with cold acetone, treated with human sera to block non-specific binding and reacted with the UL16 antiserum. Specific fluorescence became detectable in the cytoplasma of infected cells at 8 h postinfection. At 9 h postinfection, the UL16-specific fluorescence was also detected in the nucleus. A typical pattern of the staining is shown in Fig. 4. Most nuclei of infected cells contained small discrete granules stained with fluorescence and this pattern of

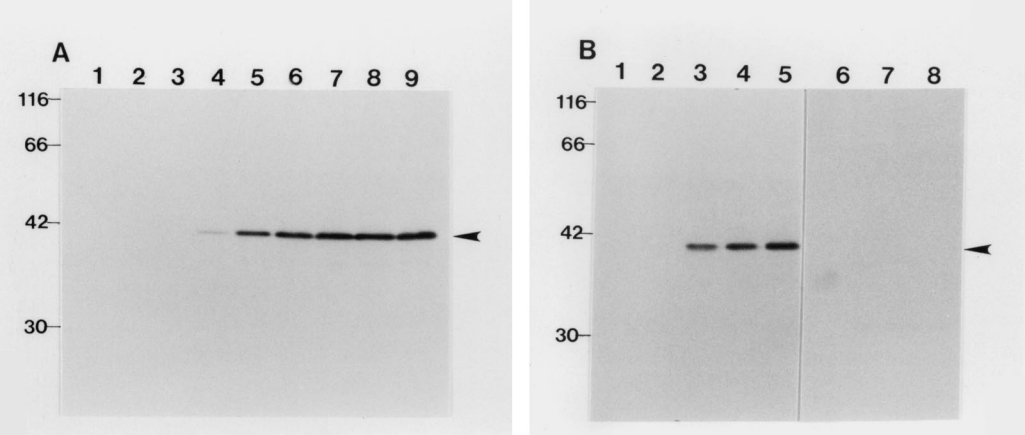

Fig. 3. Production of the UL16 protein in HSV-2-infected cells. **A** Time course of the UL16 protein expression. Vero cells were mock-infected (*1*) or infected with HSV-2 (*2–9*) at a multiplicity of 3 PFU/cell. The cells were harvested at 3 h (*2*), 6 h (*3*), 9 h (*4*), 12 h (*5*), 15 h (*6*), 18 h (*7*), 21 h (*8*) and 24 h (*1, 9*) postinfection. Proteins were separated by SDS-PAGE and analyzed by Western blotting using the anti-UL16 serum. **B** Effect of inhibition of viral DNA synthesis on the UL16 protein expression. Mock-infected (*1, 2*) and HSV-2-infected (*3–8*) cells were cultured in the absence (*1–5*) or presence of 300 μg/ml ACV (*6–8*), and harvested at 12 h (*1, 3, 6*), 18 h (*4, 7*) and 24 h (*2, 5, 8*) postinfection. Proteins were separated by SDS-PAGE and analyzed by Western blotting. Arrows indicate the position of the UL16 protein

the intranuclear staining continued during the course of infection. On the other hand, the cytoplasmic staining patterns significantly changed: at earlier times of infection, the perinuclear cytoplasmic region was diffusely stained with the UL16 antiserum but at later times of infection, the UL16 protein was mainly detected as a mass in a perinuclear region. As shown in Fig. 4A, some cells at 9 h postinfection already displayed such perinuclear cytoplasmic distribution. No specific staining was observed in mock-infected cells which were reacted with the UL16 antiserum (Fig. 4B) or in HSV-infected cells reacted with preimmune serum (data not shown).

In order to further examine the subcellular localization of the UL16 protein in infected cells, cells were fractionated into nuclear and cytoplasmic fractions by NP-40 lysis. Western blot analysis showed that approximately equal amounts of the UL16 product were distributed in the crude nuclear and cytoplasmic/membrane fractions of the infected cells at 12 h postinfection. Since the nuclear fractions obtained by NP-40 lysis contain the perinuclear filamentous structures (30, 35), an ionic detergent DOC was also used to remove the perinuclear structures from the crude nuclear fractions, and the addition of DOC reduced the amount of the UL16 protein detected in the nuclear fraction, suggesting that a significant amount of the UL16 protein was localized in a perinuclear region. These results are consistent with those of immunofluorescence microscopic analysis.

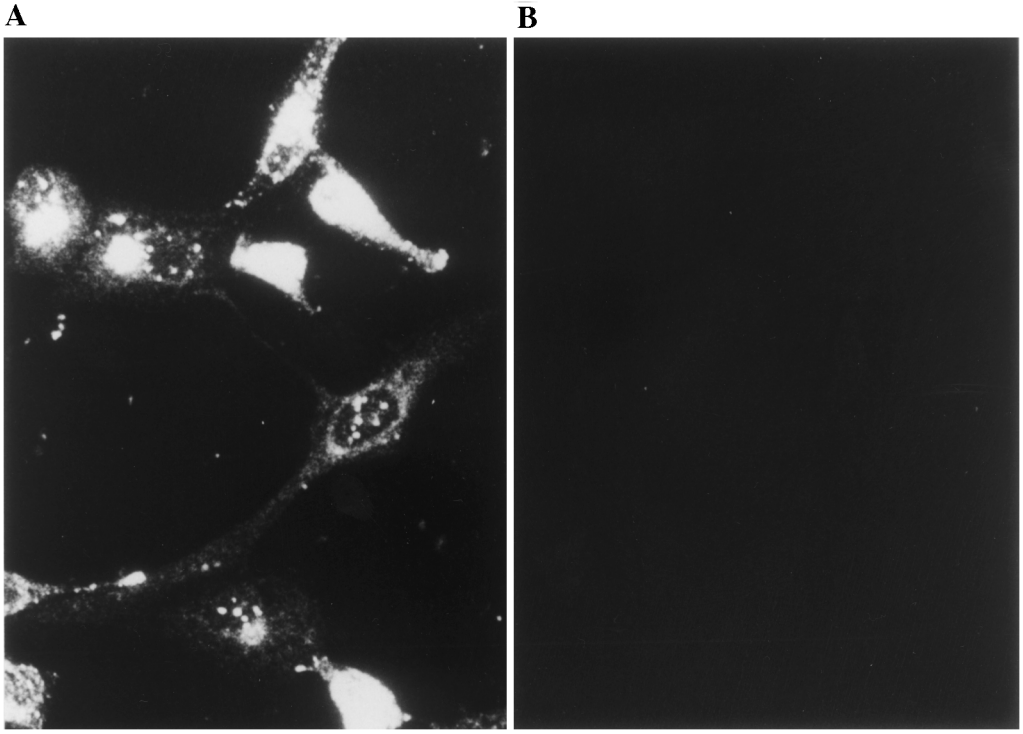

Fig. 4. Confocal microscopic images of the UL16 protein in HSV-2-infected cells. HSV-2-infected (**A**) and mock-infected (**B**) Vero cells were fixed with cold acetone at 9 h postinfection for immunofluorescence staining as described under Materials and methods, and examined with the Bio-Rad MRC-1024 confocal imaging system

To examine the nature of association of the UL16 protein with the nucleus, isolated nuclei from the infected cells were treated with DNase and then washed with LM buffer. As shown in Fig. 5B, the UL16 protein was found to be almost completely removed from the nuclear fraction by treatment with DNase and washing with LM buffer.

Association of the UL16 product with intracellular capsids

To determine if the UL16 protein is associated with intracellular capsids, cell lysates were prepared from HSV-2-infected cells at 15 h postinfection, subjected to sucrose density gradient centrifugation and fractions were collected from the bottom to the top. Each fraction was applied to SDS-PAGE and stained with silver to identify the positions 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 (20, 24). As shown in Fig. 6A, the peak fraction of B capsids was readily 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 seemed to be Fractions 10, 8 and 4, respectively.

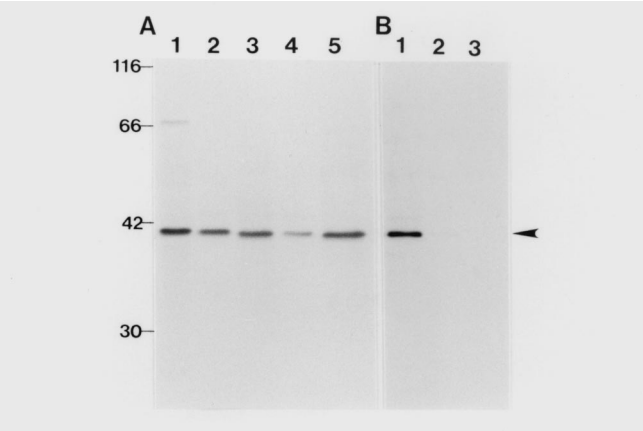

Fig. 5. Western blot analysis of subcellular localization of the UL16 protein. **A** HSV-2-infected Vero cells (*1*) were harvested at 12 h postinfection and were separated into the nuclear (*2, 4*) and cytoplasmic (*3, 5*) fractions by using 0.5% NP-40 (*2, 3*) or 0.5% NP-40 and 0.5% DOC (*4, 5*), as described under Materials and methods. The purity and morphology of the isolated nuclei were examined with a microscope after staining with toluidine blue. **B** Effect of DNase treatment on the association of the UL16 protein with the nucleus. The nuclear fraction (*1*) was treated with DNase and washed once with LM buffer (*2*). The DNase-treated nuclear fraction was further washed with LM buffer three times and pelleted by centrifugation (*3*). The arrow indicates the position of the UL16 protein

Each fraction of the same preparation was also examined by Western blotting with the UL16 antiserum. The results indicate that the UL16 product was associated with C capsids but not with B and A capsids (Fig. 6B). In these experiments, the intracellular capsids were isolated in the presence of 0.1 M NaCl, a relatively low concentration of salt for capsid isolation [7]. We then examined the association of the UL16 protein with C capsids in the presence of 0.5 M NaCl (Fig. 6D). No detectable amount of the UL16 protein was associated with C capsids as well as B and A capsids. Whereas the UL16 protein was detected in the top fractions of sucrose density gradient. The results indicate that the interaction of the UL16 product with C capsids was not tight.

Association of the UL16 product with virions

The above results suggest that the UL16 protein is a component of HSV-2 virions. To test this possibility, extracellular virions were purified from culture media harvested at 36 h postinfection. The viral particles were pelleted by centrifugation at 87 000 **g** for 1 h and purified by Ficoll density gradient centrifugation. Fractions were collected from the bottom to the top and the protein composition of each fraction was analyzed by PAGE followed by silver staining. By using Ficoll gradient centrifugation, virions can be separated into H and L particles, and H particles are characterized by the presence of two proteins, 155 kDa and 37 kDa. In our experiments, we could not identify fractions of typical L particles which lack both 155 kDa and 37 kDa. However, fractions of the particles which lacked 37 kDa

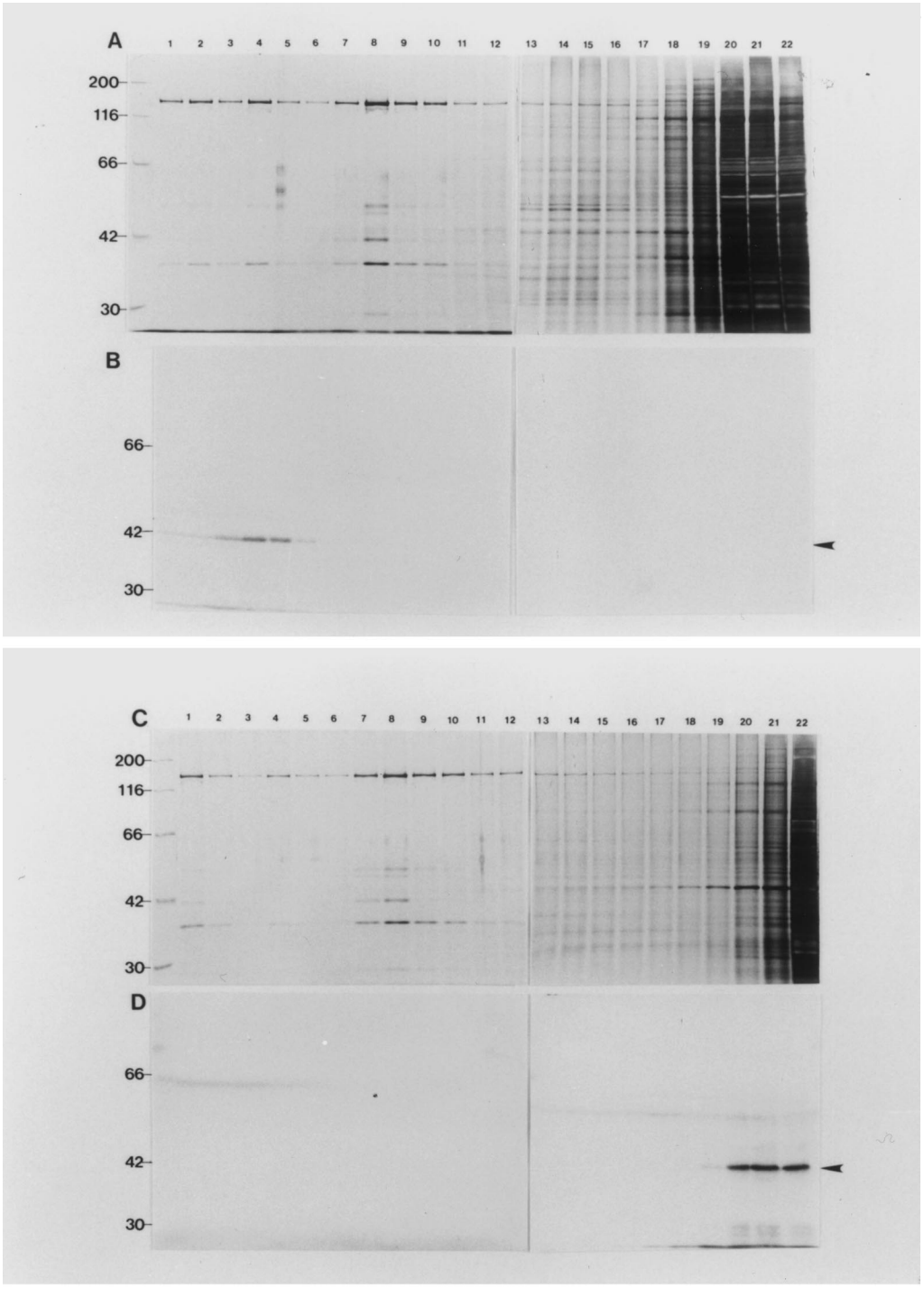

Fig. 6. Association of the UL16 protein with intracellular viral capsids. HSV-2-infected cells were harvested at 15 h postinfection. Cell lysates were prepared in lysis buffer I containing 0.1 M NaCl (**A, B**) or lysis buffer II (**C, D**) containing 0.5 M NaCl, as described under Materials and methods, 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 analyzed by Western blotting (**B, D**). Arrows indicate the position of the UL16 protein

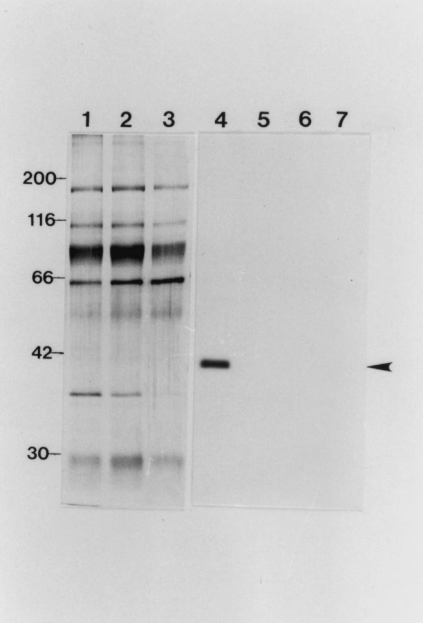

Fig. 7. Association of the UL16 protein with purified virions. HSV-2 virions were collected from extracellular fluid of infected cells and purified by 5–15% Ficoll gradient centrifugation as described under Materials and methods. Fractions were collected from the bottom to the top, and the positions of H (*1, 5*) and putative L (*3, 7*) particles were determined by SDS-PAGE followed by silver staining. A intermediate fraction (*2, 6*) between H and L particles as well as infected cell lysates (*4*) was also shown. Proteins were stained with silver (*1–3*) and analyzed by Western blotting (*4–7*). The arrow indicates the position of the UL16 protein

were detected above the fractions containing H particles. The peak fractions of these particles and its intermediate fractions were probed for the UL16 antiserum in Western blots. Unexpectedly, no detectable level of the UL16 product was observed in either H and putative L particles (Fig. 7). The results suggest that the UL16 product was not a stable component of HSV-2 virions.

DNA-binding activity of the UL16 product

Wing et al. have reported that the UL16 product of HSV-1 and the homologs including HCMV UL94 and VZV 44 contain a potential zinc finger domain, which suggests a role in DNA binding. To examine if the HSV-2 UL16 protein has a DNA-binding activity, the protein was partially purified by column chromatography. The cellular extract, prepared from HSV-2-infected Vero cells 24 h after infection, was adsorbed to an appropriate amount of phosphocellulose and protein was eluted with a linear gradient of NaCl and fractions were probed for the UL16 antiserum. The UL16 protein was eluted as a single peak at 0.41 M NaCl. The peak fractions were pooled, dialyzed against buffer A, and then applied to a ssDNA agarose column. As shown in Fig. 8B, there was no detectable amount of the UL16 protein in the flow through fractions, and the product was eluted at 0.20 M of NaCl. Control experiments showed that the UL16 protein did not adsorb to agarose (data not shown). The results indicate that the UL16 product could directly or indirectly bind ssDNA.

Analysis of amino acid sequences of the HSV-2 UL16

We have determined the nucleotide sequence of the UL16 region of the genome from a plasmid clone containing a Hind III B fragment of HSV-2 strain 186

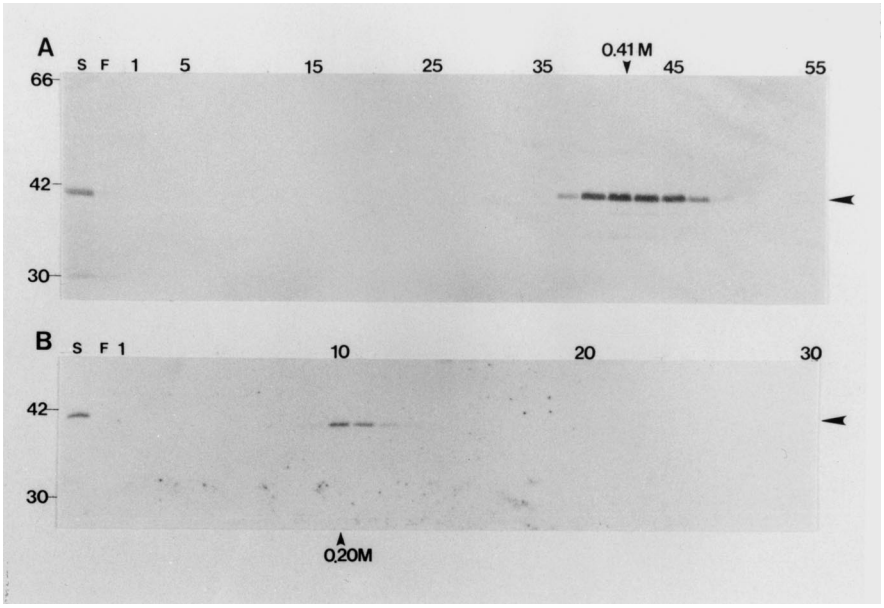

Fig. 8. Chromatographical behavior of the UL16 protein. HSV-2-infected cells were harvested at 24 h postinfection. The cellular extracts were prepared as described under Materials and methods, applied to a phosphocellulose column (**A**), and proteins were eluted with a linear gradient from 0.0 to 0.5 M NaCl. The peak fractions of the UL16 protein were collected, further applied to a ssDNA agarose column (**B**) and proteins were eluted with a linear gradient from 0.0 to 0.5 M NaCl. Each fraction as well as applied samples (*S*) and flow through fractions (*F*) was subjected to SDS-PAGE and analyzed by Western blotting. The position of molecular weight markers are indicated at the left. Arrows indicate the position of the UL16 protein

inserted into pACYC184 [29], as described under Materials and methods. Surprisingly, no ATG was found at the putative initiation site for translation of the HSV-2 UL16, at which CTG was found. The sequence around the CTG was CCAACCTGG, which was at least satisfied with the Kozak's consensus sequence for translation except C in the initiation codon. Since there was no ATG codon between -200 and $+200$ nucleotides from the putative initiation site and also since the UL16 product of HSV-2 was almost the same with HSV-1 counterpart in molecular weight, we conclude that the CTG acted as the initiation codon for the HSV-2 UL16. Figure 9 presents the amino acid sequences in the one-letter notation of the polypeptide predicted for the HSV-2 UL16. The UL16 protein of HSV-2 consisted of 372 amino acids while the HSV-1 counterpart consists of 373 amino acids. The calculated isoelectric point was 7.69 that was slightly higher than that of the HSV-1 counterpart. The overall amino acid homology between the HSV-1 and HSV-2 UL16 proteins was 75.5%. As reported previously [32], the HSV-2 UL16 product, like its homologs, contained cystein-rich amino acid sequences which were similar to those seen in zinc-finger proteins although any of these sequences were not identical to well-characterized zinc-finger motifs [6].

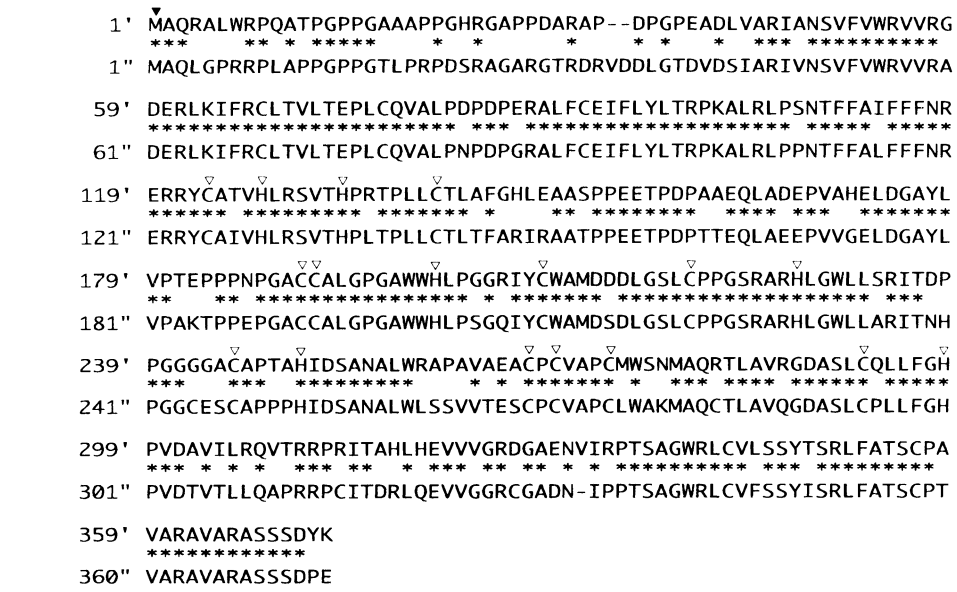

Fig. 9. The predicted amino acid sequence of the HSV-2 UL16 protein. The sequences are presented in the single-letter code and amino acids are numbered at the left end of each line. The HSV-1 UL16 sequence is presented in the lower line and the amino acid residues identical to those of the HSV-2 UL16 protein are indicated by asterisks. The putative initiation codon of HSV-2 UL16 was CTG, which must be translated as leucine according to the codon usage, and therefore the N-terminal methionine of HSV-2 is marked by a closed triangle. Open triangles indicate conserved cysteine and histidine residues which might be involved in zinc and/or nucleic acid binding

Discussion

The present study demonstrates that the UL16 product of HSV-2 was a protein of 41 kDa synthesized at a late phase of infection. At 9 h postinfection, the protein was localized both in the nucleus and in the cytoplasma, and the specific fluorescence in the nucleus was primarily detected within a granular structure. These observations are consistent with those of Nalwanga et al. [19], in which the HSV-1 UL16 protein is located within a discrete region of the infected cell nucleus that also contained HSV capsid proteins. Since this region coincides with so called “assemblons” in which capsid assembly or DNA cleavage/packaging may occur [31], they suggested a role for the UL16 protein of HSV-1 in capsid or virion assembly. Our analysis of intracellular capsids clearly demonstrated that the UL16 protein of HSV-2 was associated with C capsids which contain viral DNA. However, the protein was not detectable in B and A capsids. Previous studies have shown that B capsids may be precursors to DNA-containing C capsids and empty A capsids, the former eventually becoming enveloped and released from infected cells as mature virions [14, 24, 27]. Moreover, some DNA cleavage/packaging proteins such as the UL6 and UL15 proteins have been shown to be associated with B and C capsids [16, 22, 36]. Considering these observations

with our results, it seems reasonable to assume that the UL16 product plays a role in capsid or virion maturation.

From the presence of the UL16 product in C capsids, it was expected that the protein would be detected in mature virions of HSV-2, however, no detectable amount of the UL16 protein was observed in extracellular virions purified from the culture medium of HSV-2-infected cells. This observation was inconsistent with that by Nalwanga et al. [19] who showed that the UL16 protein of HSV-1 is a component of mature virions. When C capsids of HSV-2 were isolated by sucrose density gradient in the presence of 0.5 M NaCl, the capsids were free from the UL16 protein, which was detected at the top of the gradient, indicating that the association of the UL16 protein with C capsids was not tight. It appears that the UL16 protein transiently and weakly interacted with viral capsids in HSV-2-infected cell. Our data do not rule out the possibility that a trace amount of the UL16 protein is associated with the HSV-2 virions, but we consider that the incorporation of the UL16 product into mature virions is not a intrinsic nature of the protein.

At the late stage of infection, the UL16 protein of HSV-2 could be primarily detected as a mass in the perinuclear region of the cytoplasma and a similar staining pattern was also reported in HSV-1-infected cells [19]. Since the UL16 protein of HSV-1 was mainly distributed in the perinuclear region of the cytoplasma when singly expressed in Vero cells (data not shown), such distribution might be explained simply by the accumulation of the UL16 protein at the late phase of infection, or since the UL16 protein of HSV-2 was associated with C capsids, this may be due to the intracellular transport of C capsids from the nucleus to the cytoplasma. At the perinuclear site, the UL16 protein might come off from the capsids and accumulate as a mass.

We have determined the nucleotide sequence of the UL16 gene of HSV-2. One of the important features of the HSV-2 UL16 gene was that there was no ATG at the putative initiation site for translation, at which CTG was found. This feature was not strain-specific but type-specific since recent data from the GenBank have shown that the UL16 gene of HSV type 2 strain HG52 has also CTG at the putative initiation site (GenBank accession no. Z86099). Kozak [15] has proposed a consensus sequence PyPyPuPyAUGG for the initiation of translation in multicellular eukaryotes. The sequence around the putative initiation site of the HSV-2 UL16 gene was CCAACCTGG (PyPyPuPuPyCTGG), which differed in two site from the Kozak sequence. Although the putative initiation site of the HSV-2 UL16 does not contain the initiation codon ATG, the two important positions flanking the codon were conserved, that is, the sequence had A in position -3 and G in position $+4$. In the vertebrate system, only a small number of mRNA are known to be initiated at a non-AUG codon such as ACG, CUG or GUG and such mRNAs that use a nonstandard initiation codon have GC-rich leader sequence in most cases [15]. Indeed, the upstream of the HSV-2 UL16 had GC-rich sequences. However, it is all the same to that of HSV-1 UL16. It is also known that translation at such non-AUG codons is inefficient, but there was no marked difference between types 1 and 2 in the amounts of the UL16 proteins synthesized,

judging from the data of Western blotting. At present, the reason why the HSV-2 UL16 gene conserves non-AUG at the putative initiation site remains unknown. Another feature was that the amino acid sequence of the HSV-2 UL16 contained cysteine and histidine residues arranged in a manner that is reminiscent of those seen in zinc fingers [6, 12, 13]. Although the patterns were not in accord with those of well-characterized zinc-finger motifs, it is possible that these conserved residues are involved in binding zinc and DNA. In fact, the partially purified UL16 protein of HSV-2 could bind ssDNA. Since the UL16 protein was eluted as a single peak in both phosphocellulose and ssDNA agarose, the binding of the UL16 protein to ssDNA may be due to its own activity. However, the possibility that the UL16 protein indirectly bound DNA through a protein tightly associated with the UL16 protein can not be ruled out.

As described earlier, the UL16 gene is present within the intron of the UL15 gene and is transcribed antisense to the UL15 gene [9, 17]. Moreover, both UL15 and UL16 genes are highly conserved among members of the herpesvirus family [1, 8, 11, 17]. In these viruses the UL16 homologs are also located within the introns of the UL15 homologs. Recently, the UL15 product of HSV-1 has been suggested to play a role in DNA cleavage and packaging as a terminase [2, 23, 36]. Although the precise function of the UL16 product remains still obscure, our observations on its intranuclear localization, association with C capsids and DNA-binding activity have suggested a possible, supplemental role of the UL16 protein in capsid maturation including DNA packaging/cleavage.

Acknowledgements

We thank E. Iwata and T. Tsuruguchi for their technical assistance. This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS-RFTF97L00703) and also by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References

1. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrel PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Segin C, Tuffnell PS, Barrell BG (1986) DNA sequence and expression of the B95–8 Epstein-Barr virus genome. Nature 310: 207–211
2. Baines JD, Poon A P, Rovnak J, Roizman B (1994) The herpes simplex virus 1 U_L 15 gene encodes two proteins and is required for cleavage of genomic viral DNA. J Virol 68: 8 118–8 124
3. Baines JD, Roizman B (1991) The open reading frames U_L 3, U_L 4, U_L 10, and U_L 16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J Virol 65: 938–944
4. Balan P, Davis-Poynter N, Bell S, Atkinson H, Browne H, Minson T (1994) An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex type 1 lacking glycoproteins gG, gE, gI or the putative gJ. J Gen Virol 75: 1 245–1 258
5. Berg JM (1986) Potential metal-binding domains in nucleic acid binding proteins. Science 232: 485–487
6. Berg JM (1990) Zinc fingers and other metal-binding domains. J Biol Chem 265: 6 513–6 516

7. Booy FP, Newcome WW, Trus BL, Brown JC, Baker TS, Steven AC (1991) Liquid-crystalline, phage-like particles of eucapsidated DNA in herpes simplex virus. Cell 64: 1 007–1 015
8. Chee MS, Bankier AT, Bohni R, Brown CM, Cherny R, Hiorsnell T, Hutchinson CA, Kousarides T, Martignette JA, Preddie E, Satchwell SC, Tomlinson P, Weston KM, Barrel BG (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus AD169. Curr Top Microbiol Immunol 154: 124–169
9. Costa RH, Draper KG, Kelley TJ, Wagner EK (1985) An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus DNA. J Virol 54: 317–328
10. Daikoku T, Yamashita Y, Tsurumi T, Maeno K, Nishiyama Y (1993) Purification and biochemical characterization of the protein kinase encoded by the US3 gene of herpes simplex virus type 2. Virology 197: 685–694
11. Davison AJ, Scott JE (1986) The complete DNA sequence of varicella-zoster virus. J Gen Virol 67: 1 759–1816
12. Evans RM, Hollenberg SM (1988) Zinc fingers: gilt by association. Cell 52: 1–3
13. Freemont PS, Hanson IM, Trowsdale J (1991) A novel cysteine-rich sequence motif. Cell 64: 483–484
14. Gibson W, Roizman B (1972) Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J Virol 10: 1 044–1 052
15. Kozak M (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. J Cell Biol 115: 887–903
16. Lamberti C, Weller SK (1996) The herpes simplex virus type 1 UL6 protein is essential for cleavage and packaging but not for genomic inversion. Virology 226: 403–407
17. McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P (1988) The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J Gen Virol 69: 1 531–1 574
18. Meignier B, Longnecker R, Mavromara-Nazos P, Sears AE, Roizman B (1988) Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus type 1. Virology 162: 251–254
19. Nalwanga D, Rempel S, Roizman B, Baines JD (1996) The U_L 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. Virology 226: 236–242
20. Nicholson P, Addison C, Cross AM, Kennard J, Preston VG, Rixon FJ (1994) Localization of the herpes simplex virus type 1 major capsid protein VP5 to the cell nucleus requires the abundant scaffolding protein VP22a. J Gen Virol 75: 1 091–1 099
21. Nishiyama Y, Yamada Y, Kurachi R, Daikoku T (1992) Construction of a US3 lacZ insertion mutant of herpes simplex type 2 and characterization of its phenotype in vitro and in vivo. Virology 190: 256–268
22. Patel AH, Rixon FJ, Cunningham C, Davison AJ (1996) Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL16 gene. Virology 217: 111–123
23. Poon AP, Roizman B (1993) Characterization of a temperature-sensitive mutant of the UL15 open reading frame of herpes simplex virus 1. J Virol 67: 4 497–4 503
24. Rixon FJ (1993) Structure and assembly of herpesviruses. Semin Virol 4: 135–144
25. Rixon FJ, Addison C, McLauchlan J (1992) Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. J Gen Virol 73: 277–284
26. Roizman B (1996) The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors. Proc Natl Acad Sci USA 93: 11 307–11 312

27. Roizman B, Sears AE (1996) Herpes simplex viruses and their replication. In: Fields BN, Knipe DM, Howley PM (eds) Virology. Lippincott-Raven, New York, pp 2 231–2 295
28. Szilagyi JF, Cunningham C (1991) Identification and characterization of a novel non-infectious herpes simplex virus-related particle. J Gen Virol 72: 661–668
29. Tsurumi T, Maeno K, Nishiyama Y (1986) Molecular cloning of herpes simplex virus type 2 DNA. J Biochem 99: 981–984
30. Tsutsui Y, Nishiyama Y, Yoshida S, Maeno K, Hoshino M (1983) Role of the nuclear matrix in the growth of herpes simplex virus type 2. Arch Virol 77: 27–38
31. Ward PW, Ogle WO, Roizman B (1996) Assemblons: nuclear structures defines by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. J Virol 70: 4 623–4 631
32. Wing BA, Lee GY, Huang E (1996) The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. J Virol 70: 3 339–3 345
33. Yamada Y, Kimura H, Morishima T, Daikoku T, Maeno K, Nishiyama Y (1991) The pathogenicity of ribonucleotide reductase-null mutants of herpes simplex virus type 1 in mice. J Infect Dis 164: 1 091–1 097
34. Yamashita S, Wada K, Horikoshi M, Gong DW, Kokubo T, Hisataka K, Yokotani N, Malik S, Roeder RG, Nakatani Y (1992) Isolation and characterization of cDNA encoding Drosophila transcription factor TFIIB. Proc Natl Acad Sci USA 89: 2 839–2 843
35. Yamauchi M, Nishiyama Y, Fujioka H, Isomura S, Maeno K (1985) On the intracellular transport and the nuclear association of human cytomegalovirus structural proteins. J Gen Virol 66: 675–684
36. Yu D, Sheaffer AK, Tenney DJ, Weller SK (1997) Characterization of ICP6: lacZ insertion mutants of the UL15 gene of herpes simplex virus type 1 reveals the translation of two proteins. J Virol 71: 2 656–2 665

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Received October 31, 1997