Nucleolar localization of the UL3 protein of herpes simplex virus type 2

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A rabbit polyclonal antiserum was raised against a recombinant 6×His–UL3 fusion protein expressed in *Escherichia coli* and used to examine the intracellular localization of the UL3 protein of herpes simplex virus type 2 (HSV-2). The antiserum reacted specifically with 31 and 34 kDa proteins in HSV-2 186-infected Vero cells and with 31 and 35 kDa proteins in UL3-expressing COS-7 cells. The UL3 protein localized both in the cytoplasm and in five to ten bright fluorescent granules in the nucleus close to the nuclear membrane at 4 h post-infection (p.i.). These structures became bigger at 5 h p.i. and showed doughnut-like forms at 6 h p.i. In transfected Vero cells, the UL3 protein localized exclusively in the nucleoplasm and specifically in the nucleolus. Five deletion mutants of the UL3 protein were constructed for transfection assays and the results showed that the region containing amino acids 100–164 was important for nucleolar localization. Moreover, green fluorescent protein (GFP)-targetting experiments showed that the region containing amino acids 100–164 was able to transport non-nucleolar GFP to the nucleolus as a fusion protein.

**Introduction**

Herpes simplex virus (HSV) is a large DNA virus, the genome of which encodes approximately 80 genes (Dolan *et al.*, 1998). Recent studies have shown that approximately half of these genes are not essential for replication of the virus in cell culture (Roizman & Sears, 1996). These dispensable gene products are, however, thought to be important for virus growth and spread in the natural host.

This report focuses on the product of the UL3 gene of HSV type 2 (HSV-2). The UL3 gene of HSV-2 is predicted to encode a 233 amino acid protein with a molecular mass of 26 kDa (McGeoch *et al.*, 1991). Homologues of the UL3 protein are encoded only among alphaherpesviruses (Davison & Scott, 1986; McGeoch *et al.*, 1988; Telford *et al.*, 1992, 1998; Dean & Cheung, 1993; Yoshida *et al.*, 1994; Khattar *et al.*, 1995). There have been several reports concerning the UL3 protein, which have revealed that: (i) open reading frames (ORFs) UL3, UL4, UL10 and UL16 are dispensable for the replication of HSV-1 in cell culture (Baines & Roizman, 1991); (ii) the UL3 protein of HSV-2 is a nuclear-localizing phosphoprotein and is not a glycoprotein (Worrad & Caradonna, 1993); and (iii) the UL3 protein of HSV-1 is a phosphoprotein and is not a glycoprotein (Ghiasi *et al.*, 1996). However, the function of the UL3 protein of HSV remains unknown.

In this study, we examined the intracellular localization of the UL3 protein in infected and transfected cells. The results demonstrate that the UL3 protein has a nucleolar-localizing property and that the region containing amino acids 100–164 is important for this nucleolar localization.

**Methods**

- **Cells and viruses.** Vero cells were grown in Eagle's minimum essential medium supplemented with 5% calf serum. COS-7 cells were grown in Dulbecco's minimum essential medium supplemented with 5% foetal bovine serum. Wild-type HSV-1 strain KOS and HSV-2 strain 186 were used in this study. Viruses were propagated and titrated on Vero cells. Vero cells were infected at an m.o.i. of 3 p.f.u. per cell.

- **Generation of polyclonal antiserum in rabbits.** The UL3 ORF is located at the left end of the U3 region of the HSV-2 genome (McGeoch *et al.*, 1991). The UL3 coding sequence was amplified by PCR from HSV-2 186 HindIII fragment B (Tsurumi *et al.*, 1986). The 5′ and 3′ primers used for the amplification were 5′ TTCGAATTCTAGGTAAATCTCGGCTCTCA 3′ and 5′ AATCTCGACTGCTTAAGTCTGTTCCGT-AGA 3′, respectively. EcorI and Xhol sites were incorporated into the 5′ and 3′ primers, respectively, to facilitate cloning. PCR amplification was carried out as described previously (Yamada *et al.*, 1997). The PCR product was digested with EcorI and Xhol and cloned in-frame.
downstream of the region encoding the initiating ATG plus six histidine residues in the *Escherichia coli* expression vector pET-28a (Novagen) to give the plasmid pET28-UL3. Expression of this fusion protein is regulated by an IPTG-inducible lac operator sequence and a phage T7 promoter. Translation is expected to terminate at the stop codon of the UL3 gene. This plasmid was transformed into *E. coli* strain BL21(DE3) (Novagen) which, following induction with IPTG, expressed large quantities of 6×His–UL3 fusion protein. Purification of the UL3 fusion protein and immunization of rabbits were done as described previously (Yamada et al., 1998). Both preimmune and immune antisera 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).

### Construction of plasmids

The wild-type form and deletion mutants of the UL3 protein were made as shown in Fig. 4(a). Nomenclature of the constructs indicates deleted amino acids; for example, MEagΔ81/174 indicates a truncated UL3 protein containing amino acids 1–80 fused to amino acids 175–236 (i.e. deletion of amino acids 81–174). Different parts of the UL3 coding sequence were obtained by PCR and PCR fragments were cloned into the EcoRI and XhoI sites of the eukaryotic expression vector pCR3 (Invitrogen). The N-terminal deletion mutant MN1Δ99 was made by using a 5′ primer encoding an internal initiation codon. All C-terminal deletion mutants were introduced by a termination codon. In the case of the mutant MEagΔ81/174, the plasmid pET28-UL3 was digested with EagI and religated (pET28-UL3EagI). The PCR fragment containing the region encoding amino acids 1–80 fused to that encoding amino acids 175–236 was obtained by using pET28-UL3EagI as a template. UL3–green fluorescent protein (GFP) fusion proteins were made as shown in Fig. 6(a). Different parts of the UL3 coding sequence were obtained by PCR as described above and PCR fragments were cloned into the XhoI and PstI sites of pEGFP-N1 vector (Clontech). The correctness of the constructs was analysed by sequencing.

### Western blotting

Proteins were transferred electrophoretically from SDS–PAGE gels to PVDF transfer membranes as described by Towbin et al. (1979). Bound primary antibodies were detected by using horseradish peroxidase-linked sheep anti-rabbit IgG (Amersham) and ECL Western blotting detection reagents (Amersham).

### Cell transfection

Vero and COS-7 cells were transfected with 2 mg plasmid DNA by using TransIT transfection reagents LT1 (PanVera) according to the manufacturer's instructions.

### Indirect immunofluorescence

The indirect immunofluorescence assay was performed essentially as described by Ward et al. (1996). A goat polyclonal antibody to the B23 protein was purchased from Santa Cruz. For secondary antibodies, we used TRITC-conjugated swine anti-rabbit IgG (Dako) and FITC-conjugated donkey anti-goat IgG (Santa Cruz). Fluorescent images were viewed and recorded with the Bio-Rad MRC-series confocal imaging system. Nucleolar shape was confirmed under the phase-contrast microscope.

## Results and Discussion

### Preparation and specificity of anti-UL3 protein antisera

As a first step towards the study of the UL3 protein, rabbit polyclonal antisera specific to this protein were raised by using an *E. coli*-produced recombinant UL3 fusion protein as antigen. The plasmid pET28-UL3 was constructed for this purpose. When expressed in *E. coli*, this plasmid expresses the entire UL3 ORF with a 6×His tag attached to the N terminus. High levels of expression of the resulting 46 kDa fusion protein were obtained in *E. coli* following induction with IPTG (Fig. 1a, lane 2). The induced fusion protein was purified (lane 3) and was used for immunization of rabbits.

To examine the reactivity and specificity of UL3 antisera, Western blotting experiments were performed. Vero cells were mock-infected or infected with HSV-1 KOS or HSV-2 186 at an m.o.i. of 3 p.f.u. per cell. Fig. 1(b) shows that one of the UL3 antisera reached with two bands with apparent molecular masses of 31 and 34 kDa in HSV-2-infected Vero cells (lane 3). It has been reported that two major bands (31 and 33 kDa) and one minor band (28 kDa) are detected in HSV-2-infected cells and that both 33 and 31 kDa proteins are phosphorylated (Worrard & Caradonna, 1993). Thus, we thought that the 31 and 34 kDa proteins in our studies corresponded to the phosphorylated 31 and 33 kDa proteins. The antisera reacted with 31 and 35 kDa proteins in UL3-expressing COS-7 cells (lane 4). However, these bands were not detected in mock- or HSV-1-infected Vero cells (lanes 1, 2). These results indicate that the 31 and 34 kDa proteins are the products of the HSV-2 UL3 gene. The reactivity of this antisera with the 31 and 34 kDa bands was clearly eliminated by preadsorption of the antisera with lysates of *E. coli* expressing the UL3 fusion protein (not shown), but there was no significant change in the reactivity after preadsorption with lysates of standard *E. coli* (not shown). The preimmune serum did not react with any
specific proteins in HSV-2-infected cells (not shown). The other two UL3 antisera showed the same results on Western blotting (not shown). Therefore, we used this polyclonal antiserum for further experiments to characterize the UL3 protein of HSV-2.

**Intracellular localization of the UL3 protein**

The intracellular localization of the UL3 protein in HSV-2-infected Vero cells was analyzed by immunofluorescence assay. As shown in Fig. 2(b), the UL3 protein localized both in the cytoplasm and in five to ten bright fluorescent granules close to the nuclear membrane in the nucleus within the first 4 h of infection. These structures became bigger (Fig. 2c) and showed doughnut-like forms at about 6 h post-infection (p.i.) (Fig. 2d). By approximately 9 h p.i., cytoplasmic staining had disappeared in almost all cells (Fig. 2e). These structures were absent from mock-infected cells (Fig. 2a), and no significant fluorescence was observed with the preimmune serum (not shown). In addition, our preliminary experiments (not shown) showed that the UL3 protein co-localized with ICP8 at the stage of infection when it formed doughnut-like structures, as shown in Fig. 2(d, e).

Next, intracellular localization of the UL3 protein expressed from a eukaryotic expression vector under the control of the cytomegalovirus (CMV) immediate-early promoter was examined. For analysis of intracellular localization in transfected cells, we used Vero cells instead of COS-7 cells to avoid overexpression of the protein, which could lead to abnormal localization. Vero cells were transfected and analyzed by immunofluorescence assay. We used a goat polyclonal antibody specific to B23 as a marker for the nucleolus. B23 is a major nucleolar phosphoprotein that shuttles between the nucleus and the cytoplasm and binds to Rev of human immunodeficiency virus type 1 (HIV-1) (Borer et al., 1989). The Rev protein transports HIV RNA from the nucleolus to the cytoplasm (Fankhauser et al., 1991). The UL3 protein co-localized with B23 in the nucleolus in transfected cells, with the rest of the nucleus staining more weakly 20 h after transfection (Fig. 3a–c). At later times after transfection (40 h), the nucleolus of UL3-expressing cells was enlarged and the UL3 protein localized at the periphery of B23 (Fig. 3d–f). Enlarged and deformed nucleoli were also observed in Rev-expressing cells (Miyazaki et al., 1995). Rev infiltrates throughout these nucleoli and co-localizes with B23, accumulating markedly, while the UL3 protein remained at the periphery of the nucleolus.

Little is known about how cellular nucleolar proteins are influenced by HSV infection. It has been reported that ICP4 interacts with a nucleolar-ribosomal protein, EAP, and translocates it from the nucleolus to the nucleoplasm (Leopardi & Roizman, 1996), and we observed that B23 was dispersed throughout the infected cells and did not form distinct structures (not shown). We think that a cellular protein(s), which interacts with the UL3 protein and is responsible for its nucleolar localization in transfected cells, may be translocated in punctate structures close to the nuclear membranes in the nucleus together with the UL3 protein at early times p.i.
Fig. 3. Intracellular localization of the UL3 protein in transfected Vero cells. UL3-expressing Vero cells were fixed 20 (a–c) or 40 h (d–f) after transfection and double-labelled with the rabbit UL3 antiserum (a, d) and with a goat polyclonal antibody to the nucleolar protein B23 (b, e). Single-colour images were captured separately and are shown in (a–b) and (d–e). The two colours were then merged and are shown in (c, f). The yellow colour seen in the merged images represents colocalization of green and red fluorescence. Magnification x1450 (a, d), x1050 (b, e).

Fig. 4. (a) A summary of intracellular localization of the UL3 protein and its deletion mutants. pCR3 vector was used to express all the constructs. Bars represent translated amino acids. Thin lines represent deleted amino acids. These are not shown to scale. N, nucleus; C, cytoplasm. (b) Western blotting analysis, using the UL3 antiserum, of the UL3 protein and its deletion mutants expressed in COS-7 cells and harvested 30 h after transfection. Wild-type UL3 (lane 1) and the mutants MN1Δ1/99 (2), MC1Δ196/233 (3), MC2Δ188/233 (4), MC3Δ165/233 (5) and MEagΔ142/205 (6) were expressed.

The nucleolus is a highly structured and specialized organelle that functions as the site of both the synthesis of rRNA and the assembly and processing of preribosomal ribonucleoprotein particles (Mélèse & Xue, 1995). Electron microscopic studies have shown that the nucleolus is subdivided into at least three morphologically distinct components: the fibrillar centre, the dense fibrillar component (DFC) and the granular component (GC). Early steps of rRNA processing and subsequent steps of assembly and maturation of ribosomal subunits occur in the first two components. The
The UL3 protein of HSV-2

(a) (b) (c) (d) (e)

Fig. 5. Intracellular localization of deletion mutants of the UL3 protein. Vero cells expressing mutants MN1Δ1/99 (a), MC1Δ196/233 (b), MC2Δ188/233 (c), MC3Δ165/233 (d) and MEagΔ142/205 (e) were processed for immunofluorescence 24 h after transfection. Magnification ×1750 (a–c), ×780 (d–e).

GC is thought to be connected with later processes of rRNA maturation (Scheer & Weisenberger, 1994). It has been shown that both Rev and Tat of HIV-1 localize to the DFC and GC and co-localize with B23 in transfected cells (Miyazaki et al., 1995). The Tat protein, which is required for efficient viral transcription by stimulating transcription directed by the long terminal repeat sequence, has a basic region that binds to the trans-activation region of the viral RNA and is required for its nucleolar localization (Hauber et al., 1989).

Expression and intracellular localization of mutant UL3 proteins

Two potential nuclear localization signals (NLSs) are found between amino acids 143 and 147 (NLS1) and 188 and 192 (NLS2) of HSV-2 UL3, with the sequences RQRKR and RKPRK, respectively (Worrad & Caradonna, 1993). To study the roles of these two potential NLSs and the amino acid sequence(s) essential for nucleolar localization, we constructed five deletion mutants as described in Methods (Fig. 4a). To examine the expression of the five deletion mutants, COS-7 cells were transfected with plasmids expressing either the wild-type or the mutant versions of the UL3 protein. The transfected cell extracts were analysed by Western blotting. As shown in Fig. 4(b), the wild-type UL3, the three C-terminal and one internal deletion mutants were expressed as stable proteins of the expected sizes (lanes 1, 3–6). However, the N-terminal deletion mutant MN1Δ1/99 was not detected by Western blotting (lane 2).

An immunofluorescence assay was used to analyse the intracellular localization of deletion mutants of the UL3 protein in transfected Vero cells 24 h after transfection. Fig. 5 shows representative immunofluorescence data from each deletion mutant. Vero cells expressing MN1Δ1/99 at low efficiency showed bright nucleolar staining (Fig. 5a). The pattern of intranuclear localization of the mutant MC1Δ196/233 was very similar to the wild-type UL3 protein (Fig. 5b). The potential NLS2-deletion mutant MC2Δ188/233 also accumulated in the nucleolus (Fig. 5c). Although the deletion mutant MC3Δ165/233 was present in both the nucleus and the cytoplasm, it accumulated in the nucleolus (Fig. 5d). These results suggested that the region containing amino acids 100–164 of the UL3 protein, which is common to all four terminal deletion mutants, may be important for nucleolar localization. The internal deletion mutant MEagΔ142/205, which has neither potential NLS1 nor NLS2, was present both in the nucleus and the cytoplasm, and was excluded from the nucleolus (Fig. 5e), suggesting a role of the potential NLS1 for nucleolar localization. These results implied that the region containing amino acids 142–164, which is common to the region 100/164 and the internal deletion of MEagΔ142/205 and includes the NLS1, was responsible for nucleolar localization of the UL3 protein.
So far, several nucleolar targeting sequences have been identified and some common features have been found: (i) the signal is extremely rich in basic residues, especially arginine; and (ii) the signal is linked to the NLS, forming an ‘extended’ NLS (Siomi et al., 1988; Dang & Lee, 1989). In most cases, however, nucleolar localization results from specific protein–protein or protein–nucleic acid interactions, not from a general targeting sequence (Peculis & Gall, 1992; Rikkonen et al., 1992; Schmidt-Zachmann & Nigg, 1993).

Expression and intracellular localization of UL3–GFP fusion proteins

To determine whether the region 100/164 would be sufficient to target a non-nucleolar protein to the nucleolus, the wild-type and deletion-mutant forms of the UL3 protein were fused to the N terminus of GFP, a well-characterized protein (Fig. 6a). To examine the expression of the proteins synthesized from the hybrid genes, COS-7 cells were transfected...

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**Fig. 6.** (a) A summary of intracellular localization of GFP fusion proteins containing portions of the UL3 protein. pEGFP-N1 vector was used to express all the constructs. Bars represent translated amino acids. Thin lines represent deleted amino acids. These are not shown to scale. N, nucleus; C, cytoplasm. (b) Western blotting analysis, using the UL3 antisera, of GFP fusion proteins containing portions of the UL3 protein expressed in COS-7 cells and harvested 30 h after transfection. UL3–GFP (lane 1), MΔ1/99–GFP (2), MΔ1/164–GFP (3), M100/164–GFP (4) and MΔ142/205–GFP (5) were expressed.

**Fig. 7.** Intracellular localization of GFP fusion proteins containing portions of the UL3 protein. Vero cells expressing GFP (a) or fusion proteins UL3–GFP (b), MΔ1/99–GFP (c), MΔ1/164–GFP (d), M100/164–GFP (e) or MΔ142/205–GFP (f) were processed for immunofluorescence 24 h after transfection. Magnification ×900 (a), ×1750 (b–c), ×650 (d–f).
with each plasmid under the control of the CMV immediate-early promoter. Equal aliquots of cell lysates were analysed by Western blotting. As shown in Fig. 6(b), a fusion protein of the expected size was produced in substantial amounts from each plasmid. These results demonstrated that the GFP portion did not affect the accumulation of the fusion proteins. Although the wild-type UL3–GFP fusion protein exhibited two bands, the N-terminal mutant MA1/99–GFP exhibited one band. Thus, it seems that one phosphorylation site is contained in the region containing amino acids 1–99.

An immunofluorescence assay was used to analyse the intracellular localization of these UL3–GFP fusion proteins in transfected Vero cells 24 h after transfection. GFP expressed alone was distributed almost equally throughout the cell except for the nucleolus (Fig. 7a). Fusion of the wild-type UL3 protein to this protein conferred nuclear and nucleolar localization (Fig. 7b). MA1/99–GFP was present in the nucleolus (Fig. 7c) but MA1/164–GFP was not (Fig. 7d), strengthening the hypothesis that the region 100/164 of the UL3 protein is required for nucleolar localization. Although M100/164–GFP was present both in the nucleus and the cytoplasm, it accumulated in the nucleolus (Fig. 7e), indicating that the region 100/164 of the UL3 protein was sufficient for nucleolar localization. MA142/205–GFP was excluded from the nucleolus (Fig. 7f).

GFP, from the bioluminescent jellyfish _Aequorea victoria_, has been used to monitor the intracellular localization of proteins fused to it (Chalfie _et al._, 1994). It yields a bright-green fluorescence when expressed and illuminated by blue or UV light and GFP fluorescence does not require any other cofactors or substrates. Variants of GFP that improve the use of the GFP reporter have been generated and one such variant, enhanced GFP (EGFP), fluoresces 35-fold more brightly than wild-type GFP when excited with blue light (Cormack _et al._, 1996). As regards HSV, recombinant viruses have been constructed expressing this modified EGFP fused to the capsid protein VP26 or inserted in place of glycoprotein K to investigate virus entry and replication (Desai & Person, 1998; Fosler _et al._, 1998). Our GFP-targetting experiments showed that the intracellular localization of each UL3–GFP fusion protein was consistent with that of the corresponding deletion mutant of the UL3 protein and that the region 100/164, specifically the region 142/164, was important for nucleolar localization. We are examining whether the region 142/164 is able to transport non-nuclear GFP to the nucleolus as a fusion protein.

There are at least three proteins of HSV that have been demonstrated to be associated with the nucleolus. Firstly, the US11 protein localizes to the nucleoli of HSV-1-infected cells (MacLean _et al._, 1987). It binds RNA and associates with ribosomal 60S subunits (Roller & Roizman, 1992). Secondly, ICP27 possesses an RGG motif, which can function as a nucleolar targeting signal and bears similarity to a putative RNA-binding motif found in a number of cellular proteins involved in nuclear RNA processing (Mears _et al._, 1995). Thirdly, the US8.5 protein localizes to the nucleoli of HSV-1-infected cells (Georgopoulou _et al._, 1995), although its function remains unknown.

A number of other viral proteins have been identified in the nucleolus. EBNA-5 of Epstein–Barr virus, together with the hsp70 protein, translocates to the nucleolus under cell density congestion or after heat shock in transfected cells (Szekely & Semliki _et al._, 1995). Rex of human T cell leukaemia virus type 1, nsP2 of Semliki Forest virus, the matrix protein of Newcastle disease virus and pIvA2 of adenovirus localize to the nucleolus in infected and transfected cells (Siomi _et al._, 1988; Peränen _et al._, 1990; Coleman & Peeples, 1993; Lutz _et al._, 1996). The capsid protein of Semliki Forest virus and MEQ of Marek’s disease virus are also reported to localize to the nucleoli in transfected cells (Favre _et al._, 1994; Liu _et al._, 1997).

The association of the UL3 protein of HSV-2 with the nucleolus may provide new leads to uncovering its function.

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