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Articles

An Importin α/β-Recognized Bipartite Nuclear Localization Signal Mediates Targeting of the Human Herpes Simplex Virus Type 1 DNA Polymerase Catalytic Subunit pUL30 to the Nucleus†

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ABSTRACT: Although the 1235 amino acids human herpes simplex virus type 1 (HSV-1) DNA polymerase catalytic subunit, pUL30, is essential for HSV-1 replication in the nucleus of host cells, little information is available regarding its nuclear import mechanism. The present study addresses this issue directly, characterizing pUL30's nuclear import pathway for the first time using quantitative confocal laser scanning microscopy (CLSM) on living cells, and fluorescent binding assays. In addition to a previously described nuclear localization signal (NLS) located within the pUL30 binding site for the polymerase accessory protein (PAP) pUL42, that appears to be dispensable for nuclear targeting, pUL30 possesses three putative basic NLSs. Intriguingly, the core of pUL30-NLS2 (residues 1114−1120) is highly homologous to that of the recently described NLS, similarly located upstream of the PAP binding site, of the human cytomegalovirus (HCMV) DNA polymerase catalytic subunit, pUL54. Here we show for the first time that pUL30-NLS2 itself is only partially functional in terms of nuclear import due to residue P^{1118} present in position 3 of the NLS core. Intriguingly, pUL30-NLS2 together with pUL30-NLS3 (residues 1133−1136) represents a fully functional bipartite NLS (pUL30-NLSbip), required for nuclear targeting of pUL30, and able to confer nuclear localization on heterologous proteins by conferring high-affinity interaction with the importin (IMP) α/β heterodimer. Since nuclear targeting of HSV-1 proteins forming the replication fork is crucial for viral replication, the pUL30-NLSbip emerges for the first time as a viable therapeutic target.

Human herpes simplex virus type 1 (HSV-1),[1] the principal alpha herpesvirus of humans, represents a cause of severe disease in newborns and immunosuppressed individuals (*1*−*4*). Replication of its 153 kb double-stranded DNA genome

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occurs in the nucleus of infected cells and requires a set of seven viral-encoded proteins (*5*, *6*), including pUL30, the 1235 amino acids, 137 kDa, catalytic subunit of the viral DNA polymerase (*7*). As a crucial enzyme for viral replication, it is the target of acyclovir (ACV), the main antiviral drug used to combat HSV-1 (*8*). As ACV resistance is becoming a problem of increasing importance, especially in immunocompromised hosts (*9*), the development of new, effective anti-HSV drugs is highly desirable (*10*). In this context, understanding the structure and role of functional domains within pUL30 would prove invaluable in developing new anti-HSV drugs.

pUL30 can be purified from infected cells together with the 488 amino acids phosphoprotein pUL42 (*11*, *12*), a 65 kDa polymerase accessory protein (PAP) which is required to confer processivity to the DNA polymerase holoenzyme (*13*, *14*), and hence is essential for viral DNA replication (*6*).

Interaction of pUL30 with pUL42 is crucial for the HSV-1 life cycle, in that small molecules interfering with such an interaction also impair viral replication (*15*). Such interaction has been studied in detail and involves hydrogen-bonding residues at the C-terminus of pUL30 and the connector loop of pUL42 (*16*−*18*). Similarly, the C-terminus of human cytomegalovirus (HCMV) DNA polymerase catalytic subunit pUL54 interacts with the connector loop of its PAP, ppUL44 (*19*−*21*). The C-terminal domains of pUL30 and pUL54 also share the presence of nuclear targeting sequences (nuclear localization signals or NLSs) containing a hydrophobic core (NLS-hyd) (*22*), partially overlapping with the PAP binding sites and thus not accessible when complexed with the respective PAP in the DNA polymerase holoenzyme (*18*, *21*−*23*). Thus, complexes of either HCMV or HSV-1 PAP with their respective pUL54 or pUL30 molecule appear to require an additional mechanism to enable nuclear targeting, which, in the case of HCMV, is supplied by an additional basic NLS in pUL54 that is not masked when ppUL44 is bound [see below; (*23*)].

The eukaryotic cell nucleus is separated from the rest of the cell by a double membrane structure—the nuclear envelope (NE)—the only passage through which is provided by the multiprotein-constituted nuclear pore complexes (NPCs). Molecules >50 kDa need to be actively translocated into the nucleus in an NLS-dependent fashion through the action of members of the importin (IMP) superfamily of intracellular transporters (*24*), which mediate docking of the NLS-containing protein to the NPC and translocation through it into the nucleus (*25*). A well-characterized class of NLS is recognized by the IMPα/β heterodimer, where IMPα recognizes the NLS, and IMPβ facilitates the IMPα-NLS interaction by mediating a conformational change in IMPα (*26*). This class of NLSs comprises both monopartite NLSs, single clusters of basic residues, similar to that characterized extensively for the simian virus SV40 large tumor antigen (Tag) and bipartite NLSs, formed by two closely located basic clusters, reminiscent of that originally described for the *Xenopus laevis* histone chaperone nucleoplasmin (*27*, *28*).

Because of its apparent molecular weight of 137 kDa (*7*), pUL30 has to be actively translocated to the nucleus of infected cells in order to catalyze the replication of the viral DNA (*22*). We have previously reported that pUL54-NLShyd is not functional and that pUL54 is translocated to the nucleus by the IMPα/β heterodimer via the basic NLS (NLSA: PA**KKRAR**^{1159}), which is located upstream of the PAP binding site and is therefore still functional when ppUL44 is bound to pUL54 (*23*). Interestingly NLS-hyd is dispensable for pUL30 nuclear accumulation (*29*), presumably because pUL30 possesses three additional putative basic NLSs (*22*), one of which (pUL30-NLS2: PA**KR**P**R**E^{1120}) is highly homologous to the pUL54-NLS, as well as being similarly located in the protein relative to the PAP binding site (*22*, *23*). Because nuclear import of herpetic DNA polymerases represents a crucial step in viral replication, it has been the object of intense study in the past few years, which led to the identification of functional NLSs on several herpetic DNA polymerase subunits (*22*, *23*, *30*−*34*). Surprisingly, little data is available on the nuclear import of HSV-1 DNA polymerase subunits. The aim of this study was to investigate the nuclear targeting mechanism of pUL30 and, in particular, the role of the basic NLSs, and the IMPs potentially recognizing them. Using live cell imaging, quantitative confocal laser scanning microscopic (CLSM) analysis at the single cell level, and quantitative gel mobility shift assays, we report characterization of the nuclear import pathway of pUL30 for the first time, identifying a bipartite NLS (NLSbip, residues 1114−1136), encompassing two putative monopartite NLSs (NLS2 and NLS3), which is necessary for pUL30 nuclear transport, able to confer nuclear localization on GFP-β-galactosidase fusion proteins as well as mediating high affinity binding to the IMPα/β heterodimer through IMPα. We also show here by mutagenic analysis that pUL30-NLS2, which has strong homology to the pUL54 basic NLS and matches the consensus for IMPα/β binding, is not fully functional in mediating pUL30 nuclear targeting in the absence of NLS3, probably due to a proline residue in position 3 of the NLS core.

MATERIALS AND METHODS

Construction of Expression Plasmids. pUL30-GFP fusion proteins expressing vectors were generated using the Gateway system (Invitrogen, Carlsbad, CA). Primers including the attB1 and attB2 recombination sites were used to amplify the UL30 sequences of interest, with plasmid pE30 as a template (*5*). Polymerase chain reaction fragments were introduced into plasmid vector pDONOR207 (Invitrogen, Carlsbad, CA) via the BP recombination reaction, according to the manufacturer's recommendations, to generate the entry clones pDNR-UL30(2-1235), pDNR-UL30(2-1115), pDNR-UL30(2-1134), and pDNR-UL30(1114-1134). pDNR-UL30-(2-1235; NLS2m) and pDNR-UL30(2-1235; NLS3m), carrying point mutations within UL30-NLS2 (PAqas**R**ETPS-PADPPGGAS**K**P**RK**^{1136}) and UL30-NL3 (PA**KR**P**R**ETPS-PADPPGGAS**K**Psl^{1136}), respectively, were generated using

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[1] Abbreviations: HSV-1, herpes simplex virus type 1; ACV, acyclovir; PAP, polymerase accessory protein; HCMV, human cytomegalovirus; NE, nuclear envelope; NPC, nuclear pore complex; IMP, importin; Tag, large tumor antigen; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; β-Gal, β-galactosidase; Fn/c, nuclear to cytoplasmic fluorescence ratio; KHSV, Kaposi's associated human herpesvirus.

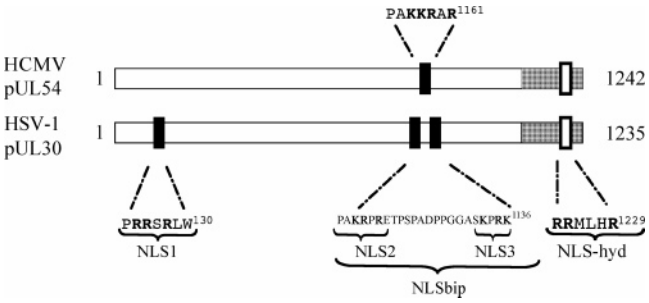


FIGURE 1: pUL30 contains three putative basic NLSs located outside the pUL42 binding site. Schematic representation of the HCMV pUL54 and HSV-1 pUL30 DNA polymerase catalytic subunit coding sequences; basic NLSs, black bars; hydrophobic NLSs, white bars; PAP binding sites, dotted boxes. The single letter amino acid code is used.

the Quickchange mutagenesis kit (Stratagene) and appropriate oligo pairs, accordingly to the manufacturer's recommendations, using vector pDNR-UL30(2-1235) as a template. These constructs, in turn, were used to perform LR recombination reactions with the Gateway system compatible expression plasmid pEPI-DEST-GFP (*35*), accordingly to the manufacturer's recommendations, in order to generate mammalian expression vectors encoding N-terminally GFP-tagged fusion proteins. Bacterially expressing construct pGFPrfB-UL30-(1114-1136), enabling the expression of the His_6GFP-UL30-(1114-1136) fusion protein in *Escherichia coli* under the control of the Ptac promoter upon induction with IPTG, was similarly generated via LR recombination reaction between pDNR-UL30(1114-1136) and the Gateway system compatible plasmid pGFPrfB for bacterial expression (*36*).

To generate mammalian cell expression constructs pHM830-UL30-NLS1(P**RRS**RLW[130]), pHM830-UL30-NLS2-(PA**KR**P**R**E[1120]), pHM830-UL30-NLS3(**K**P**RK**[1136]), pHM830-UL30-NLSbip (PA**KR**P**R**ETPSPADPPGGAS**K**P**RK**[1136]), and pHM830-UL30-NLS2mut(PA**KR**a**R**E[1120]) encoding fusion proteins containing the HSV-1 pUL30 putative NLSs inserted between the coding sequences of green fluorescent protein (GFP) and β-galactosidase (β-Gal), appropriate oligonucleotide pairs were annealed and cloned into expression vector pHM830 as previously (*33*). The mammalian cell expression constructs pHM830-nucleoplasmin NLS and pHM830-pUL54 NLS encoding fusion proteins with the nucleoplasmin bipartite NLS (**KR**PAAT**KK**AGQA**KKKK**[170]) or the HCMV pUL54 NLS (PA**KKR**A**R**[1159]) in frame between the coding sequences of GFP and β-Gal, respectively, and pEPI-GFP-UL44, encoding for full length HCMV ppUL44 fused to GFP have been described (*33*, *37*).

The integrity of all constructs was confirmed by DNA sequencing (Primm, Rome, Italy).

Cell Culture and Transfection. COS-7 and Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 50 U/mL penicillin, 50 U/mL streptomycin, and 2 mM L-glutamine.

Cells were trypsinized and seeded onto coverslips in twelve-well multiwell plates, or directly onto 22 mm glass bottom Willcodishes (Willcowells, Amsterdam, The Netherlands) 1 day before transfection, which was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's specifications.

Fluorescence Microscopy/Image Analysis. Following fixation with paraformaldehyde 4% (w/v), COS-7 cells expressing GFP-UL30(2-1235) were incubated with DAPI (1 μg/mL) for 4 min at RT to stain cell nuclei. Samples were washed with PBS, mounted on coverslips in PBS/glycerol 50% (v/v), and imaged using a Nikon Eclipse E600 microscope equipped with a Nikon DXN1200 digital camera and a Nikon Plan Fluor 40× objective (Nikon, Tokio, Japan). Live COS-7 cells grown on Willcodishes (Willcowells, Amsterdam, The Netherlands) expressing GFP-UL30 fusion proteins were imaged using a Nikon Eclipse E600 inverted microscope equipped with a Nikon DXN1200 digital camera and a Nikon Plan Fluor 40× objective (Nikon, Tokio, Japan). Subcellular localization of GFP-β-Gal fusion proteins in living COS-7 and Vero cells was visualized 16−24 h after transfection using a BioRAD MRC500 CLSM (BioRad Laboratories, Hercules, CA), and a Nikon 40 × water immersion lens in combination with a heated stage. The ImageJ 1.62 public domain software (NIH) was used as previously (*38*) to perform single cell measurements for each of the nuclear (Fn) and cytoplasmic (Fc) fluorescence, subsequent to the subtraction of fluorescence due to autofluorescence/background, to determine the nuclear to cytoplasmic fluorescence ratio (Fn/c).

Expression and Purification of His_6 and Glutathione S-Transferase-Tagged Fusion Proteins. His_6GFP-UL54-(1125-1242) and His_6GFP-UL30(1114-1136) were purified from *E. coli* strain BL21 carrying plasmid pREP4, mediating expression of high levels of the LacI repressor, and the pGFPfrB-UL54(1125-1242) or pGFPrfB-UL30(1114-1136) bacterial expression constucts, respectively, by inducing expression for 6 h with isopropyl β-D-thiogalactoside (1 mM) at 28 °C, using nickel affinity chromatography as previously (*33*). Expression and purification of glutathione *S*-transferase (GST)-tagged mouse IMPs α1 and β1 were as described (*39*).

Gel Mobility Shift Assay. To test the ability of IMPs to bind pUL30, native polyacrylamide gel electrophoresis (PAGE)/fluorimaging was performed as previously (*39*). Briefly, GFP fusion proteins (2 μM) were incubated for 15 min in PBS at room temperature with increasing amounts of mouse IMPα-GST or precomplexed IMPα/IMPβ-GST (*39*). Sucrose was added to reactions to a final concentration of 15% (w/v), and the mixture was electrophoresed at 30 V for 8 h on a native PAGE 4−20% gradient gel (Gradipore, Frenchs Forest, New South Wales, Australia) run in TBE buffer. The gels were visualized using a Wallac Arthur 1422 multiwavelength fluorimager (Perkin-Elmer, Boston, MA) using side illumination and exposure times of 0.1−5 s.

Sequence Alignments. The sequence of human HSV-1 pUL30-NLSbip was aligned to the sequence of other α-herpesviruses using the software BLASTp (NCBI), in order to identify novel bipartite NLSs homologues to that of human HSV-1 pUL30.

RESULTS

pUL30 Localizes to the Nucleus in the Absence of Other HSV-1 Proteins. In addition to a weak hydrophobic NLS identified in its C-terminal PAP binding site (*22*), pUL30 possesses three additional putative basic NLSs: NLS1 (P**RRS**RLW[130]), NLS2 (PA**KR**P**R**E[1120]), and NLS3 (**K**P**RK**[1136]). The core of pUL30-NLS2 is highly homologous to that of NLS of HCMV pUL54, which is analogously located relative to its respective PAP binding site [(*22*, *23*);

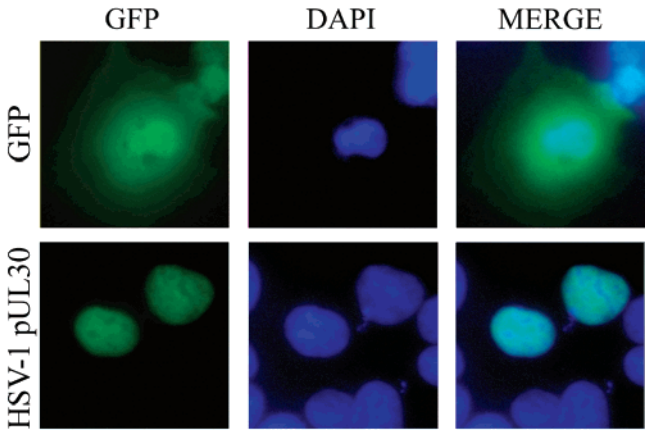


FIGURE 2: pUL30 nuclear localization does not require additional viral proteins. Fluorescent images of COS-7 cells, fixed as described under Materials and Methods 16−24 h after transfection to express GFP-UL30, or GFP alone as a control. The green (GFP) channel is shown in the left panels, and a merged image with the cell nuclei (DAPI, middle panels) is shown in the right panels.

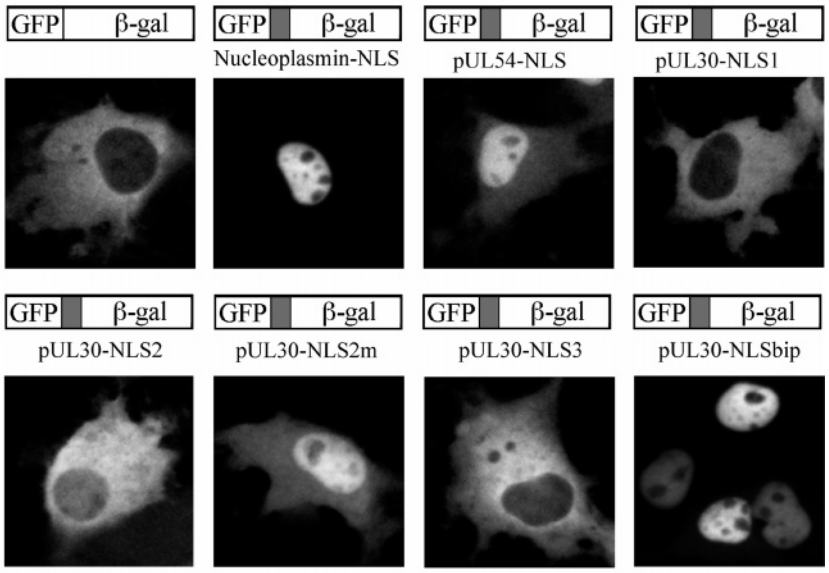
Figure 1]. Moreover, pUL30-NLS2 is located 13 residues upstream of pUL30-NLS3, suggesting that these two sequences could form a bipartite NLS resembling that of nucleoplasmin (*40*). Since pUL30-NLShyd is not required for nuclear accumulation of pUL30 in a cellular context when expressed in the absence of other viral proteins (*29*), we decided to test the functionality of these basic NLSs in living cells using green fluorescent protein (GFP) as a fluorescent tag. We transfected COS-7 cells to express a GFP-UL30-(2−1235) fusion protein, and analyzed its subcellular localization by fluorescence microscopy. As expected GFP alone showed diffuse localization throughout the nucleus and cytoplasm, whereas GFP-UL30(2-1235) accumulated in the nucleus of COS-7 cells when transiently expressed in the absence of other viral proteins (Figure 2), as previously reported for untagged pUL30 (*41*, *42*), clearly showing that tagging pUL30 with GFP does not interfere with its nuclear localization properties.

The C-Terminus of pUL30 Contains a Functional Bipartite NLS. To test the functionality of pUL30's putative basic NLSs, we generated several constructs encoding GFP-UL30-NLSs-β-Gal fusion proteins within expression vector pHM830, where the exogenous sequences are inserted in-frame between the coding sequences of GFP and β-Gal (*37*). We transiently expressed the fusion proteins in COS-7 (Figure 3A) cells and quantitatively analyzed their subcellular localization by live-cell CLSM (Figure 3B), to calculate the nuclear to cytoplasmic ratio (Fn/c). We also expressed GFP-β-Gal and GFP-UL54-NLS-β-Gal as well as GFP-nucleoplasmin-NLS as negative and positive controls for nuclear accumulation, respectively. Both GFP-UL30-NLS1-β-Gal and GFP-UL30-NLS3-β-Gal localized exclusively in the cytoplasm of COS-7 cells, in similar fashion to GFP-β-Gal (Fn/c ca. 0.2), whereas GFP-UL30-NLS2-β-Gal was able to enter the nucleus (Fn/c ca. 0.9). However, the nuclear accumulation of GFP-UL30-NLS2-β-Gal was much weaker compared to that of the positive controls GFP-nucleoplasmin-NLS-β-Gal (Fn/c > 40) and GFP-UL54-NLS-β-Gal (Fn/c > 5), encouraging us to speculate that its activity could be enhanced by the neighboring pUL30-NLS3, located only 13 residues downstream. Expression of GFP-UL30-NLSbip-β-Gal, which contains both pUL30-NLS2 and NLS3 between GFP and β-Gal, resulted in strong nuclear localization (Fn/c of ca. 50). Similar results were obtained in Vero cells (Figure 3C). Our results suggest that pUL30-NLS2 and pUL30-NLS3 form a functional bipartite NLS which, intriguingly, is extremely well conserved in human HSV-2 and in cercopithecine HSV-1, 2, and 16 (See Figure 4). Although UL30-NLS2 matches the consensus for IMPα/β binding [K-(K/R)-X-(K/R)] (*43*), it only exhibited partial NLS activity. We speculated that pUL30-NLS2 reduced activity could partially be attributable to the P residue in position 3 of the core, which is likely to confer extreme rigidity to the domain (*44*, *45*). To verify this, we analyzed the subcellular localization of a GFP-β-Gal fusion protein containing a derivative point mutant of UL30-NLS2, where the P residue was substituted by an A residue (UL30-NLS2m: PA**KR**a**R**E^{1120}). Intriguingly, GFP-UL30-NLS2m-β-Gal localized to the nucleus more efficiently than GFP-UL30-NLS-β-Gal (Fn/c increase of 3- and 2-fold in COS-7 and Vero cells, respectively), suggesting that indeed the P residue interferes with the activity of that NLS. Results were thus consistent with the idea that pUL30 residues 1114−1136 (pUL30-NLSbip) represent a functional bipartite NLS.

The pUL30-NLSbip Is Recognized with High Affinity by the IMPα/β Heterodimer. To identify the cellular receptor responsible for recognition of pUL30-NLSbip and thus characterize the nuclear import pathway of pUL30, gel mobility shift assays were performed with His_6GFP-UL30-(1114-1136) fusion protein and bacterially expressed IMPs, using the His_6GFP-UL54(1125-1242) fusion protein (*23*) and His_6GFP alone (*39*) as positive and negative controls, respectively. Incubation of the GFP fusion proteins with increasing amounts of the IMPα/β heterodimer or IMPα alone (Figure 5) confirmed that GFP alone does not specifically interact with IMPs (not shown, see ref *39*) and revealed that pUL30 binds IMPα/β with higher affinity than IMPα (K_ds of 600 and 1000 nM, respectively), consistent with autoinhibition of NLS binding by IMPα in the absence of IMPβ1 (*26*), and in a fashion reminiscent of pUL54-IMP binding. Results were thus consistent with the idea that pUL30 is translocated to the nucleus by the IMPα/β heterodimer.

The pUL30-NLSbip Is Required for UL30 Nuclear Targeting. Our results suggested that pUL30-NLSbip is a functional bipartite NLS able to mediate nuclear targeting via IMPα/β binding. To test its role in the nuclear import of pUL30 we analyzed the subcellular localization of several pUL30 mutant derivatives (see Figure 6A and Materials and Methods) as compared to the wild-type, when transiently expressed in mammalian cells. COS-7 cells were transfected to express the GFP-UL30 fusion protein, as well as GFP-UL30(2-1115), that lacks both sequences forming the pUL30-NLSbip, GFP-UL30(2-1134), lacking the C-terminal NLS3, and the point mutant derivatives GFP-UL30(2-1235; NLS2m) and GFP-UL30(2-1235; NLS3m), carrying mutations within NLS2 and NLS3, respectively (see Materials and Methods). GFP-UL44-(2-433), GFP-β-gal, and GFP alone were also expressed as controls for nuclear, cytoplasmic, and ubiquitous subcellular localization, respectively. As expected, both GFP-UL44(2-433) and GFP-UL30(2-1235) localized exclusively in the nucleus of COS-7 cells. Deletion of UL30 C-terminal domain, containing NLSbip, resulted in retention of GFP-UL30(2-1115) in the cytoplasm, in similar fashion to the

A



B

COS-7 cells

insert into GFP-β-gal fusions	*NLS / putative NLS*	*Fn/c*
none	-	0.2 ± 0.1 (35)
Nucleoplasmin-NLS	**KR**PAATKKAGQA**KKKK**^{170}	40.6 ± 6.4 (29)
pUL54-NLS	PA**KKRAR**^{1165}	6.4 ± 0.4 (25)
pUL30-NLS1	P**RRSR**LW^{130}	0.2 ± 0.1 (41)
pUL30-NLS2	PA**KRPR**E^{1120}	0.9 + 0.1 (90)
pUL30-NLS2m (P1118A)	PA**KRaR**E^{1120}	2.9 ± 0.2 (92)
pUL30-NLS3	**KPRK**^{1136}	0.3 ± 0.1 (32)
pUL30-NLSbip	PA**KRPR**ETPSPADPPGGAS**KPRK**^{1136}	46.6 ± 8.9 (29)

C

Vero cells

insert into GFP-β-gal fusions		*Fn/c*
none	-	0.2 + 0.1 (34)
Nucleoplasmin-NLS	**KR**PAATKKAGQA**KKKK**^{170}	112.2 ± 21.1 (15)
pUL54-NLS	PA**KKRAR**^{1165}	30.2 ± 4.5 (63)
pUL30-NLS1	P**RRSR**LW^{130}	0.2 + 0.1 (54)
pUL30-NLS2	PA**KRPR**E^{1120}	1.4 ± 0.2 (64)
pUL30-NLS2m (P1118A)	PA**KRaR**E^{1120}	2.9 ± 0.2 (42)
pUL30-NLS3	**KPRK**^{1136}	0.3 ± 0.3 (50)
pUL30-NLSbip	PA**KRPR**ETPSPADPPGGAS**KPRK**^{1136}	74.3 ± 8.9 (43)

FIGURE 3: pUL30 residues 1114−1136 represent a functional bipartite NLS. (A) Images of COS-7 cells imaged live by CLSM 16−24 h after transfection to express the indicated GFP-*β*-Gal fusion proteins; the NLS/putative NLS inserts in each case are listed in panel B. (B) Results for the determination of the nuclear to cytoplasmic ratio (Fn/c) for the respective fusion proteins from images such as those shown in panel A. The number of cells analyzed is indicated in parentheses. (C) Results for the determination of the Fn/c for the respective fusion proteins when transiently expressed in HSV-1 infection permissive Vero cells. The number of cells analyzed is indicated in parentheses.

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HUMAN HSV-1:                PAKRPRETPSPADPPGGASKPRK1136
HUMAN HSV-2:                PAKRPRETPSHADPPGGASKPRK1141
CERCOPIHTECINE  HSV-1:      PAPKRPHGRQAGESEAKKRK1148
CERCOPIHTECINE  HSV-2:      PAPPKRPRGQQPGDPEPKRRR1126
CERCOPIHTECINE  HSV-16:     PAPPKRPRGRQPGDPEPKRRR1143
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FIGURE 4: Human HSV-1 pUL30-NLSbip is conserved among other α-herpesvirus homologues. Putative bipartite NLSs are indicated using the single letter amino acid code. Basic residues are in bold type. The two stretches of basic amino acids constituting the NLSbip are underlined.

GFP-*β*-gal fusion (Figure 6B). Mutation of pUL30-NLS2 had a strong effect in reducing nuclear accumulation: GFP-UL30(2-1235; NLS2m) localized with a largely diffuse pattern inside the cell, resembling that of GFP alone. Both GFP-UL30(2-1134) and GFP-UL30(2-1235; NLS3m), lacking a functional NLS3, localized mainly to the nucleus of transfected cells, but with a significant amount of protein in the cytoplasm (Figure 6B). Thus, mutation of either NLS2 or NLS3 is sufficient to impair pUL30 nuclear targeting, with removal of NLS2 having the most dramatic effect (Figure 6B,C). Removal of both NLS2 and NLS3 resulted in cytoplasmic localization, demonstrating that the pUL30-NLSbip is required for nuclear targeting of pUL30 in the absence of other viral proteins.

DISCUSSION

Nuclear import of the HSV-1 DNA polymerase catalytic subunit pUL30 is a critical step in the viral life cycle, this study being the first to examine pUL30 nuclear localization in a live cell system and to identify sequences necessary for the process. Herpesvirus DNA polymerases comprise a catalytic subunit and a PAP, with their nuclear import still poorly understood. We have recently demonstrated that the HCMV DNA polymerase pUL54 is translocated to the nucleus by the IMP*α*/*β* heterodimer, which recognizes a basic NLS located at the C-terminus of the protein (*23*).

Here we show that pUL30 nuclear import is mediated by a bipartite NLS, which is conserved among other α-herpesviruses (see Figure 4), and similarly able to mediate IMP*α*/*β*-dependent nuclear import. Analysis of UL30 fusion protein derivatives revealed residues 1114−1136 to be required for

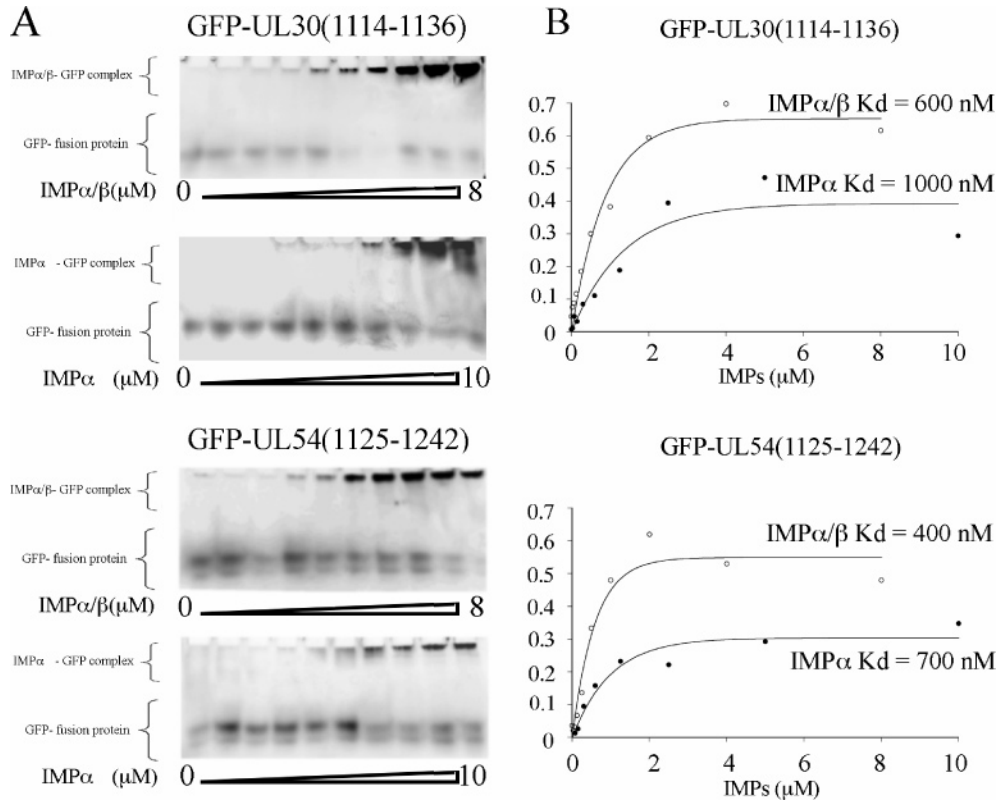
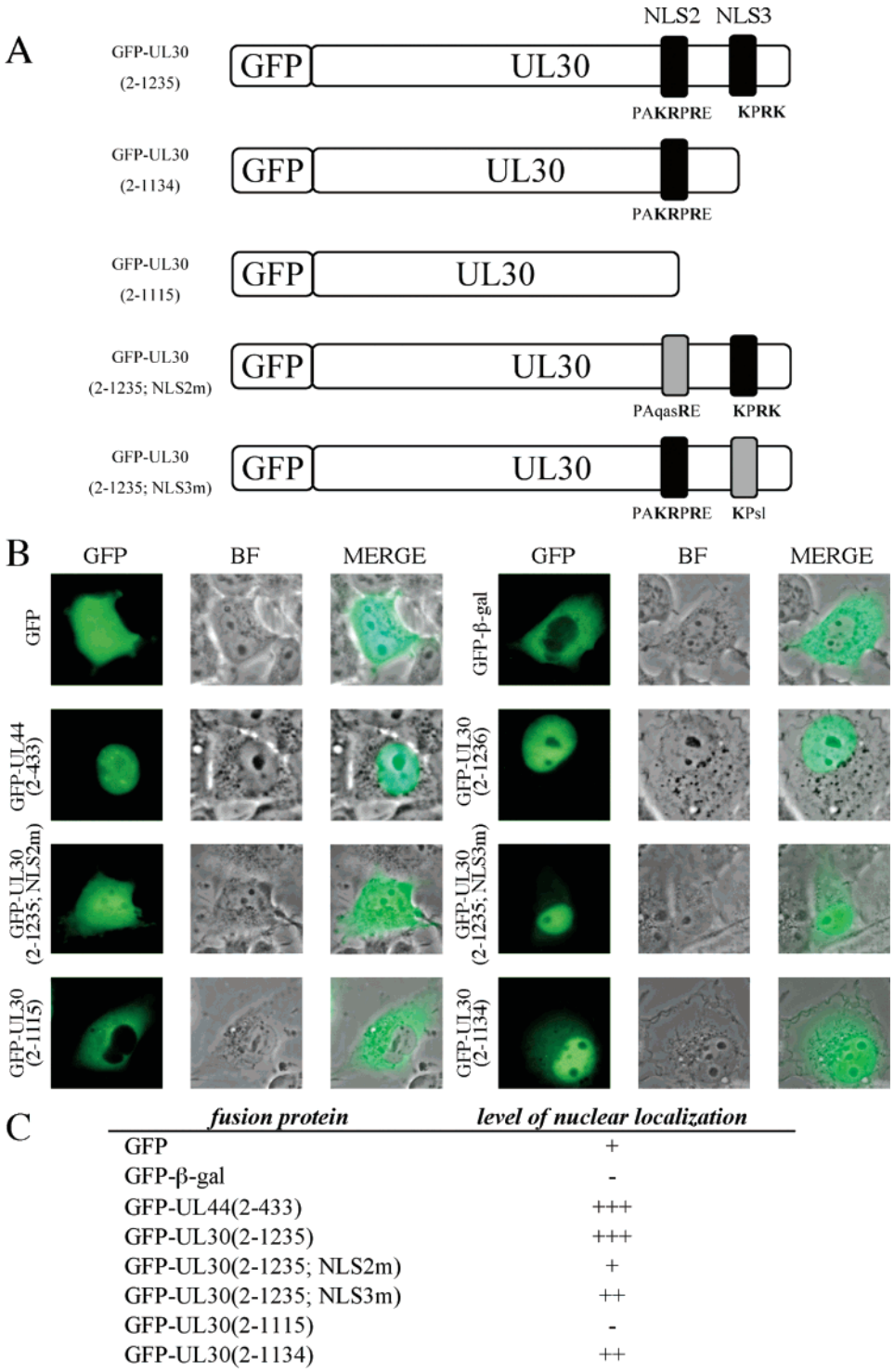


FIGURE 5: pUL30 residues 1114−1136 confer IMPα/*β* heterodimer binding abilities to GFP. (A) Fluorescent images of native gels after electrophoresis for 8 h at 80 mA. The position of the control molecule His_6GFP-UL54(1125-1242) or of His_6GFP-UL30(1114-1136) is shown in the absence (left lane) or presence of increasing amounts of GST-mouse IMPs prior to native PAGE. (B) Results of quantitation of specific fluorescence from panel A for the relative amount of His_6GFP-UL54(1125-1242) and of His_6GFP-UL30(1114-1136) shifted fluorescence due to IMPs binding to the total fluorescence, with indicated the values for the apparent K_ds.

nuclear targeting of UL30 (see Figures 1 and 6), and to be sufficient to confer nuclear accumulation to GFP-*β*-Gal (Figure 3) and IMPs binding abilities to GFP (Figure 5). The ability of pUL30-NLSbip to confer nuclear targeting to the 580 kDa tetrameric fusion protein GFP-*β*-Gal implies that it is sufficient to mediate active nuclear import (Figure 3), because molecules larger than 60−100 kDa cannot diffuse passively through the NPC (*24*). Our quantitative gel mobility shift assay results show that bacterially expressed His_6GFP-UL30(1114-1136) is recognized by IMPα/*β* with higher affinity than by IMPα alone, suggesting that pUL30 is likely to be translocated to the nucleus via IMPα/*β*, although it is not formally possible to exclude the possibility that other members of the IMP superfamily may also contribute to its nuclear transport.

The amino terminal portion (PA**KR**P**R**E^{1120}) of pUL30-NLSbip shares a strong positional and sequence homology with the pUL54-NLS (PA**KKR**A**R**^{1159}), matches the consensus motif for IMPα/*β* binding [K-(K/R)-X-(K/R)] (*43*), and is partially functional (see Figures 1−3 and 6). The P1118A substitution restores some degree of NLS function (Fn/c increase of ca. 2- and 3-fold when fused to GFP-*β*-Gal, in Vero and COS-7 cells, respectively), suggesting that the presence of a P residue in position 3 of the NLS core might negatively affect the efficiency of IMP binding. This result is of importance in better defining the optimal consensus for IMPα binding by monopartite NLSs to [K-(K/R)-X′-(K/R)] with X′ any amino acid except for P. This idea is consistent with previously published data (*42*), showing that the sequence **KR**P**R** is not sufficient to promote nuclear import of reporter proteins. The detrimental effect of the P residue can be overcome by additional basic amino acids surrounding the sequence, which is the case in the polycomb protein M33 (*42*), or by an additional basic sequence placed 10/12 amino acids downstream, as we report here for HSV-1 pUL30. Our findings have implications for the evolution of such nuclear targeting sequences within viral DNA polymerases and PAPs in general (see also Figures 4 and 7), as well as facilitating the identification of bipartite NLSs similar to that identified here for pUL30. For example, a functional NLS has been identified on the C-terminus of PAPs from several herpesviruses but not on pUL42 (*23*, *30*−*34*); intriguingly, a putative bipartite NLS, reminiscent of the pUL30-NLSbip, is also present within the C-terminus of pUL42 from both HSV-1 and HSV-2 (**KR**G**R**SGGEPARAPTAL**KK**P**K**^{413} and **KRR**HPGAEVVPAPPAT**KR**P**K**^{404}, respectively). Direct experimentation will of course be required to verify the importance of the aforementioned NLSs on pUL42 nuclear import.

Different herpesviruses appear to have evolved different strategies to ensure the proper subcellular localization of their DNA replicating enzymes. In the case of Kaposi's associated human herpesvirus (KHSV), the catalytic subunit Pol-8 is incapable of nuclear targeting in the absence of its PAP PF-8, and the DNA polymerase holoenzyme is therefore believed to translocate to the nucleus as a complex (*34*). On the other hand, HCMV DNA polymerase catalytic subunit and PAP are both able to translocate to the nucleus independently through IMPα/*β* dependent processes, as well as being able to be imported as a complex (*23*, *33*). In fact, the basic NLS



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fusion protein	*level of nuclear localization*
GFP	+
GFP-β-gal	-
GFP-UL44(2-433)	+++
GFP-UL30(2-1235)	+++
GFP-UL30(2-1235; NLS2m)	+
GFP-UL30(2-1235; NLS3m)	++
GFP-UL30(2-1115)	-
GFP-UL30(2-1134)	++

FIGURE 6: pUL30 residues 1114−1136 are essential for optimal UL30 nuclear targeting. (A) Schematic representation of the pUL30 mutant derivatives expressed to investigate the role of NLS2 and NLS3 in pUL30 nuclear import. Wild-type NLSs are indicated as black boxes, mutant NLSs as gray boxes. The NLS sequences are also indicated using the single letter amino acid code, with basic residues in bold type. (B) Fluorescent images of live COS-7 cells, 16−24 h after transfection to express the indicated GFP fusion proteins. The green (GFP) channel is shown in the left panels, and a merged image with the bright field (BF, middle panels) is shown in the right panels. (C) Classification of the degree of nuclear localization of the indicated GFP fusion proteins on the basis of their subcellular localization as shown in panel B: +++ (only nuclear); ++ (mainly nuclear with cytoplasmic staining); + (diffuse in cytoplasm and nucleus); − (cytoplasmic).

of pUL54 is located upstream of the ppUL44 binding site, enabling pUL54 to bind simultaneously to both ppUL44 and the IMPα/β heterodimer (*23*). Our data show clearly that HSV-1 pUL30 is also able to localize to the nucleus independently of its PAP, consistent with the findings of others (*41*, *46*). A recent study showed that pUL42 is also able to localize to the nucleus independently of pUL30 (*47*). Notably, infection of Vero cells with a mutant virus lacking the C-terminal third of pUL42, encompassing its putative NLS, resulted in efficient viral replication and nuclear localization of pUL42 (*47*). In this context, our finding that pUL30-NLSbip, similarly to HCMV pUL54-NLS, is located

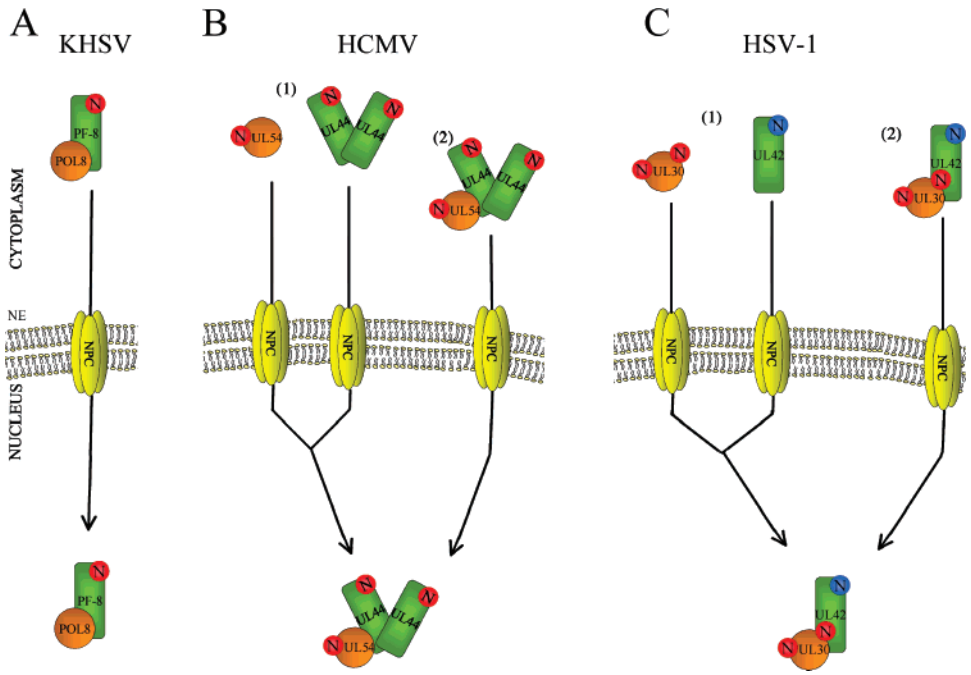

FIGURE 7: Schematic representation of nuclear import pathways for herpesviruses DNA polymerase holoenzyme subunits. (A) KHSV DNA polymerase holoenzyme nuclear import relies on the NLS present on the processivity factor PF-8, since the catalytic subunit pol-8 lacks a functional NLS, and hence the two subunits are targeted into the nucleus as a complex. (B) HCMV DNA polymerase catalytic subunit (pUL54) and processivity factor (ppUL44) can independently localize to the nucleus because they both possess functional NLSs (*1*); additionally, pUL54 and ppUL44 can associate in the cytoplasm to form the holoenzyme, in which both pUL54 and ppUL44 NLSs are functional and able to confer IMPα/β dependent nuclear targeting (*2*). (C) In a similar fashion to HCMV, as suggested by this study, the HSV-1 DNA polymerase subunits can independently localize to the nucleus (*1*); the presence of a functional NLS on pUL30 outside the pUL42 binding domain also suggests that they could be imported as a holoenzyme (*2*). Red circles represent functional NLSs, and blue circles represent the pUL42 bipartite putative NLS.

upstream to the UL42 binding site further strengthens the hypothesis that the HSV-1 DNA polymerase holoenzyme could be translocated to the nucleus as a complex, in similar fashion to the HCMV and KHSV homologues (*23*, *34*). As mentioned, a previous study identified a functional, although dispensable, NLS within the pUL30 binding site for pUL42 [(*22*); see Figure 1], so that it seems likely that pUL30, as HCMV UL54, has evolved to contain multiple NLSs to ensure optimal nuclear targeting both as a single protein and when in a complex with its PAP, during viral infection. Figure 7 represents a model of this, highlighting the idea that the HCMV and HSV viruses are very similar in this regard, in contrast to KHSV, where the polymerase catalytic subunit only appears to be translocated to the nucleus as a holoenzyme complex. The similarities of the findings for the enveloped DNA viruses HCMV and HSV underline the importance of nuclear localization of the respective polymerase subunits and thereby the potential to devise strategies to hinder this. The bipartite NLS we characterize here is clearly a starting point to develop therapeutics to attempt to inhibit HSV-1 replication, by preventing nuclear localization of pUL30 complexed to pUL42, as well as of pUL30 itself. These intriguing possibilities are the focus of future work in this laboratory.

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