RNA-binding of the human cytomegalovirus transactivator protein UL69, mediated by arginine-rich motifs, is not required for nuclear export of unspliced RNA

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ABSTRACT

The human cytomegalovirus protein pUL69 belongs to a family of regulatory factors that is conserved within the Herpesviridae and includes the proteins ICP27 of herpes simplex virus type 1 and EB2 of Epstein–Barr virus. ICP27 and EB2 have been shown to facilitate the nuclear export of viral mRNAs via interacting with the cellular mRNA export factor REF. Furthermore, direct RNA-binding of these proteins was found to be essential for their stimulating effects on mRNA export. Recently, we demonstrated that pUL69 shares common features with ICP27 and EB2 such as (i) nucleocytoplasmic shuttling and (ii) stimulation of nuclear RNA export via binding to the cellular mRNA export machinery. Here, we demonstrate that pUL69 can also interact with RNA both *in vivo* and *in vitro* via a complex N-terminal RNA-binding domain consisting of three arginine-rich motifs. Interestingly, the RNA-binding domain of pUL69 overlaps with both the NLS and the binding site of the cellular mRNA export factors UAP56 and URH49. While the deletion of the UAP56/URH49-binding site abolished pUL69-mediated RNA export, an RNA-binding deficient pUL69 mutant which still interacts with UAP56/URH49 retained its RNA export activity. This surprising finding suggests that, in contrast to its homologues, RNA-binding is not a prerequisite for pUL69-mediated nuclear RNA export.

INTRODUCTION

One of the characteristic features of eukaryotes is the spatial and temporal separation of gene transcription and mRNA translation by the nuclear membrane. These essential cellular processes are connected by the mRNA export pathway. Currently, the majority of metazoan mRNAs appears to be exported to the cytoplasm by the heterodimeric TAP-p15 transport receptor (1–4). Although TAP is able to interact directly with cellular RNA *in vitro* in a sequence-non-specific fashion, several lines of evidence suggest that additional factors are needed to bridge the interaction between TAP and mRNA *in vivo* (5). Proteins that function as adaptors between mRNAs and TAP-p15 have been identified. Among these are several RNA-binding proteins and in particular members of the REF protein family. REF proteins shuttle between the nucleus and the cytoplasm and bind directly to both mRNA and TAP (6). Another important mRNA export factor is the putative DExD/H-box RNA helicase UAP56 which has been implicated in the recruitment of REF onto mRNAs (7,8). Recently, a protein with >90% amino acid identity to UAP56, termed URH49, has been identified in mammalian cells and is supposed to have similar functions to UAP56 (9). Both REF and UAP56 additionally interact with components of the splicing machinery thus coupling splicing to mRNA export. This has been proposed as a mechanism to enhance the nuclear export of RNAs derived from spliced genes (10,11).

While most metazoan mRNAs undergo splicing, viruses often contain intronless genes (e.g. herpesviruses) or are dependent on the nuclear export and translation of unspliced messages (e.g. retroviruses). As a consequence, several viruses that replicate in the nucleus have evolved transactivator proteins to promote the nuclear export of otherwise inefficiently exported viral mRNAs via utilizing distinct nuclear export pathways (12,13). For instance, complex retroviruses like HIV-1 encode sequence-specific RNA-binding proteins such as HIV-1 Rev to recruit the CRM-1 nuclear export receptor onto incompletely spliced and unspliced viral RNAs to facilitate their nuclear export (14). In contrast, herpesviruses express a group of homologous regulatory proteins which

Table 1. Sequences of oligonucleotides used for PCR, cloning reactions and sequencing

Oligonucleotide	Sequence (5′ to 3′)
UL69156R	TATGCTCGAGAGTCGTAAGACTTACCGTAG
BamH50F	GTCAGGATCCTCTCGACACGGAACGGAGCAG
BamH100F	GTCAGGATCCCTCCCCTTCCACCCTCC
BamH156F	GTCAGGATCCTCAACGATGACGGCGAACCG
Xho370R	GTCACTCGAGGCCCACCGTTCCAGATAG
UL69aa1-16F	GATCCCCATGGAGCTGCACTCACGCGGCCGTCACGACGCTCCGTCGTTATCTTCCG
UL69aa1-16R	AATTCGGAAGATAACGACGGAGCGTCGTGACGGCCGCGTGAGTGCAGCTCCATGGG
EcoR31F	GTCAGAATTCTGTCTGGATTACGAACC
EcoR35R	GTCAGAATTCTTCGTAATCCAGACAGAAG
EcoR50F	GTCAGAATTCTCGACACGGAACGGAGCAG
EcoR122R	GTCAGAATTCGGTGCCCATGATGATGTG
UL69aa140-156F	AATTCGCCGCGTCCGCCTCGGTGCCCCTTAACCCCCACTACGGTAAGTCTTACGACC
UL69aa140-156R	TCGAGGTCGTAAGACTTACCGTAGTGGGGGTTAAGGGGCACCGAGGCGGACGCGGCG
UL69Sal35	ATACGTCGACACGGGTTCGTAATCCAG
Eco140F	ATATGAATTCGCCGCGTCCGCCTCGGTG
3UL69 Xho I	GATCCTCGAGTTAGTCATCCATATCATCGCT
5Ul69Bam	GATCGGATCCATGGAGCTGCACTCACGCGGC
CAT5	CGTTGATATATCCCAATGGC
CAT3	GCATGATGAACCTGAATCGC
698GAP5	GTACGTCGTGGAGTCCACTG
698GAP3	TCCACCACCCTGTTGCTGTA
pGEX5-seq	GGGCTGGCAAGCCACGTTTGGTG

are thought to facilitate the nuclear export of viral intronless RNAs by exploiting components of the cellular mRNA export pathway (13).

The best-characterized herpesviral mRNA export factor is the protein ICP27, which is encoded by the alpha-herpesvirus herpes simplex virus type 1 (HSV-1). It has been demonstrated that ICP27 recruits the adaptor protein REF and hence the mRNA export receptor TAP onto a set of intronless viral mRNAs thus recruiting these transcripts to the cellular mRNA export pathway (15–18). Additionally, a direct interaction between ICP27 and TAP has been detected recently (16). Subsequently, it was described that several homologues of ICP27 encoded by members of the gamma-herpesvirus subgroup could also bind to REF suggesting a conserved mechanism of herpesviral mRNA export. An interaction with REF was detected for the Epstein–Barr virus (EBV) protein EB2 (19), the Kaposi's sarcoma-associated herpesvirus (KSHV) protein ORF57 (20) and the Herpesvirus saimiri (HVS) protein ORF57 (21). In addition to protein contacts with REF, a direct RNA-binding activity of some of these herpesviral mRNA export factors was found to be essential for their stimulatory effects on mRNA export, suggesting that they serve as adaptor proteins bridging the interaction between viral mRNA, REF and hence the TAP-p15 exporter (18,22).

Human cytomegalovirus, the prototype of the beta-herpesvirus subfamily, also encodes a transactivator protein with homology to ICP27, termed pUL69 (23). The UL69 protein is a tegument phosphoprotein (24) which was first described as a pleiotropic transactivator of both viral and cellular gene expression (23). Recently, we reported that pUL69 shares several activities with its herpesviral homologues such as (i) nucleocytoplasmic shuttling activity (25) (ii) the ability to promote the nuclear export of unspliced mRNA and (iii) binding to cellular proteins involved in mRNA export (26). However, in contrast to ICP27, EB2 and ORF57, pUL69 was not able to bind to REF but stimulated the export of unspliced messages through an interaction with the DExD/H-box RNA helicase UAP56 and/or its close relative URH49 (26). Apart
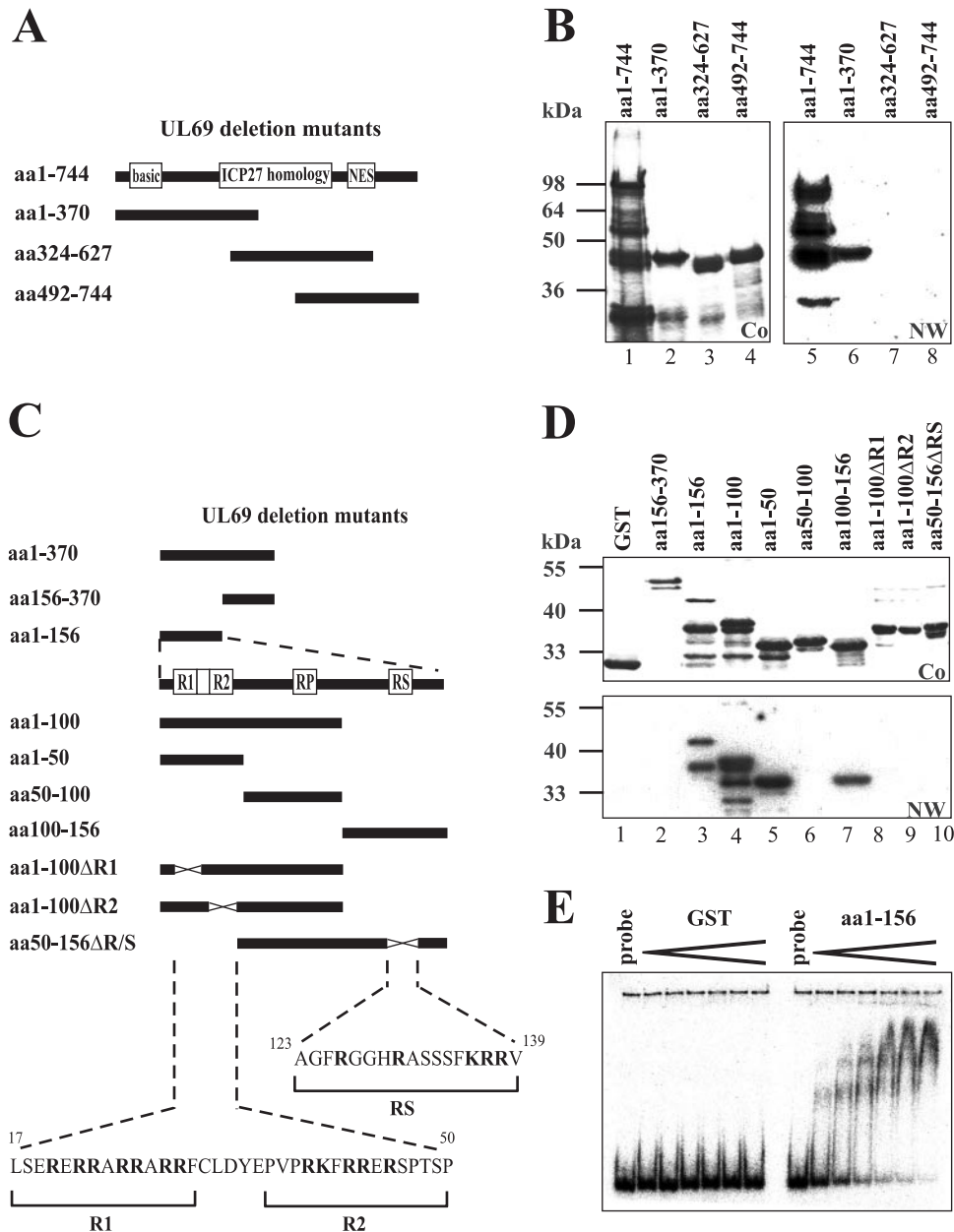
from this, pUL69 has also been shown to interact with hSPT6, a cellular factor which is implicated in transcription elongation and chromatin remodelling, thus suggesting a function of pUL69 upstream of mRNA export (27).

Based on the functional similarity between the herpesviral mRNA export factors we asked whether pUL69 directly binds to RNA as has been demonstrated previously for its homologues in alpha- and gamma-herpesviruses. Here, we report that pUL69 directly interacts with RNA both *in vitro* and *in vivo*. Mapping of the domain required for RNA-binding identified a complex arginine-rich RNA-binding domain located at the N-terminus of the protein. Surprisingly, an RNA-binding deficient pUL69 mutant was still able to stimulate the cytoplasmic accumulation of an unspliced CAT reporter RNA suggesting that, in contrast to its homologues, RNA-binding is not a prerequisite for pUL69-mediated nuclear mRNA export.

MATERIALS AND METHODS

Oligonucleotides and plasmids

Oligonucleotides were obtained from Biomers.net GmbH (Ulm, Germany) or Sigma-ARK (Darmstadt, Germany). The sequences of oligonucleotides (5′–3′ sequences) used in this study are listed in Table 1. The prokaryotic expression plasmids for His-tagged (pHM164) and GST-tagged pUL69 (pHM185) have been described previously (23). For prokaryotic expression of His-tagged pUL69 deletion mutants vectors of the pQE series were used (Qiagen, Hilden, Germany) (Figure 1A). Mutant aa1–370 was made by a PCR using oligonucleotides pGEX5-seq and Hind370R as well as pHM185 as a template. The fragment was inserted into the BamHI site of vector pQE32 and the construct was designated pHM2282. Mutant aa324–627 (pHM2283) was created by inserting the BglII fragment of pHM164 into the BamHI-digested vector pQE32. Mutant aa492–744 (pHM2284) was generated by subcloning the PstI–HindIII fragment of pHM164 into pQE31. GST-tagged aa1–50 (pHM2219) was

A

UL69 deletion mutants

aa1-744   basic | ICP27 homology | NES

aa1-370

aa324-627

aa492-744

B

C

UL69 deletion mutants

aa1-370

aa156-370

aa1-156

R1 | R2 | RP | RS

aa1-100

aa1-50

aa50-100

aa100-156

aa1-100ΔR1

aa1-100ΔR2

aa50-156ΔR/S

123   139
AGF**R**GGH**R**ASSSF**KRR**V

RS

17   50
LSE**RERR**A**RR**A**RR**FCLDYEPVP**RK**F**RRER**SPTSP

R1   R2

D

E

Figure 1. N-terminal regions of pUL69 mediate direct binding to RNA *in vitro*. (**A**) Schematic representation of pUL69 (aa1–744) and its truncated versions that were expressed as His-tag fusion proteins and then used in northwestern assays. The localization of the arginine-rich region (basic), the nuclear export signal (NES) and the ICP27 homology region of pUL69 are indicated. (**B**) His-tagged full-length (aa1–744) and truncated derivatives of pUL69 were prokaryotically expressed and purified by metal chelate affinity chromatography. An aliquot of each preparation was analysed by SDS–PAGE and Coomassie blue staining (lanes 1–4, Co). For the northwestern assay (NW) the same amount of proteins was separated by SDS–PAGE, blotted onto a nitrocellulose membrane, renatured and incubated with a ^{32}P-labeled RNA probe which was generated by *in vitro* transcription from plasmid pHM127 coding for the IE1/2 region of HCMV. The molecular masses of the molecular weight marker proteins are indicated at the left in kilodaltons (kDa). (**C**) Schematic representation of the overlapping GST-tagged polypeptides derived from amino acids 1 to 370 of pUL69. The arginine-rich sequences within aa1–156 are indicated as R1, R2, RP and RS. Deletions of R1 and R2 in aa1–100 remove amino acids 17–30 (aa1–100ΔR1) and amino acids 36–50 (aa1–100ΔR2), respectively. Deletion of RS in aa50–156 removes amino acids 123–139 (aa50–156ΔRS). Amino acid sequences of the arginine-rich regions removed from the respective pUL69 fragments are shown. (**D**) GST and the GST-tagged polypeptides described in (C) were prokaryotically expressed and purified by GST affinity chromatography. Equal amounts of each protein were subjected to Coomassie blue staining (Co) as well as northwestern assay (NW) in the same manner as described in (B). (**E**) Electrophoretic mobility shift assays were performed by using increasing amounts of affinity-purified GST or the GST-tagged N-terminal region of pUL69 comprising the RNA-binding domain (aa1–156). The proteins were incubated with a ^{32}P-labeled, 150 nt RNA fragment of the UL69 gene and the RNA–protein complexes were separated by electrophoresis on a 6% native polyacrylamide gel and detected by autoradiography.

made by inserting the BamHI–SalI fragment of pHM185 into the prokaryotic expression vector pGEX-5X-1 (Pharmacia Biotech, Freiburg, Germany). Five overlapping UL69 DNA fragments encoding polypeptides derived from amino acids 1

to 370 of pUL69 (Figure 1C) were inserted into BamHI and XhoI sites of pGEX-5X-1. These fragments were generated by PCR using the following oligonucleotides and pHM185 as a template (the designation of the constructs expressing

the respective pUL69 polypeptide as GST fusion protein is indicated in brackets): BamH156F and Xho370R (aa156–370, pHM2274), pGEX5-Seq and Xho100R (aa1–100, pHM2270), pGEX5-Seq and UL69156R (aa1–156, pHM2271), BamH50F and Xho100R (aa50–100, pHM2272), BamH100F and UL69156R (aa100–156, pHM2269). GST-tagged pUL69 mutant aa1–100ΔR1 (pHM2275) expressing amino acids 1–100 of pUL69 carrying a deletion of residues 17–30 was constructed by ligation of three DNA fragments: oligonucleotides UL69 aa1–16F and UL69 aa1–16R were annealed, vector pGEX-5X-1 was digested by BamHI and XhoI and a fragment was made by PCR using oligonucleotides EcoR31F and Xho100R as well as pHM185 as a template followed by digestion with EcoRI and XhoI. The ligation of these three DNA fragments resulted in pHM2275. GST-tagged pUL69 mutant aa1–100ΔR2 (pHM2276) expressing amino acids 1–100 of pUL69 with a deletion of residues 36–50 was constructed as follows: two fragments were made by PCR using oligonucleotides pGEX5-Seq/EcoR35R and EcoR50F/Xho100R as well as pHM185 as a template. Then, these PCR fragments were cleaved either by BamHI and EcoRI or by EcoRI and XhoI, respectively, and ligated with BamHI–XhoI-digested vector pGEX-5X-1 which resulted in pHM2276. GST-tagged pUL69 mutant aa50–156ΔRS (pHM2278) expresses amino acids 50–156 of pUL69 with a deletion of residues 123–139. To construct this plasmid, oligonucleotides UL69 aa140–156F and UL69 aa140–156R were annealed and a DNA fragment was made by PCR using pHM185 as a template as well as oligonucleotides BamH50F and EcoR122R followed by digestion with BamHI and EcoRI. The resulting fragments were then cloned into BamHI and XhoI sites of vector pGEX-5X-1 resulting in pHM2278. Plasmids for prokaryotic expression of the following pUL69 in-frame deletion mutants (pHM2279, 2280, 2281, 2320, 2321) were constructed by using vector pGEX-5X-1 in which the proteins are N-terminally tagged with GST. ΔR1 (pHM2279) expresses pUL69 with an internal deletion of amino acids 17–30. The BamHI–SalI part of pHM185 was replaced by the BamHI–SalI fragment of pHM2275 resulting in pHM2279. ΔR2 (pHM2280) expresses pUL69 with an internal deletion of amino acids 36–50. pHM2280 was constructed by replacing the BamHI–SalI part of pHM185 with a fragment made by PCR using oligonucleotides pGEX5-Seq and UL69Sal35 as well as pHM185 as a template. ΔRS (pHM2281) encodes pUL69 with an internal deletion of amino acids 123–139. For this, two fragments were made by PCR using pHM185 as a template and oligonucleotides pGEX5-Seq and EcoR122R as well as Eco140F and 3UL69XhoI. These PCR fragments were then cleaved by BamHI and EcoRI or EcoRI and XhoI, respectively and ligated with BamHI–XhoI-digested vector pGEX-5X-1 which resulted in pHM2281. ΔR1ΔRS (pHM2320) encodes pUL69 carrying deletions of amino acids 17–30 and amino acids 123–139. pHM2320 was constructed by replacing the BamHI–SalI region of pHM2281 with the BamHI–SalI part of pHM2275. ΔR2ΔRS (pHM2321) contains pUL69 carrying deletions of amino acids 36–50 and amino acids 123–139. pHM2321 was created by replacing the BamHI–SalI region of pHM2281 with the BamHI–SalI part of pHM2276. The N-terminally FLAG-tagged pUL69 mutants for eukaryotic expression were produced by vectors FLAG-pcDNA3 and FLAG-NLS-pcDNA3 (28). Mutants ΔR1 (pHM2322), ΔR2 (pHM2323), ΔRS (pHM2324), ΔR1ΔRS (pHM2325) and ΔR2ΔRS (pHM2326), fused to the FLAG epitope, were constructed by inserting BamHI/XhoI PCR fragments of pHM2279, pHM2280, pHM2281, pHM2320 and pHM2321, respectively, into vector FLAG-pcDNA3. In addition, pUL69 mutants ΔR2 and ΔR2ΔRS were also subcloned into FLAG-NLS-pcDNA3 to express them as FLAG and NLS fusion proteins resulting in plasmids N-ΔR2 (pHM2328) and N-ΔR2ΔRS (pHM2330). The eukaryotic expression plasmids coding for FLAG-tagged pUL69, myc-tagged UAP56, myc-tagged URH49, FLAG-SPT6-HA (provided by Dr H. Handa), CFN-βGal as well as the CAT reporter plasmid pDM128/CMV/RRE were described previously (25,26,29,30). Plasmids pHM127, pHM149, pHM1584 and pHM2119 used in the *in vitro* transcription reaction to generate RNA probes for the RNA–protein binding assays were also described elsewhere (26,28,31).

Protein expression and purification

Prokaryotic expression and purification of pUL69 and its deletion mutants as histidine- and GST-tagged proteins were performed as described previously (25,32). To purify genuine and mutated pUL69 as FLAG fusion proteins from mammalian cells, 3×10^7 HEK 293T (human embryonic kidney) cells were transfected with plasmids expressing the respective protein by using the standard calcium phosphate co-precipitation method. The fusion proteins were purified from crude cell lysates by immunoprecipitation with an anti-FLAG M2 affinity gel according to the manufacturer's instructions (Sigma, Deisenhofen, Germany). Briefly, cells were harvested 48 h after transfection and lysed in 10 ml of lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min at 4°C followed by centrifugation. The cleared lysate was incubated with anti-FLAG M2 agarose on a rotating wheel for 4 h at 4°C. The immunoprecipitates were then washed with TBS500 (50 mM Tris–HCl, pH7.4, 500 mM NaCl and 1 mM PMSF) five times for 5 min and with TBS150 (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1 mM PMSF) three times. The anti-FLAG M2 agarose-bound proteins were eluted with TBS150 containing 100 μg/ml FLAG peptide. The immunoselected proteins were analysed by SDS–PAGE followed by Coomassie blue staining, silver staining and western blot analysis.

In vitro transcription and RNA–protein binding assays

^{32}P-labeled RNA probes for the northwestern blotting assays were generated by *in vitro* transcription. To generate templates for the IE1/2 and CAT RNA probes, plasmids pHM127 and pHM2119 were linearized by cleavage with SalI. The templates for the CREB and UL82 RNA probes were prepared by cleavage of plasmid pHM149 with HindIII and pHM1584 with XbaI. *In vitro* transcription was performed as described previously (33). Northwestern blotting assays were carried out as described by Mears and Rice (34) with some modifications. The purified proteins were subjected to SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane followed by three washing steps with NW buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1× Denhardt's

solution and 2 mM DTT). The transferred proteins were renatured *in situ* for 12 h at 4°C in NW buffer. The membranes were incubated at room temperature (20–25°C) for 2 h in NW buffer containing 100 µg/ml yeast tRNA and then, after the addition of 5×10^6 c.p.m. of ^{32}P-labeled RNA probe, incubation was continued for 6 h. Then, the membranes were washed three times in NW buffer (10 min each) at room temperature and subsequently analysed by autoradiography.

For RNA electrophoretic mobility shift assays the affinity-purified proteins were incubated with 5×10^4 c.p.m. of a 150 nt ^{32}P-labeled RNA probe for 30 min at 4°C in binding buffer (10 mM Tris–HCl, pH 7.4, 100 mM KCl, 2 mM DTT and 1% Triton X-100). The RNA probe was derived from the 5′ end of the UL69 gene. The RNA–protein complexes were then analysed by electrophoresis on a 6% native polyacrylamide gel followed by autoradiography.

Indirect immunofluorescence analysis and interspecies heterokaryon assays

For these experiments HeLa cells were transfected by the standard calcium phosphate co-precipitation method. Indirect immunofluorescence analysis and the interspecies heterokaryon assays were carried out as described earlier (25). To detect UL69 proteins and CFN-β-Gal, a pUL69 polyclonal serum and an anti-β-galactosidase monoclonal antibody (Roche, Mannheim, Germany) were used in combination (25).

CAT RNA export assay and co-immunoprecipitation analysis

For the CAT RNA export assay HEK293T cells were plated in 6-well dishes at 4.5×10^5 cells per dish the day before transfection. DNA transfection was performed by the standard calcium phosphate co-precipitation method using 150 ng of the reporter plasmid pDM128/CMV/RRE and 500 ng of the co-transfected plasmid expressing FLAG-pUL69 or the FLAG-tagged pUL69 mutants. The cells were lysed 48 h after transfection and CAT protein expression was analysed by a CAT enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Molecular Biochemicals). Equal aliquots of each transfection were saved for monitoring the protein expression by western blotting. Each transfection was performed in triplicate and repeated at least three times. Co-immunoprecipitation analyses and detection of the co-precipitated proteins by western blotting was performed exactly as described previously (26).

RNA immunoprecipitation

HEK 293T cells (3×10^6 cells) were transfected with the CAT reporter vector pDM128/CMV/RRE along with plasmids expressing either FLAG-UL69 or its different mutants using the standard calcium phosphate co-precipitation method. RNA immunoprecipitation was performed essentially as described by Niranjanakumari *et al*. (35) except that no covalent cross-linking was performed. Anti-FLAG M2 affinity gel (Sigma, Deisenhofen, Germany) was used to precipitate the respective FLAG-tagged proteins. One-tenth of the RNA purified from the immunoprecipitates was used as a template for RT–PCR using the Titan One Tube RT–PCR system (Roche, Mannheim, Germany). For amplification of the CAT mRNA oligonucleotides CAT5 and CAT3 were used giving rise to a

552 bp CAT fragment. Oligonucleotides 698GAP5 and 698GAP3 were used to amplify a 698 bp fragment of the GAPDH mRNA. Amplification conditions were optimized to ensure that the amplification reaction was within the linear range. To detect any vector DNA contamination of the immunoprecipitated RNA samples, one-tenth of the samples was subjected to PCR without prior reverse transcription. pDM128/CMV/RRE vector DNA (100 ng) served as a positive control in the PCR.

RESULTS

An N-terminal domain within pUL69 mediates direct binding to RNA *in vitro*

Homologues of pUL69 such as the HSV-1 protein ICP27 and the EBV protein EB2 have been shown to bind to RNA and this activity was found to be essential for their stimulatory effects on mRNA export (18,22). To determine whether pUL69 can also interact with RNA, we employed the northwestern assay since this method proved to be reliable for the demonstration of direct RNA–protein interactions. For this, His-tagged fusions of full-length pUL69 and of three overlapping fragments covering the entire protein were expressed in *Escherichia coli* and purified by affinity chromatography on Ni-NTA agarose (Figure 1A and B). Although the His-pUL69 preparations yielded major bands of the expected molecular sizes, some additional smaller bands were visible primarily when full-length pUL69 was purified (Figure 1B, lane 1). Since the full-length protein and most of the smaller derivatives reacted with both an anti-His and an anti-pUL69 antibody in a western blot analysis these bands were most probably a result of proteolysis during expression and/or purification (data not shown). Next, the purified proteins were subjected to SDS–PAGE, transferred to a nitrocellulose membrane, renatured *in situ* and incubated with ^{32}P-labeled RNA which was generated by *in vitro* transcription using a template of the HCMV IE1/2 gene region. As shown in Figure 1B, lanes 5–8, full-length pUL69 (aa1–744), several C-terminally truncated forms of full-length pUL69 as well as the N-terminal pUL69 polypeptide (aa1–370) bound RNA. We concluded from this experiment that the N-terminal part of pUL69 comprising amino acids 1–370 directly binds to RNA and hence this domain contains the putative RNA-binding motif(s).

To further identify these motifs, we constructed a series of deletion mutants spanning amino acids 1–370 of pUL69 (Figure 1C). The mutants were then expressed in *E.coli* as GST fusion proteins, purified by glutathione Sepharose affinity chromatography and subjected to the northwestern assay. We observed that the very N-terminal part of pUL69 comprising amino acids 1–156 showed RNA-binding activity whereas a mutant consisting of amino acids 156–370 did not (Figure 1D, lanes 2 and 3). Furthermore, the result shown in Figure 1D, lanes 4–7, demonstrates that the RNA-binding activity of pUL69 can be assigned to two mutants, namely aa1–50 and aa100–156. Sequence inspection revealed that mutant aa1–50 contains two arginine-rich sequences, termed R1 and R2 (Figure 1C, bottom left), mutant aa100–156 includes a region containing arginines and serines that we designated RS (Figure 1C, bottom right). Since arginines are often key residues within RNA-binding motifs we next wanted to

determine the contribution of these basic clusters to the RNA-binding activity of pUL69. For this, three internal deletion mutants lacking the indicated arginine-rich regions were constructed (Figure 1C). As shown by northwestern assays, a deletion of either R1 or R2 from mutant aa1–100 or a deletion of RS from mutant aa50–156 resulted in a loss of the RNA-binding activity (Figure 1D, lanes 8–10). The results of the northwestern assay were considered specific since neither GST alone nor GST-tagged pUL69 mutants aa156–370 or aa50–100 bound RNA in this assay (Figure 1D, lanes 1, 2 and 6). Thus, we concluded from these experiments that the arginine-rich N-terminus of pUL69 comprising amino acids 1–156 is responsible for its *in vitro* RNA-binding activity and this activity requires arginine-rich clusters termed R1, R2 and RS.

To confirm the UL69 RNA-binding activity with an independent experimental approach, we performed an RNA mobility shift assay. For this, increasing amounts of either GST alone or GST fused to mutant aa1–156 were incubated with ^{32}P-labeled RNA, followed by separation of the resulting RNA–protein complexes on a native polyacrylamide gel. As shown in Figure 1E, retarded RNA–protein complexes were visible only with the UL69 mutant aa1–156 but not with GST alone. Thus, this result is in accordance with the results obtained using the northwestern assay and confirms that the N-terminus of pUL69 encodes an RNA-binding domain.

Arginine-rich clusters within pUL69 are required for *in vitro* RNA-binding

We next wanted to specify which of the arginine-rich sequences R1, R2 or RS contributes to the RNA-binding activity of full-length pUL69. Therefore, a series of internal deletion mutants in the context of wild-type (wt) pUL69 was generated (Figure 2A). In these mutants the arginine-rich sequences were removed either separately (Figure 2A, ΔR1, ΔR2, ΔRS) or in different combinations (Figure 2A, ΔR1ΔRS,

ΔR2ΔRS). For purification purposes all mutants were expressed as GST fusion proteins. As shown by Coomassie blue staining (Figure 2B), full-length GST fusion proteins could be detected (Figure 2B, FL-UL69); additionally, truncated species of ~40, 60 and 75 kDa were visible. Western blot analysis using an anti-GST or an anti-pUL69 antibody confirmed that all fragments corresponded to C-terminally truncated derivatives of GST-UL69 (data not shown). After purification, all mutants were subjected to the northwestern assay and we observed that mutants lacking either R1 or R2 showed a substantial reduction in RNA-binding whereas the mutant lacking RS still bound RNA (Figure 2B, lanes 2–4). However, pUL69 mutants carrying a deletion of the RS sequence in combination with either R1 or R2 entirely lost their RNA-binding activity (Figure 2B, lanes 5 and 6). It is noteworthy, that in these experiments using GST-tagged, prokaryotically expressed proteins RNA-binding was detected only with the C-terminally truncated derivatives of GST-UL69 but not with the full-length proteins. A similar observation has also been reported for the two pUL69 homologues ICP27 (34) and EB2 (36) and is most probably due to an incorrect refolding of the full-length, GST-tagged pUL69 during renaturation. Taken together, these experiments suggest that the arginine-rich motifs R1 and R2 as well as the RS motif contribute to the RNA-binding activity of the UL69 protein.

Full-length pUL69, purified from mammalian cells, interacts with RNA *in vitro* but exhibits no sequence-specificity in RNA-binding

While the herpesviral mRNA export factors ICP27 and EB2 have been shown to selectively export a set of intronless viral mRNAs *in vivo* (18,37,38), they lack a distinct RNA-binding specificity *in vitro* (22,34). Based on these findings we next addressed the question whether pUL69 has any apparent RNA-binding specificity *in vitro*. For this, northwestern assays were performed using genuine and mutated pUL69 together with four ^{32}P-labeled RNAs that differed in both size and
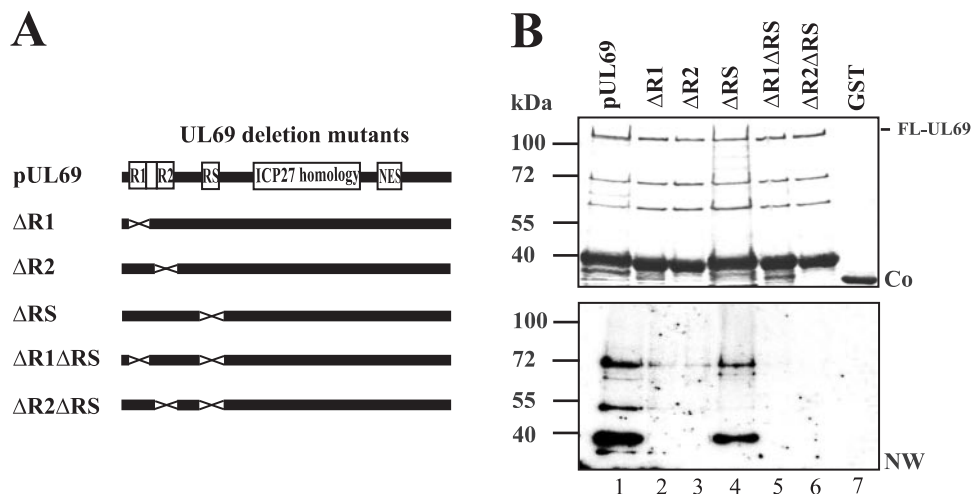
Figure 2. Internal deletions within the RNA-binding domain of pUL69 impair RNA-binding *in vitro*. (**A**) Schematic diagram of GST-tagged pUL69 in-frame deletion mutants. ΔR1, ΔR2, ΔRS, ΔR1ΔRS, ΔR2ΔRS carry deletions of amino acids 17–30, amino acids 36–50, amino acids 123–139, amino acids 17–30 and 123–139, amino acids 36–50 and 123–139, respectively. (**B**) GST and the GST-tagged proteins described in (A) were prokaryotically expressed and purified by GST affinity chromatography. Equal aliquots of each preparation were analysed by SDS–PAGE and Coomassie blue staining (Co). The localization of molecular weight markers is shown at the left in kilodaltons (kDa). The same amounts of proteins were tested in the northwestern assay (NW) using a ^{32}P-labeled RNA probe derived from the IE1/2 gene region of HCMV. FL-UL69: full-length UL69 proteins.
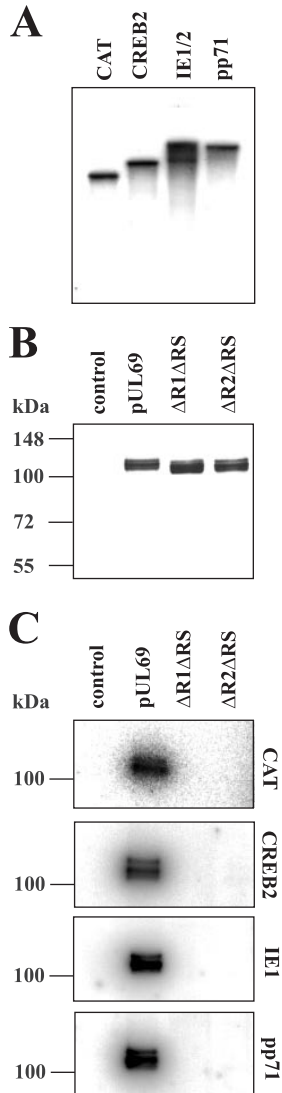
Figure 3. pUL69, immunopurified from mammalian cells, does not show sequence-specific binding to RNA *in vitro*. (**A**) ^{32}P-labeled RNA probes for northwestern blot analysis. RNA probes for the northwestern assays were generated by *in vitro* transcription in the presence of [α-^{32}P]UTP. An aliquot of each probe was analysed by 6% urea-polyacrylamide gel electrophoresis and autoradiography. CAT, antisense transcript of the chloramphenicol acetyltransferase gene; CREB2, cellular transcription factor; IE1/2, immediate early HCMV gene; pp71, early–late HCMV gene. (**B**) Immunopurification of FLAG-tagged pUL69 and the RNA-binding deficient mutants from mammalian cells. pUL69, ΔR1ΔRS and ΔR2ΔRS were expressed and purified from HEK293T cells by anti-FLAG M2 agarose affinity chromatography. Control, immunopurification was performed using mock transfected cells. Equal amounts of each protein preparation were subjected to SDS–PAGE and Coomassie blue staining. The molecular masses of the molecular weight marker proteins are indicated at the left in kilodaltons (kDa). (**C**) Northwestern assays were performed by using the same amount of proteins shown in (B) and the four different ^{32}P-labeled RNA probes (CAT, CREB2, IE1/2, pp71) shown in (A).

origin. The RNA probes used were a 0.75 kb antisense CAT probe, a 1 kb RNA corresponding to the cellular transcription factor CREB2, a 7 kb RNA generated from the IE1/2 gene region of HCMV and a 1.7 kb RNA derived from the UL82 gene of HCMV (Figure 3A). Since specific RNA-binding might require posttranslational modifications that do not occur in bacteria, all proteins used in this assay were purified

from transfected 293T cells. As shown by Coomassie blue staining (Figure 3B), we were able to purify full-length wild-type pUL69 as well as the two mutants ΔR1ΔRS and ΔR2ΔRS from mammalian cells. A closer inspection revealed the presence of three subforms of pUL69 that were originally described for pUL69 expressed in infected fibroblasts and corresponded to differentially phosphorylated protein isoforms (24). When performing the northwestern assays we noticed that full-length pUL69 (Figure 3C, pUL69) indistinguishably bound to every RNA tested, whereas both RNA-binding deficient mutants ΔR1ΔRS and ΔR2ΔRS did not bind to RNA at all (Figure 3C). Thus, these results show that the deletion of motifs R1 or R2 in combination with motif RS also abolished the RNA-binding activity of eukaryotically expressed pUL69. Furthermore, it is suggested that pUL69 demonstrates no obvious sequence-specificity in RNA-binding, at least *in vitro*. Importantly, these data are in agreement with results obtained with other herpesviral homologues of pUL69 (22,34).

An RNA-binding deficient pUL69 mutant is able to facilitate nuclear RNA export

Recently, we reported that pUL69 promotes the nuclear export of unspliced mRNAs generated from a CAT reporter plasmid (26). The UL69 protein shares this activity with some of its herpesviral homologues such as EBV EB2 (19,22,39) and KSHV ORF57 (20). Additionally, it has been demonstrated that EB2 requires its RNA-binding activity to promote the cytoplasmic accumulation of unspliced mRNA (22). To determine whether RNA-binding is also a prerequisite for pUL69-mediated mRNA export, a series of eukaryotic expression constructs encoding pUL69 mutants with various internal deletions within N-terminal motifs required for RNA-binding was generated (Figure 4A). Since the nuclear localization signal (NLS) of pUL69 is also contained within the N-terminus of the protein (25), we first examined the intracellular localization of the various pUL69 mutants in transfected HeLa cells (Figure 4B). This revealed that all mutants lacking the R2 region displayed a partial cytoplasmic localization (Figure 4B, e, f and k, l), whereas all other mutants accumulated in the nucleus as did the wild-type protein. This finding is in accordance with the assumption that the R2 region overlaps with the putative bipartite NLS of pUL69. In order to reconstitute the nuclear localization of the R2 deletion mutants the NLS of the SV40 T antigen was fused to ΔR2ΔRS and ΔR2 (Figure 4B, m, n and o, p).

Next, the RNA export activity of the indicated pUL69 mutants was compared with wt pUL69 using the pDM128/CMV/RRE reporter system (Figure 4C). In this assay, unspliced pre-mRNA encoding an intronic CAT reporter gene is transcribed from the reporter plasmid pDM128/CMV/RRE. Owing to suboptimal splice sites this transcript is a poor substrate of the spliceosome. As a consequence, the majority of unspliced pre-mRNAs expressed from this plasmid is retained in the nucleus yielding only traces of CAT protein expression. However, co-transfection of pDM128/CMV/RRE with plasmids expressing proteins that facilitate the nuclear export of the unspliced pre-mRNAs, leads to an increase in CAT expression (40). HEK293T cells were co-transfected with pDM128/CMV/RRE and plasmids expressing either wt

pUL69 or one of the mutants indicated in Figure 4A, followed by quantification of CAT protein expression (Figure 4D, lanes 1–9). In accordance with our previous results, the amount of CAT protein was significantly increased after co-expression of wt pUL69, indicating that pUL69 efficiently facilitates the nuclear export of unspliced pre-mRNAs (Figure 4D, lane 2) (26). Interestingly, the deletion of the R1 region within pUL69 abolished the RNA export activity (Figure 4D, lanes 3 and 6) whereas a deletion of R2 or RS did not cause any significant defect in the RNA export activity of pUL69 (Figure 4D, lanes 4, 5 and 7–9). Furthermore, the

RNA-binding deficient pUL69 mutants ΔR2ΔRS and N-ΔR2ΔRS could still promote the RNA export as efficiently as the wild-type protein (Figure 4D, lanes 7, 9), whereas the RNA-binding deficient pUL69 mutant ΔR1ΔRS (Figure 4D, lane 6) was inactive. This difference in the RNA export activity of RNA-binding deficient pUL69 mutants was not due to insufficient protein expression since all mutants were expressed at comparable levels (Figure 4E). It is of note that the pUL69 mutants which exhibited a partial cytoplasmic localization due to the deletion of R2 (ΔR2 and ΔR2ΔRS) showed a slight, however insignificant, reduction in mRNA export activity (Figure 4D, lanes 4 and 7); when fusing these mutants to the SV40 NLS their full RNA export activity was restored (Figure 4D, lanes 8 and 9). In summary, we observed that the RNA-binding deficient pUL69 mutants ΔR2ΔRS and N-ΔR2ΔRS were still able to promote the nuclear export of unspliced RNA indicating that, in contrast to the EBV protein EB2 (22), direct RNA interaction is not a prerequisite for the RNA export activity of pUL69.

RNA-binding of pUL69 is dispensable for its nucleocytoplasmic shuttling activity

Recently, we reported that the RNA export function of the UL69 protein depends both on its nucleocytoplasmic shuttling activity and on its ability to interact with the cellular mRNA export factors UAP56 and/or URH49 (26). Thus, we next aimed to exclude that the different behaviour of the two RNA-binding deficient mutants ΔR1ΔRS and ΔR2ΔRS (or N-ΔR2ΔRS) in the mRNA export assay was due to differences either in the ability to translocate between the nucleus and the cytoplasm or to get access to the cellular mRNA export pathway. First, we compared the shuttling activity of the two RNA-binding deficient mutants using the interspecies heterokaryon analyses. To do so, HeLa cells were co-transfected with an expression plasmid encoding either ΔR1ΔRS or N-ΔR2ΔRS and the CFN-βGal control plasmid that encodes a nucleus restricted β-galactosidase. Subsequently, the transfected cells were fused with NIH3T3 mouse fibroblasts using polyethylene
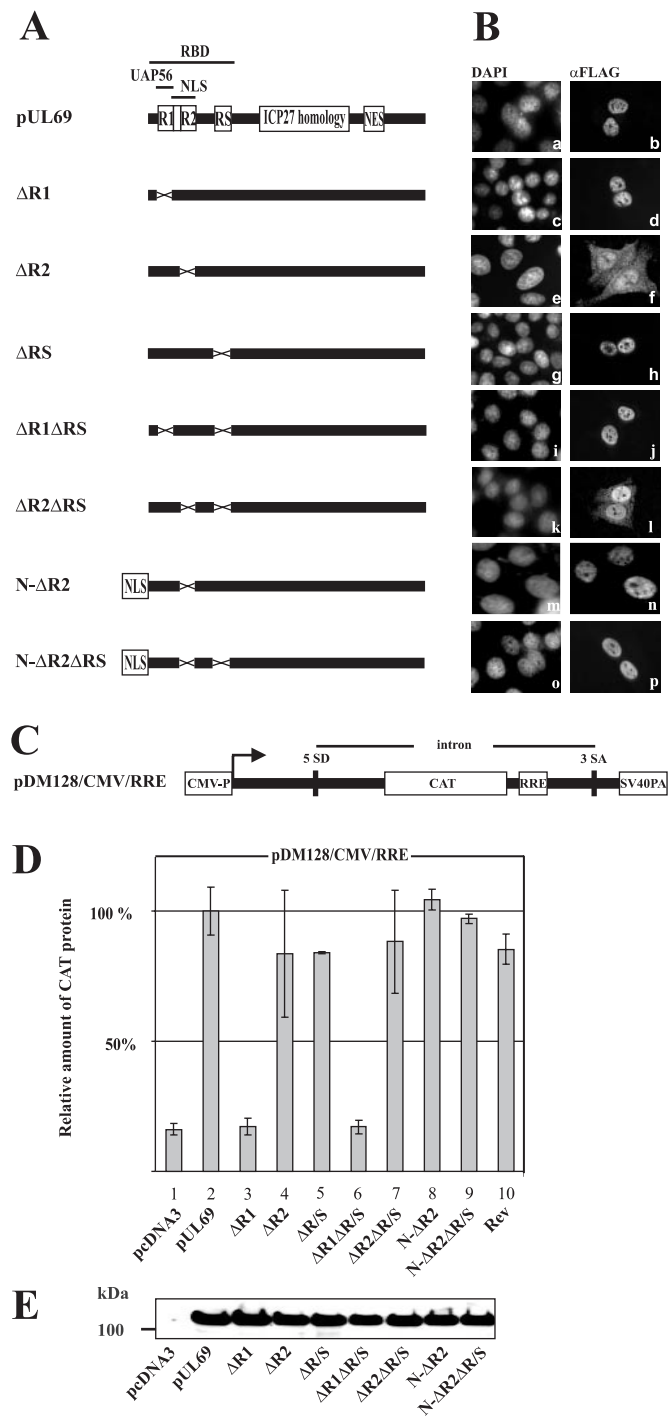

Figure 4. RNA export activity of pUL69 mutants in transfected cells. (**A**) Schematic diagram of FLAG-tagged pUL69 in-frame deletion mutants used in the CAT RNA export assay. RBD represents the RNA-binding domain of pUL69 containing arginine-rich sequences R1, R2 and RS. The binding site of the cellular mRNA export factors UAP56 and URH49 within pUL69 overlaps with the R1 region whereas the NLS of the protein overlaps with the R2 region. The localization of the ICP27 homology region and the NES of pUL69 are also indicated. NLS in N-ΔR2 and N-ΔR2ΔRS stands for SV40 TAg NLS. (**B**) Immunofluorescence analysis of the subcellular localization of the pUL69 mutants shown in (A). The proteins were transiently expressed in HeLa cells. The transfected cells were fixed by methanol 48 h after transfection and immunofluorescence staining was performed using anti-FLAG monoclonal antibody M2 and anti-mouse TRITC-conjugated secondary antibodies. (**C**) Schematic representation of the reporter plasmid pDM128/CMV/RRE used in the RNA export assay. The CAT reporter gene is inserted into an intron. The positions of the 5′ splice donor and 3′ splice acceptor sites are indicated. CMV-P, cytomegalovirus immediate early promoter; SV40PA, SV40 late poly(A) signal. (**D**) The diagram shows the relative amount of CAT proteins expressed in HEK293T cells following transfection with a mixture of plasmids including reporter plasmid pDM128/CMV/RRE and plasmids expressing either FLAG-UL69 or its mutants indicated at the bottom of the diagram or HIV-1 Rev which was used as a positive control. The relative amount of expressed CAT protein is shown as percentage relative to the sample when the reporter plasmid was co-transfected with FLAG-UL69. (**E**) Western blot analysis of HEK293T cell extracts after transfection of the FLAG-tagged proteins indicated in (D) using an anti-FLAG antibody.
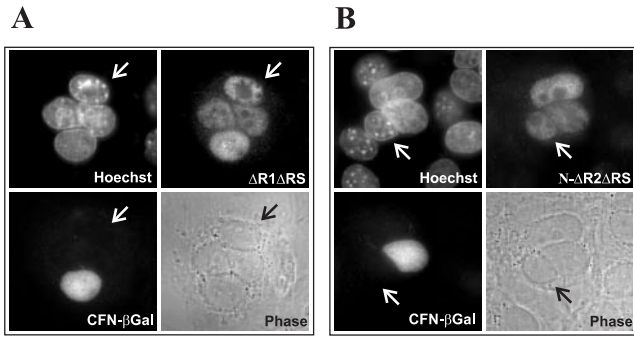
Figure 5. Nucleocytoplasmic shuttling activity of the RNA-binding deficient pUL69 mutants. Heterokaryons were generated by fusion of HeLa cells transfected with the control plasmid CFN-β-Gal and either FLAG-tagged ΔR1ΔRS or N-ΔR2ΔRS and NIH3T3 mouse cells. Cells were then immunostained both with an anti-UL69 polyclonal serum and a monoclonal antibody against β-Gal. Murine nuclei are indicated by arrows. The phase contrast images (phase) show the heterokaryons. Hoechst, staining with Hoechst 33258. (**A**) Nucleocytoplasmic shuttling of the RNA-binding deficient pUL69 mutant ΔR1ΔRS and (**B**) the RNA-binding deficient pUL69 mutant N-ΔR2ΔRS.
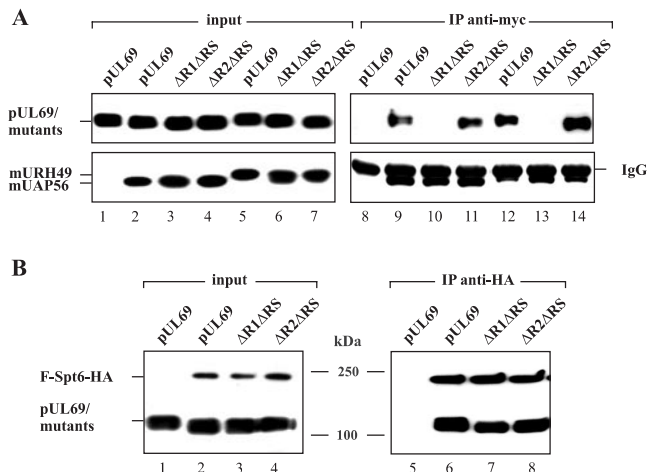

Figure 6. *In vivo* interaction of the RNA-binding deficient pUL69 mutants with cellular proteins UAP56 and URH49 as well as hSPT6. (**A**) Analysis of the interaction between RNA-binding deficient pUL69 mutants (ΔR1ΔRS, ΔR2ΔRS) and the cellular mRNA export factors UAP56 and URH49 by co-immunoprecipitation experiments. HEK293T cells were transfected with plasmids coding for FLAG-UL69 (pUL69, lanes 1, 2, 5, 8, 9 and 12) or FLAG-tagged RNA-binding mutants (ΔR1ΔRS, lanes 3, 6, 10 and 13 and ΔR2ΔRS, lanes 4, 7, 11 and 14) together with plasmids expressing either myc-UAP56 (mUAP56, lanes 2–4 and 9–11) or myc-URH49 (mURH49, lanes 5–7 and 12–14). The amount of protein in the input was analysed by western blotting (lanes 1–7). Immunoprecipitations were performed with anti-myc monoclonal antibody and the co-immunoprecipitated proteins were detected by western blot analysis using anti-FLAG antibody M2 (upper panel) or an anti-myc antibody (lower panel) (lanes 8–14). (**B**) Interaction of the RNA-binding deficient pUL69 mutants with the cellular transcription elongation factor SPT6. FLAG- and HA-tagged SPT6 (FLAG-SPT6-HA) was co-transfected with either FLAG-UL69 (pUL69, lanes 1, 2, 5 and 6) or the RNA-binding deficient pUL69 mutants (ΔR1ΔRS, lanes 3 and 7 and ΔR2ΔRS, lanes 4 and 8) into HEK293T cells. The amount of protein in the input was analysed by western blotting using anti-FLAG antibody M2 (lanes 1–4). Immunoprecipitations were performed by using anti-HA polyclonal antibody followed by western blot analysis of the co-immunoprecipitated proteins using anti-FLAG antibody M2 (lanes 5–8).

glycol. Two hours after cell fusion the cells were fixed and the subcellular localization of the respective proteins was assessed by indirect immunofluorescence analysis. As shown in Figure 5A and B, mutants ΔR1ΔRS and N-ΔR2ΔRS were

found to be present in both murine and human nuclei whereas CFN-β-Gal was detected exclusively in the human nuclei. Therefore, both RNA-binding deficient UL69 mutants were selectively and comparably transported from the transfected HeLa cell nucleus into the cytoplasm and subsequently into the murine nucleus and thus were fully capable of nucleocytoplasmic shuttling. We concluded from this set of experiments that (i) the removal of the R1, R2 or RS motif from pUL69 does not interfere with its shuttling activity and that (ii) nucleocytoplasmic shuttling of pUL69 is not dependent on its RNA-binding activity.

Discordant interaction of RNA-binding deficient pUL69 mutants with the cellular mRNA export factors UAP56 and URH49

Next, we asked whether the observed discrepancy in the RNA export activity of the two RNA-binding deficient pUL69 mutants could be explained by an impaired binding capacity of ΔR1ΔRS to the cellular mRNA export factors UAP56 or URH49. This idea is based on our previous observation that the UAP56/URH49-binding motif could be mapped by yeast two-hybrid experiments to the N-terminal amino acids 18–30 of pUL69 (26). The ΔR1 mutants carry a deletion which comprises amino acids 17–31 and hence lack the putative UAP56/URH49-binding motif entirely. Thus, in order to clarify whether the observed discrepancy in the RNA export activity is due to impaired binding to UAP56 or URH49, immunoprecipitation experiments were performed. For this 293T cells were co-transfected with expression vectors encoding either myc-tagged UAP56 or myc-tagged URH49 together with plasmids expressing the FLAG-tagged proteins UL69, ΔR1ΔRS or ΔR2ΔRS (Figure 6A). Subsequently, immunoprecipitations were carried out from whole cell extracts using an anti-myc antibody and the co-immunoprecipitated proteins were detected by western blot analysis using an anti-FLAG antibody. We found that both myc-UAP56 and myc-URH49 failed to co-immunoprecipitate the ΔR1ΔRS mutant (Figure 6A, compare lanes 3, 6 and 10, 13) while wt UL69 and the ΔR2ΔRS mutant were precipitated efficiently (Figure 6A, compare lanes 2, 4, 5, 7 and 9, 11, 12, 14). To exclude that the overall structure of one of the pUL69 mutants was seriously affected by the internal deletions, we also examined the interaction of the mutants with an additional cellular pUL69 interaction partner, the transcription elongation factor hSPT6 (Figure 6B). Hence, 293T cells were co-transfected with an expression vector for FLAG- and HA-tagged SPT6 (Figure 6B, F-Spt6-HA) and expression vectors encoding either pUL69 or one of the RNA-binding deficient mutants as FLAG fusion proteins. The immunoprecipitations were carried out using an anti-HA antibody and co-immunoprecipitated proteins were detected in a western blot analysis using an anti-FLAG antibody. As demonstrated, both RNA-binding mutants could still interact with the pUL69 interaction partner hSPT6 (Figure 6B, compare lanes 2–4 and 6–8), indicating that the 3D structure of the internal deletion mutants was not significantly affected due to the deletion of R1. Taken together, these experiments indicate that, as expected, binding of pUL69 to UAP56 or URH49 is crucial for the pUL69-mediated export of a reporter mRNA, while RNA-binding of pUL69 appears to be dispensable. In addition,
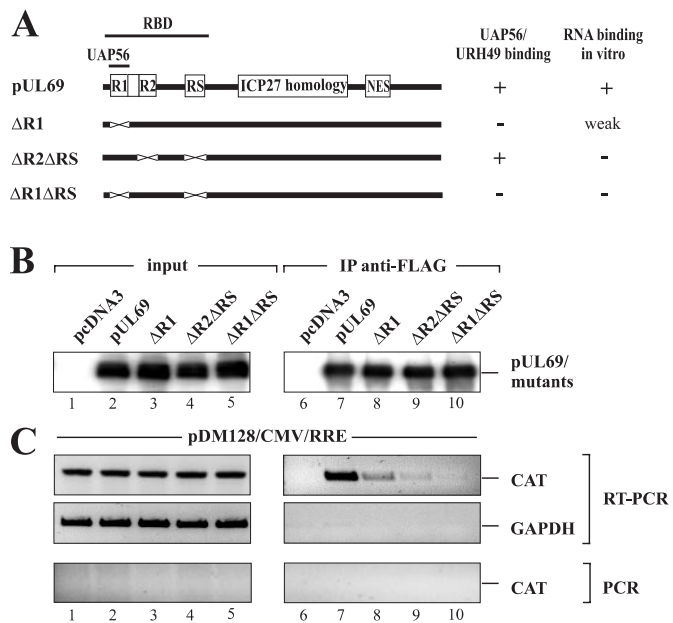
Figure 7. Association of pUL69 with CAT mRNA *in vivo*. (**A**) Schematic diagram of FLAG-tagged pUL69 and its in-frame deletion mutants used in the RNA immunoprecipitations. RBD, RNA-binding domain of pUL69 containing arginine-rich sequences R1, R2 and RS; UAP56, binding site of the cellular mRNA export factors UAP56 and URH49 within pUL69. The localization of the ICP27 homology region and the NES of pUL69 is indicated as well as the capacity of pUL69 and of the mutants to bind to UAP56/URH49 and to RNA *in vitro*. (**B**) Immunoprecipitation of pUL69 and the mutants from HEK293T cells. Cells were transfected with pDM128/CMV/RRE along with vectors expressing either pUL69 (lanes 2 and 7) or the mutants (ΔR1, lanes 3 and 8; ΔR2ΔRS, lanes 4 and 9; ΔR1ΔRS, lanes 5 and 10). The amount of protein in the input was analysed by western blotting using an anti-FLAG antibody (lanes 1–5). Immunoprecipitation was performed by an anti-FLAG antibody followed by a western blot analysis of the precipitated proteins using an anti-FLAG antibody (lanes 6–10). (**C**) Co-immunoprecipitation of CAT mRNA bound by pUL69 and the mutants *in vivo*. RNA was extracted from cell extracts and the immunoprecipitates described in (B). Thereafter, RT–PCR was performed using sequence-specific primers for the intronic CAT reporter gene and GAPDH. The amount of both CAT and GAPDH mRNA was evaluated by RT–PCR both in cell extracts (input, lanes 1–5) and in the immunoprecipitates (lanes 6–10). Control PCRs were also performed to detect any reporter vector contamination of the RNA samples used for RT–PCR (PCR, 1–10).

we confirmed our previous observation that binding of pUL69 to UAP56 or URH49 is not bridged via RNA.

pUL69 associates with unspliced CAT mRNA *in vivo*

Internal deletions within the RNA-binding domain of pUL69 resulted in three different types of mutants depicted in Figure 7A: (i) a UAP56/URH49-binding deficient mutant termed ΔR1, (ii) an RNA-binding deficient mutant termed ΔR2ΔRS and (iii) mutant ΔR1ΔRS incapable of both UAP56/URH49- and RNA-binding. Although it was confirmed with both prokaryotically and eukaryotically expressed proteins that ΔR1ΔRS and ΔR2ΔRS are incapable of interacting with RNA *in vitro*, we considered the possibility that these mutants might still be able to bind RNA under *in vivo* conditions. In order to investigate this, an RNA-immunoprecipitation analysis was performed. HEK293T cells were transfected with plasmids encoding the FLAG-tagged proteins pUL69, ΔR1, ΔR2ΔRS or ΔR1ΔRS along with the CAT reporter plasmid pDM128/CMV/RRE

(Figure 7A). Cell lysates were prepared 48 h after transfection and western blotting was performed to ensure that all transfected proteins were expressed at a comparable level (Figure 7B, lanes 1–5). Additionally, RT–PCR was used to detect the amount of co-transfected CAT mRNA in each of these cell lysates (Figure 7C, lanes 1–5; CAT). In parallel, an anti-FLAG immunoprecipitation was performed followed by the extraction of bound RNAs from the immunoprecipitates. Subsequently, the presence of unspliced CAT mRNA in the immunoprecipitates was monitored by RT–PCR using primers with specificity for the intronic CAT sequence (Figure 7C, lanes 6–10). As expected, CAT mRNA could not be amplified from an immunoprecipitate derived from cells transfected with vector pcDNA3 (Figure 7B and C, compare lane 6). However, after precipitation of pUL69 we could easily detect co-precipitated CAT mRNA (Figure 7C, lane 7) demonstrating that CAT mRNA associates with pUL69 within the transfected cells. In contrast, when the two RNA-binding deficient mutants ΔR2ΔRS and ΔR1ΔRS were immunoprecipitated, coprecipitating CAT mRNA was barely or not detectable (Figure 7C, lanes 9 and 10) emphasizing the importance of the arginine-rich regions for *in vivo* RNA-binding of pUL69.

To investigate whether the interaction of pUL69 with the cellular mRNA export factors UAP56 and URH49 contributes to the *in vivo* association of pUL69 with CAT mRNA, the ΔR1 mutant lacking the UAP56/URH49-binding site was also tested in the RNA-immunoprecipitation analyis (Figure 7C, lane 8). It is shown that a significantly reduced amount of CAT mRNA could be immunoprecipitated by the ΔR1 mutant (Figure 7C, lane 8). This finding is consistent with our *in vitro* results which also revealed a reduced RNA-binding activity for ΔR1 in comparison to wt pUL69. This could indicate that the R1 motif might be involved in directly contacting RNA *in vivo* as well. However, the fact that mutant ΔR2ΔRS, which was found to be inactive in *in vitro* RNA-binding assays, could be co-immunoprecipitated with low amounts of CAT mRNA, whereas the RNA- and UAP56-binding deficient mutant ΔR1ΔRs was negative suggests that the association with UAP56 contributes to the *in vivo* association of pUL69 with CAT mRNA (Figure 7C, lanes 9 and 10).

Finally, we asked whether pUL69 binds non-specifically to any arbitrary RNA within the transfected cells. Therefore, we analysed the immunoprecipitates for the presence of GAPDH mRNA, an abundant cellular transcript. Although GAPDH mRNA was easily detectable in total cell extracts (Figure 7C, lanes 1–5; GAPDH) it was not present in pUL69 immunoprecipitates (Figure 7C, lanes 6–10; GAPDH), demonstrating that the association of CAT mRNA with pUL69 *in vivo* can not be considered to be completely non-specific. In summary, we conclude from these experiments that pUL69 associates with unspliced CAT mRNA within living cells and that this pUL69–RNA complex can be disrupted by deleting distinct arginine-rich regions which have also been shown to mediate RNA-binding of pUL69 in *in vitro* assays.

DISCUSSION

In this study we demonstrate that the beta-herpesviral protein encoded by the open reading frame UL69 of HCMV is capable

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B
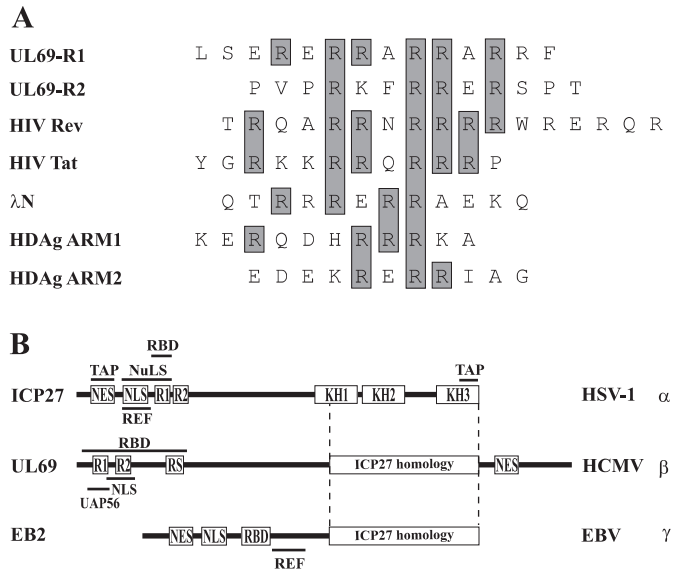
Figure 8. pUL69 contains two RNA-binding ARMs which can be found at the non-conserved N-teminus of the protein. (**A**) The ARMs of pUL69 (UL69-R1 and -R2) in comparison with conserved ARMs in representative viral RNA-binding proteins. The arginine residues are highlighted. Abbreviations: HIV Rev, the viral mRNA export factor of human immunodeficiency virus; HIV Tat, HIV antiterminator protein; λN, bacteriophage λ antiterminator N protein; HDAg ARM1 and ARM2, two ARMs in hepatitis delta antigen of hepatitis delta virus. (**B**) Schematic comparative diagram of the functional domain organization at the N-terminus of pUL69 and its counterparts from α, β and γ-herpesvirus subfamilies. ICP27, viral mRNA export factor of HSV-1 from the α-subfamily. EB2, viral mRNA export factor of EBV from the γ-subfamily. Domains supposed to be important for their function as viral mRNA export factors are shown. NES, nuclear export signal; NLS, nuclear localization signal; RBD, RNA-binding domain; REF, TAP and UAP56, the binding sites of the cellular mRNA export factors REF, TAP and UAP56/URH49, respectively, within the viral proteins. KH1, KH2 and KH3, regions with homology to KH RNA-binding motifs. Other domains are also shown such as NuLS for nucleolar localization signal and ICP27 homology region.

of directly interacting with RNA, an activity that is shared with its homologous proteins in alpha- and gamma-herpesviruses (18,22,34,41). The RNA-binding activity could be assigned to the N-terminus of pUL69 and was further narrowed down to three arginine-rich motifs termed R1, R2 and RS. Both R1 and R2 resemble arginine-rich RNA-binding motifs (ARMs) which have been identified in several viral RNA-binding proteins like the HIV-1 Rev and Tat proteins, the phage λ N or the hepatitits delta virus (HDV) HDAg proteins (Figure 8A) (42–45). The ARM sequence is short (10–20 amino acids) and characterized by a preponderance of arginine residues but little other sequence similarity suggesting that ARMs have arisen independently throughout phylogeny (46). With regard to herpesviruses, it has been demonstrated that RNA-binding of the EBV mRNA export factor EB2 also involves an ARM motif (22). In contrast, RNA-binding of HSV-1 ICP27 is mediated by an RGG box motif *in vitro* and *in vivo* (18,34,41) but three additional KH-like RNA-binding domains were proposed to be of additional importance for interacting with a subset of mRNAs *in vivo* (47,48). For pUL69, R1 and R2 together appear to be required for strong RNA-binding *in vitro* suggesting that they may function as a bipartite ARM which was first identified in the HDV delta antigen (44). Deletion of RS in combination with either R1 or R2 abolished the RNA-binding activity

of pUL69 *in vitro* and disrupted the efficient association with RNA *in vivo*.

Although one might assume that protein domains with numerous arginine residues bind RNA non-specifically, sequence-specific interactions were demonstrated for the majority of ARM containing proteins investigated so far which includes the HIV-1 proteins Rev and Tat or the phage λ N protein (42,43,45). However, the RNA target sequences of ARM proteins appear to be complex consisting of secondary structures such as stem–loops (phage λ N protein), internal loops (HIV-1 mRNA export factor Rev), bulges (HIV-1 Tat antiterminator protein) or rod-like structures (hepatitis D virus HDAg protein) (49,50). In addition, it was shown that some ARMs exhibit promiscuity in RNA-binding which was described as 'chameleon-like' behaviour (51). For instance, the Jembrana disease virus (JDV) Tat protein can bind both the BIV and HIV-1 TAR RNAs via different secondary protein structures indicating that RNA architectures can dictate the folds of ARM peptides (51).

As shown by our *in vitro* RNA-binding assays we were not able to obtain evidence for sequence-specific RNA-binding by pUL69 although we used full-length protein purified from mammalian cells thus representing the three differentially phosphorylated isoforms of pUL69 (Figure 3). This is similar to results obtained for ICP27 (34) and EB2 (22). Nevertheless, several lines of evidence suggest the existence of a mechanism that confers specificity to the recognition of RNA by these herpesviral RNA export factors, at least *in vivo*: (i) by UV-crosslinking experiments using virus infected cells it was shown that ICP27 selectively binds to HSV-1 intronless RNAs but not to HSV-1 RNAs that undergo splicing (18); (ii) EB2 selectively mediates the nuclear export of mRNAs *in vivo* as demonstrated for a set of EBV replication gene mRNAs and most of the late mRNAs (38,52); and (iii) we could show in this study by RNA-immunoprecipitation experiments that pUL69 efficiently associates with CAT mRNA but not with the cellular transcript for GAPDH. At present, it is not clear which factors determine the selective association of pUL69 and its homologues with mRNA *in vivo*. Since the herpesviral RNA export factors interact with several viral and cellular proteins, it is conceivable that some of these interactions may be needed to confer specificity to RNA recognition. In addition, the secondary structure of RNA may be crucial for its correct recognition which could be altered by proteins associated with the mRNAs in cells. Further detailed investigations will be required in order to clarify these points.

We have recently shown that pUL69 facilitates the nuclear export of unspliced mRNA from a CAT reporter gene (26). Consequently, we investigated whether RNA-binding of pUL69 would be required for its ability to promote RNA export. Surprisingly, discordant results were obtained when we analysed the activity of two different RNA-binding deficient mutants of pUL69: while mutant ΔR2ΔRS could still promote the nuclear export of unspliced CAT reporter RNA, mutant ΔR1ΔRS was inactive. In addition, we observed that mutant ΔR1, which was still able to bind RNA, could also no longer stimulate mRNA export. The discrepancy in the RNA export activity of these mutants turned out to be due to deletion of the binding site for the cellular RNA export factors UAP56 and URH49, which overlaps with the R1

motif (Figure 8B). This result confirms our previous observation that the binding of UAP56 or URH49 to pUL69 is a prerequisite for pUL69-mediated RNA export (26). For ΔR2ΔRS we detected a residual RNA-binding activity *in vivo* by RNA-immunoprecipitation analysis but this might be due to an indirect association with RNA via its interaction with the cellular mRNA export factors UAP56 or URH49. This is also supported by the observation that the UAP56/URH49-binding deficient mutant ΔR1ΔRS is totally devoid of RNA-binding both *in vitro* and *in vivo*. In summary, we conclude from these results that direct RNA-binding by pUL69 is dispensable for its activity to stimulate the nuclear export of unspliced CAT mRNA.

This finding seems to be contradictory to previous assumptions that the RNA-binding of herpesviral mRNA export factors is essential for their function, since it was proposed that both ICP27 and EB2 bind directly to specific herpesviral RNAs, ultimately recruiting these transcripts to the cellular mRNA export pathway via interacting with REF (15,17,19). However, it has to be mentioned that EB2 interacts with REF *in vivo* in an RNase sensitive manner since RNase treatment completely abolished the immunoprecipitation of EB2 with REF and TAP from both transfected and infected cells (19). This was also observed for ICP27 (19) although other studies reported on an RNase insensitive interaction with REF (15). This may suggest that RNA is a stabilizing component of the complex between REF and EB2 or ICP27 within cells and contributes to the RNA export activity of these factors. In contrast, binding of pUL69 to UAP56 and URH49 has been shown previously to be insensitive to RNase treatment (26) which is also supported by results from this study demonstrating that the RNA-binding deficient pUL69 mutant ΔR2ΔRS is still able to interact with UAP56 or URH49. Since the UAP56/URH49-binding site overlaps with the RNA-binding domain within pUL69 (Figure 8B), it is even conceivable that RNA-binding and UAP56/URH49-binding might be mutually exclusive as has been reported for the influenza virus nucleocapsid protein (NP) (53). For influenza virus NP it was shown that UAP56 facilitates the formation of the NP–RNA complex suggesting that UAP56 may function as a chaperon for loading of free NP onto RNA thus leading to enhanced influenza virus RNA synthesis (53). It is tempting to speculate that the cellular mRNA export factor UAP56 could also function as a carrier to transfer pUL69 onto the pre-mRNA incorporating it into the nascent mRNP where pUL69 may become a stable component of mRNPs by interacting with additional mRNP proteins. Further studies are required to clarify this hypothesis.

Furthermore, RNA-binding of pUL69 might play a role for the packaging of RNAs into HCMV virions. A subset of viral and cellular mRNAs has been shown previously to be incorporated into HCMV virions by an as yet unknown mechanism (54,55). Since the UL69 protein is also contained within viral particles it is conceivable that its RNA-binding activity might contribute to the packaging of RNAs into virions.

In summary, we identified and characterized a complex, arginine-rich RNA-binding domain within the N-terminus of pUL69 which directly interacts with RNA in a sequence non-specific manner in *in vitro* assays but may selectively associate with RNA *in vivo*. Additional studies using recombinant HCMVs which express RNA-binding deficient

pUL69 mutants will be required to further clarify the role of pUL69 RNA-binding for viral replication.

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