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ARTICLE

RNA binding activity of HSV-2 ICP27 protein

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Herpes Simplex virus (HSV) protein ICP27 is a multifunctional gene expression regulator, which assumes various roles during the course of the viral infection. Previously we identified ICP27 switches RNA isoforms expression of particular genes such as PML (promyelocytic leukemia). Although ICP27 protein contains several RNA binding domains, it remains unclear what domain of ICP27 protein is critically important for RNA binding activity to both host and viral transcripts. Here we characterized the RNA binding activity of ICP27 protein in vitro. We found that R2 domain and three KH domains in the C terminal region of ICP27 protein bound to PML pre-mRNA and also suppressed retention of PML intron 7a, indicating that the C-terminal region contributes to such alternative splicing regulation of host gene. Notably C-terminal region of ICP27 protein efficiently binds to poly G, poly U and poly C, although the N-terminal region preferentially binds to poly G. In addition, the mutant proteins that lack either R2 or KH domains decreases RNA binding activity to poly U. We also employed Cross-Linking and Immuno-Precipitation (CLIP)-PCR approach in HSV-2 infected cells and identified 39 RNA binding targets of ICP27 protein in the viral genome that is G-rich. Finally, we showed that loss of either the N or C terminal regions of ICP27 significantly impaired RNA binding to such viral RNAs. Overall our results suggest that combination of ICP27 protein domains determines specificity of RNA binding to host and HSV-2 RNAs.

Keywords: HSV-2; ICP27; RGG box; KH domain; RNA binding

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Abbreviations: HSV, Herpes Simplex virus; PML, promyelocytic leukemia; Cross-Linking and Immuno-Precipitation, CLIP; IE, immediate early; ICP, Infected-cell protein; DE, delayed early; EMSA; Electrophoretic Mobility Shift Assay; MOI, multiplicities of infection; hpi, hours post infection; PNK, polynucleotide kinase; TnT, Transcription and Translation; FLA, phosphorimage analyzer; FITC, fluorescein isothiocyanate; KH, K-homology; FMRP, fragile X mental retardation protein.

Introduction

Herpes Simplex Virus (HSV)-2 is a nuclear replicating DNA virus that causes many diseases [1-3]. HSV-2 hijacks host factors to facilitate its productive infection while it efficiently shuts down host gene expression. A tegument

protein VP16 recruits the host transcription machinery to express immediate early (IE) gene products, including Infected-cell protein (ICP)0, ICP4, ICP27, and ICP47 [3,4]. ICP27 is a multifunctional regulatory protein that is conserved throughout the Herpesviridae [5,6]. A primary function of ICP27 is regulation of delayed early (DE) and late (L) gene expression. For this purpose, ICP 27 utilizes several gene expression mechanisms, including transcription promotion through interacting with the C terminus of RNA polymerase II [7], modulating pre-mRNA splicing by interacting with splicing factors [8-10], regulation of transcription termination and 3' end processing [11-14], and export of viral mRNAs from the nucleus to the cytoplasm [9,15,16].

Previously we found that the expression of promyelocytic leukemia (PML) splicing isoforms was switched during HSV-2 infection by alternative splicing [10]. Our group has established a splicing reporter capable of visualization of alternative splicing events *in vivo* [17-22] and we have developed a virus-sensitive splicing reporter whose fluorescent protein expression is changed in HSV-2-infected cells [10]. We identified ICP27 as an alternative splicing regulator for PML pre-mRNA and we found that it suppressed intron 7a removal presumably by modulating 3' splice site recognition of the cellular trans-acting factor [10]. In addition, we found that RGG box of ICP27, which was assumed to be a major RNA binding domain of ICP27, was not required for PML-splicing regulation and KH domains at carboxy-terminus of ICP27 are critical for this step [10]. These results strongly suggested that carboxy-terminus of ICP27 including R2 domain and KH domains plays important roles in recognition of the ICP27 target RNAs including viral and host RNAs.

Here we focused on RNA binding and splicing regulation activity of HSV-2 ICP27 protein. Our data show that ICP27 is capable to bind PML pre-mRNA through its C-terminal domain and modulate its alternative splicing without RGG box. *In vitro* ribohomopolymer binding assays reveal that both R2 domain and KH domains in C-terminal region confer RNA binding activity. Furthermore, CLIP analysis demonstrates that ICP27 binds to many of HSV-2 mRNA. Electrophoretic Mobility Shift Assay (EMSA) demonstrated that both N-and C-terminus regions of ICP27 can bind to viral RNAs, but less efficiently than the full-length protein. Our results strongly suggest that ICP27 binds to its target RNA through RGG box, R2 domain and KH domains cooperatively.

Materials and Methods

Plasmid Construction

Construct expressing Flag-tagged ICP27 proteins prepared by inserting a PCR product of a total genome of HSV-2 recovered from infected Vero cells into the pcDNA3-Flag vector [10]. To construct Flag-tagged amino- and carboxyl- terminal deletion mutants, which are called as ICP27 (1-239) and ICP27 (240-512), respectively, the fragments containing corresponding regions of ICP27 were prepared by PCR and cloned them into pENTR-D/TOPO. Subsequently, ICP27 (1-239) and ICP27 (240-512) in pENTR were converted to pDEST-pcDNA3-Flag destination vector by the LR reaction (invitrogen). The carboxyl-terminal deletion mutants, Flag-ICP27 (1-168), Flag-ICP27 (N:1-152) and Flag-ICP27(1-137) were prepared by PCR-based mutation (deletion) using QuikChange II XL kit (Stratagene). The amino-terminal deletion mutants, Flag-ICP27 (C:153-512) and Flag-ICP27(168-512) were prepared in the same way. The amino- and carboxyl-terminal deletion mutants Flag-ICP27 (153-450), Flag-ICP27 (153-367), Flag-ICP27 (153-281) and Flag-ICP27 (nls-C) were prepared in the same way. Flag-ICP27 (RGG) and Flag-ICP27 (M15) were previously described [10]. The primers used for cloning are shown in the supplementary Table S1.

Viruses and Antibodies

HSV-2 strain G was used at multiplicities of infection (MOI) based on their plaque-forming unit titers in Vero cells. Anti-Flag antibodies, anti-ICP27 (8.F.137B) and Normal mouse IgG were purchased from Sigma, Abcam and Santa Cruz, respectively.

Cross-linking Immunoprecipitation (CLIP)-PCR of ICP27

For a CLIP-PCR experiment of ICP27 in HSV-2 infected HeLa cells, we used the CLIP method reported by Ule et al [23,24]. HSV-2 infected HeLa cells (6hpi (hours post infection)) were UV crosslinked for a total of 400mJ/cm2 using FUNA-CV-LINKER FS-800 (FUNAKOSHI, Japan) to covalently bond proteins and nucleic acids. To remove DNA, the soluble extract was treated with 30 U RQ DNase I (Promega) for 15 min at 37℃, and then incubated with RNaseA (4x10-5U/ul) to digest RNA for 10 min at 37℃. Subsequently, the extract was centrifuged at 60,000rpm using an Optima L-70k ultracentrifuge (BECKMAN COULTER, USA) in an NVT 65.2 rotor for 20 min at 4℃. The supernatant was mixed with either ICP27 antibody (abcam) or non-specific mouse IgG antibody (nacalai tesque, Japan) bound to Protein G Dynabeads (invitrogen). ICP27-RNA were immunoprecipitated from extract under stringent conditions (1 x PBS, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40). Purified ICP27-RNA complexes were modified using alkaline phosphatase (Takara Bio, Japan)

followed by ligation of an RNA linker sequences (5'-OH AGGGAGGACGAUGCGG3'-OH) (Dharmacon) and end-labeling with [γ-^{32}P] ATP by polynucleotide kinase (PNK) (Takara Bio, Japan). Subsequently, samples were size-separated by 10% Nu-PAGE (Invitrogen) and transferred to nitrocellulose membranes. RNA species covalently bound to proteins on nitrocellulose membranes were identified by monitoring radioactivity. To purify RNA, small strips of nitrocellulose membranes corresponding to the highest signal were cut out and treated with proteinase K, and then precipitated overnight in ethanol with GlycoBlue (Ambion). Subsequently, a 5' RNA linker (5'-P GUG UCA GUC ACU UCC AGC GG-3' puromycin) was attached to the purified RNA. PCR was performed using primers specific for each RNA linker to amplify regions associated with ICP27. PCR products between 80-90 base pairs were then cut out of an acrylamide gel and cloned into pCR-Blunt II-TOPO vector (invitrogen) and sequenced.

In Vitro Ribohomopolymer Binding Assay

In vitro translated proteins were synthesized using TnT (Transcription and Translation)-coupled reticulocyte lysates (Promega) and ^{35}S-labeled methionine. Radiolabeled proteins were incubated with ribohomopolymers conjugated to agarose beads (SIGMA) 10 min at 4 °C in a binding buffer (10 mm Tris-HCl, pH 7.4, 2.5 mm $MgCl_2$, 0.5% Triton X-100, 100 mm NaCl) and washed with the binding buffer. Finally, the bound proteins were analyzed by electrophoresis on SDS-PAGE and visualized by autoradiography [25-27].

EMSA (Electrophoretic Mobility Shift Assay)

The ^{32}P-labeled HSV-2 RNA probes containing CLIP tag were prepared by *in vitro* transcription with [α-^{32}P] GTP and T7 RNA polymerase (Takara Bio, Japan) as described [10]. The PCR products of HSV-2 T7-UL27(2480-2579, 100nt), T7-UL42(516-615, 100nt) and T7-UL26(1559-1658, 100nt) were used as the DNA templates for T7 transcription. The PCR primers are shown in supplementary Table S2.

Ten femtomoles of each radiolabeled RNA probe were incubated with 500 fmoles of Flag-tagged ICP27 purified from T-Rex293 cells expressing Flag-ICP27 in RNA-binding buffer I(20 mM HEPES-KOH at pH 7.9, 320 mM KCl, 5% glycerol, 1% Triton X-100, 1 mM DTT) with yeast tRNA for 30 min at 30 ℃. Each sample was separated on a nondenaturing polyacrylamide gel and visualized with a phosphorimage analyzer (FLA-3000G; Fuji Film).

RNA immunoprecipitation

Whole cell extracts were prepared from HEK293 cells transfected with plasmids expressing Flag alone, Flag-ICP27(WT), Flag-ICP27(N) and Flag-ICP27(C). ^{32}P-labeled US12(1-261), UL27(2379-2678) and UL42(467-716) of HSV-2 RNA were prepared by *in vitro* transcription with [α-^{32}P] GTP and T7 RNA polymerase (Takara Bio, Japan). PML exon 7-7b was prepared by *in vitro* transcription with [α-^{32}P] UTP and T7 RNA polymerase (Takara Bio, Japan). Gel purified RNA was incubated for 30 min at 30 ℃ with each nuclear extract in 20 μl of RNA binding buffer II [20 mM HEPES–KOH (pH 7.9), 100 mM KCl, 5% Glycerol, 1% Triton X-100, 2 mM DTT and 0.2 mM PMSF] supplemented with 0.4 U of an RNase inhibitor (Promega), and immunoprecipitation was then performed with Flag-M2 antibody. Each RNA was purified by the protein removal and ethanol precipitation. The purified RNAs were analyzed by denaturing PAGE and imaging using a phosphorimage analyzer (FLA-3000G; FUJIFILM).

Confocal immunofluorescence microscopy

Twenty-four hours after the transfection, cells on 15-mm glass coverslips were fixed with 4% paraformaldehyde for 10 min, permeabilized with 1% Triton X-100 for 20 min, blocked with the blocking solution (0.2% Gelatin, 1% BSA, 0.05% Tween 20 in PBS) for 1h and reacted with a diluted primary antibody (rabbit anti- Flag polyclonal antibody) in the blocking solution for 12 h at 4 ℃. After incubation, cells were washed extensively with a washing buffer (0.05% Tween 20 in PBS (pH 8.0)), incubated for 2 h at room temperature with the appropriate secondary antibody (fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG) diluted in the blocking solution, and then washed three times with the washing solution. The cells were analyzed under a confocal microscope (Olympus FV1000).

Results

ICP27 can bind to PML pre-mRNA without RGG box

In order to examine whether ICP27 protein without RGG box can associate with PML pre-mRNA or not, we performed RNA immunoprecipitation assay with ICP27 WT, ICP27 N, ICP27 C and ICP27 RGG proteins. The ICP27 N protein contains N-terminal 152 amino acids that include RGG box, whereas ICP27 C protein contains R2 and three KH domains (C-terminal 360 amino acids). The ICP27 RGG protein lacks RGG box (Figure 1A). PML pre-mRNAs containing from exon 7 to exon 7b region were synthesized by *in vitro* transcription in the presence of [α-

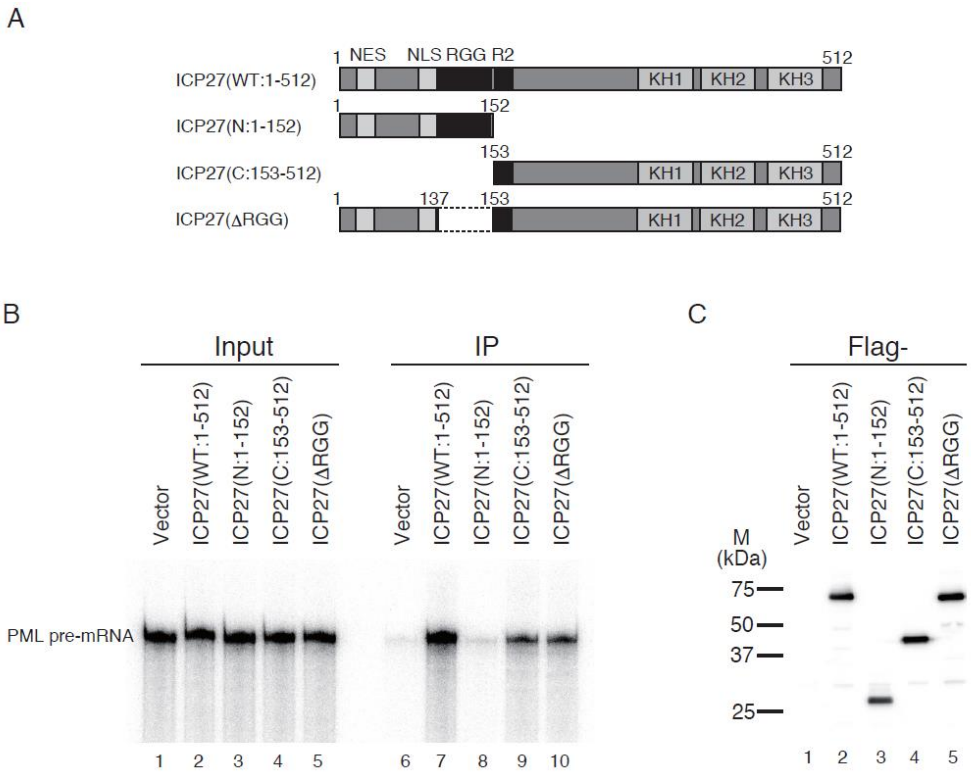


Figure 1. Binding activity of ICP27 wild type and the truncation mutants to the PML pre-mRNA. (A) Schematic representation of the ICP27 wild type and mutant proteins. (B) Immunoprecipitations were carried out after incubation of ^{32}P-labeled PML pre-mRNA under *in vitro* splicing reaction condition. For inclusion of Flag-ICP27 in the reaction, HeLa nuclear extract was added with total cell extract from HEK293T cells that had been transfected with control plasmid (Flag-pcDNA3, vector, lanes 1 and 6) or Flag-ICP27 wild type (lanes 2 and 7) or various deletion mutants (lanes 3-5 and 8-10). Flag monoclonal antibody M2 was used for immunoprecipitations. (C) Western blotting of the extracts from the cells transfected with control plasmid (vector) or Flag-ICP27 wild type and mutant proteins (Flag-ICP27 WT, N, C or DRGG). The Flag-ICP27 proteins were detected using monoclonal antibody to Flag.

^{32}P] UTP, and then RNAs were mixed with the whole cell extracts from HEK293 cells expressing Flag tagged proteins complemented by HeLa cell nuclear extracts. The expression level of those proteins was determined by western blotting with anti-Flag tag antibody and we found that all proteins were almost equally expressed (Figure 1C). Following incubation, RNAs were immunoprecipitated with anti-Flag antibody. The results in Figure 1B showed that Flag-ICP27 WT was able to precipitate PML pre-mRNA, while the negative control (vector transfected) was not (lanes 1 and 6). Interestingly, Flag-ICP27 C and ICP27 RGG which do not have an RGG box also precipitated PML pre-mRNA (lanes 9 and 10). Surprisingly, Flag-ICP27 N that contains RGG box was not able to precipitate with PML pre-mRNA (Figure 1B, lane 8). These results indicate that ICP27 is able to associate with PML pre-mRNA without RGG box.

C-terminal region of ICP27 can modulate the PML pre-mRNA alternative splicing

Next we have examined whether the C-terminal region of ICP27 that does not have RGG box was able to alter PML alternative splicing or not. The E6-7b reporter, which was previously described [10], was transfected into HEK293 cells together with wild type or several mutants of ICP27 (Figure 2A). Both wild type and mutants of ICP27 were confirmed their nuclear localization by expressing them in HeLa cells, followed by immunofluorescence with anti-Flag-tag antibody (Figure 2C). Alternative splicing pattern was determined by RT-PCR analysis of RNAs recovered from HEK293 cells (Figure 2B, upper panel). As we previously reported, overexpression of ICP27 wild type and RGG switch PML alternative splicing from intron 7a removal to retention (Figure 2B, lanes 3 and 6), while M15 that has a point mutation in KH3 domain of ICP27 failed to switch the alternative splicing of PML pre-mRNA (Figure 2B, lane 7) [10]. Interestingly, both nls-C and RGG mutants retained the promotion activity for intron 7a retention same as ICP27 WT (Figure 2B, lanes 5 and 6). The effect of this PML splicing by ICP27 N was still inconclusive, since the

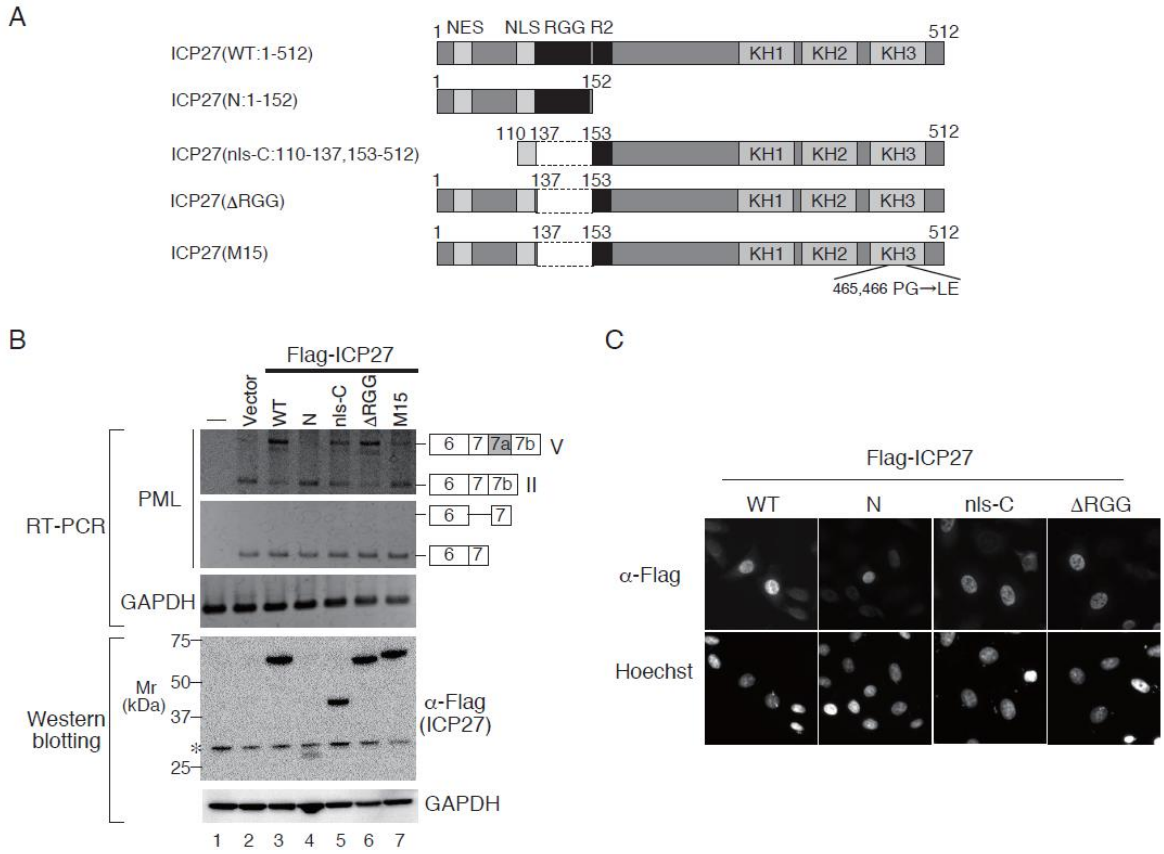


Figure 2. Effects of ICP27 mutants on PML pre-mRNA splicing in cells. (A) Schematic representation of the ICP27 wild type and mutant proteins. (B) Upper panel: RT-PCR analysis of HEK293 cells transfected with Flag-tagged ICP27 wild type or mutant plasmids in combination with PML splicing reporter plasmid. GAPDH was used as a control. Lower panel: Western blot analysis of HEK293 cell whole cell extracts transfected with Flag-tagged ICP27 wild-type or mutant plasmids. Flag-tagged proteins were detected by anti-Flag monoclonal antibody. GAPDH protein was employed as a loading control. (C) Immunofluorescence of HeLa cells transfected with either Flag-tagged ICP27 wild-type or the mutant plasmids with anti-flag antibody. Hoechst staining indicates HeLa cell nuclei.

expression level of this protein was much lower than others (Figure 2B, lower panel, lane 4). These results indicate that C-terminal region of ICP27 containing R2 and KH domains has an important role in the regulation of PML alternative splicing.

Binding properties of ICP27 wild type and truncation mutants to RNA homopolymers

The results described above strongly suggested that R2 domain and KH domains of ICP27 have an RNA binding activity. In order to test whether C-terminal region of ICP27 harbors a general RNA binding activity or not, we took advantage of *in vitro* ribohomopolymers binding assays. This assay was commonly used for testing RNA binding activity of several RNA binding proteins [28,29]. For the binding assays, full-length and truncated ICP27 proteins (Figure 3A) were synthesized by *in vitro* transcription and translation in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The radiolabeled proteins were incubated with RNA homopolymers beads, and bound proteins were analyzed by SDS-PAGE. The full length ICP27 protein bound to poly G and poly U and weakly to poly C (Figure 3B, lanes 2-5). ICP27 N bound strongly to poly G, but weekly to poly U and C (Figure 3B, lanes 7-10). ICP27 C was then tested its binding properties with this assay, and we found that it bound poly G and poly U and slightly weakly to poly C, which is quite similar to those of the full length ICP27 (Figure 3B, lanes 12-15). These results indicate that downstream region of RGG box in ICP27 have an RNA binding activity that prefers poly G and poly U. To further identify the critical region for RNA binding activity of ICP27 C, we also analyzed the ability of N-terminal truncated mutants of ICP27 C to bind ribohomopolymers (Figure 3C). For these assays we used poly G and poly U ribohomopolymers, since ICP27 WT binds strongly to them. As well as wild type, ICP27 C bound strongly to poly G and poly U ribohomopolymers (Figure 3D, lanes 5 and 6). Deletion of R2 domain caused the loss of binding activity to poly U (Figure 3D, lane 9). The deletion of KH domains from ICP27 C also abolished binding activity to poly U (Figure 3D, lane12). Taken together, these results indicate

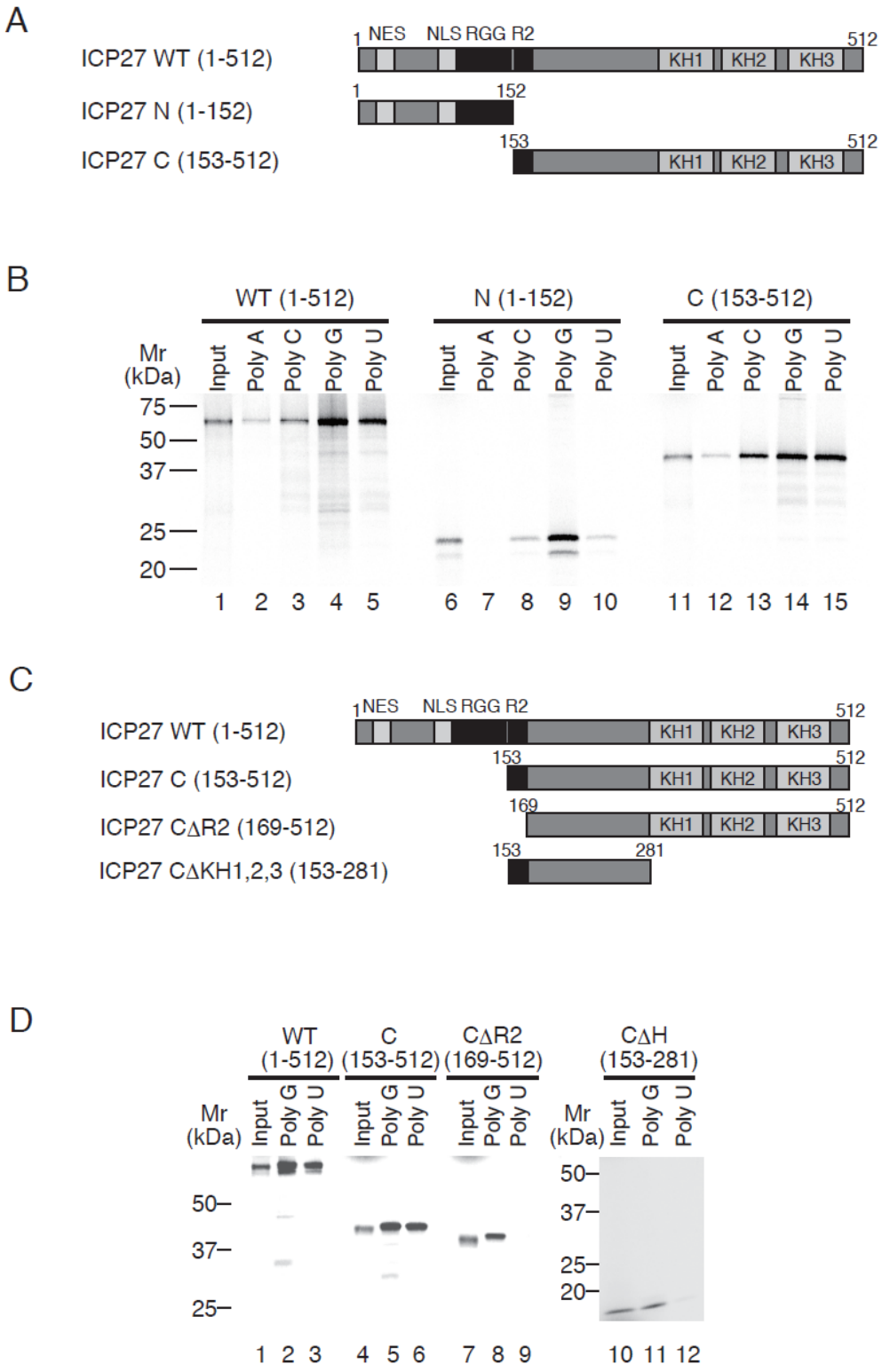
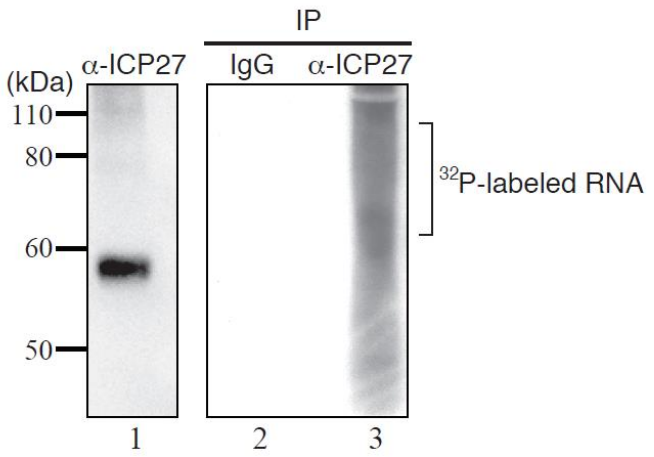


Figure 3. RNA-Binding properties of ICP27 wild type and truncated mutant proteins. (A) Schematic diagram of ICP27 and the N- and C-terminal truncated mutants used for *in vitro* binding experiments with ribonucleotide homopolymers. (B) Binding of the ICP27 protein to ribonucleotide homopolymers. Flag-tagged ICP27 proteins were synthetized by *in vitro* transcription and translation in rabbit reticulocyte lysate programmed with cDNA encoding ICP27 wild type and truncation mutants in the presence of [^{35}S] methionine. Labeled proteins bound to the indicated ribohomopolymers (lanes A, C, G and U) were analyzed by SDS-PAGE followed by fluorography. An amount equivalent to 10% of the material used for each binding reaction is shown in the lanes marked Input. The positions of molecular mass markers are indicated on the left side of the panel. (C) Schematic representation of ICP27 and the various C-terminal mutants used for *in vitro* binding experiments with ribonucleotide homopolymers. (D) Binding of the ICP27 WT and mutant proteins to poly G and poly U homopolymers. Bound proteins were analyzed as in (A).

A



B

Gene	Kinetic class	Gene ID	Position	sequence	length	# Seq. App
RL2	α	1487289	1737-1757	CTTTGTCTCCTCCTCCTCCGC	21	1
RL2	α	1487289	2224-2246	AAGAGGGCGGCGCCGCGCCGGGC	23	1
RS1	α	1487291	3072-3092	GGGCGTGCTGCTGCTGTCCAC	21	1
US1	α	1487350	527-551	AGATGCGGAGCGGAGCCGCCTGGAC	25	1
US12	α	1487353	141-157	GGAGCTCCCGCTGCTGT	17	2
UL23	β	1487307	432-451	CGGGGGGGAGGCTGTGGGCC	20	1
UL29	β	1487314	2522-2545	AGTGGTTCTGGACCGCCCTCCAGC	24	2
UL30	β	1487316	1069-1087	ATGTGTGACCTGCCGGCCT	19	1
UL30	β	1487316	1895-1917	AGAAGGGCTTCATCCTGCCGGAC	23	2
UL30	β	1487312	2860-2883	AAGATGCTCATCAAGGGCGTGGAT	24	1
UL39	β	1487325	1360-1377	AAGGAGGTGGACCTGGAC	18	1
UL39	β	1487325	2023-2042	AGAATGAAGGGCGTCCTCGC	20	1
UL42	β	1487329	556-582	ACGAAGGTGGTGAACGCCGTCGGGGAC	27	1
US2	γ	1487354	477-501	GGGGGAACCGTGGCCCCTGCCCGAT	25	1
US3/ US4	γ	1487355/ 1487356	2192-2211/ 639-658	GGAGGGCGGCCCGTGCGTCC	20	1
US3/ US4	γ	1487355/ 1487356	3371-3391/ 1818-1838	AGGAATTCTTGGGCCGCTCGC	21	1
US8/ US9	γ	1487360/ 1487362	2018-2037/ (-)7-13	GTCCGCGATGACCTCCCGGC	21	1
US10	γ	1487351	672-690	GTGGCCCTGGCGGACGGCC	19	1
UL15	γ	1487298	937-962	CGGGTGGACCACGTCAAGGGGGAAAC	26	1
UL18	γ	1487301	364-390	GAGGGCCAGGCGACGGACGTGCGCCTG	27	1
UL19	γ	1487302	421-440	TGCCCTGCTCACGGGGGAGGC	20	1
UL19	γ	1487302	823-840	GACGGGGTGCTGGTCACC	18	1
UL19/ UL20	γ	1487302/ 1487304	4250-4270/ 5230-5250	AGGGGGGCTTGGACTGGGAGC	21	1
UL21	γ	1487305	337-360	GAAGTGCTGGACGAATGCCTGGCC	24	1
UL21	γ	1487305	956-980	AGGCGTGGAAGCTGTTTGGGTCGGT	25	1
UL24	γ	1487308	1101-1124	GGAGCGCCGTCGTCGCCCGCCCGC	24	1
UL26/ UL26.5	γ	1487310/ 1487311	1059-1082/ 135-158	GTGCGGCCTGCCGGCCGCGGGGAC	24	1
UL26/ UL26.5	γ	1487310/ 1487311	1598-1615/ 674-691	AGGCCGTCTATCTGCCGC	18	1
UL27	γ	1487312	2509-2528	GAGGCCAAGTTGGCCGAGGC	20	1
UL38	γ	1487324	33-59	GGCGTGGGCCGAGAGTGCCGTGGAAAC	27	1
UL41	γ	1487328	32-52	AGACTCACCATCTGGTGAAGC	21	1
UL48	γ	1487335	1261-1278	CTGGGAGACGAACTCCGC	18	1
UL55	γ	1487344	297-319	AGAGGATAAGCGTGGGGGGCGGC	23	1

Figure 4. Identification of ICP27-bound RNA by CLIP. (A) (Left panel) Immunoblot analysis of ICP27 IP using anti-ICP27 antibody. (Right panel) HeLa cells infected by HSV-2 were irradiated with or without UV. Protein–RNA complexes were immunoprecipitated with anti-ICP27 antibody, and the 5' ends of immunoprecipitated RNAs were radiolabeled with ^{32}P. Proteins cross-linked with radiolabeled RNAs were separated by SDS-PAGE and visualized by autoradiography. RNA–protein complexes (indicated by a bracket) are seen with ICP27 immunoprecipitants dependent on UV irradiation but not control and IgG immunoprecipitation. (B) Annotated list of ICP27 CLIP tags derived from viral RNAs.

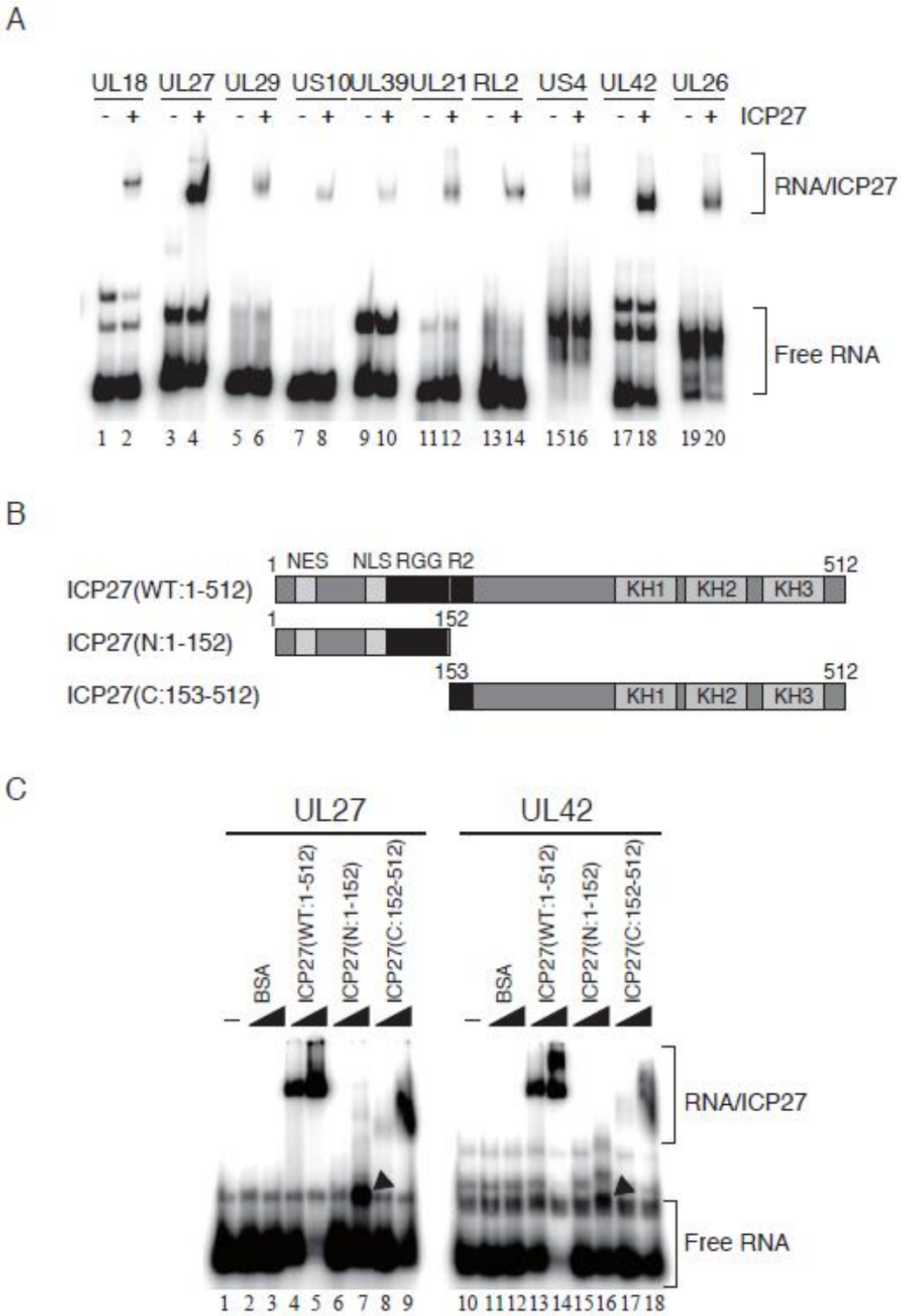


Figure 5. Both N- and C-terminal regions of ICP27 bind to viral RNAs. (A) EMSA with Flag-ICP27 proteins and in vitro synthesized viral RNAs. Ten fmol of radiolabeled RNA probes were incubated with 500 fmol of either BSA or recombinant Flag-ICP27 WT protein. Each mixture was separated on a nondenaturing polyacrylamide gel and visualized with a phosphorimage analyzer (FLA-3000G; Fuji Film). (B) Schematic representation of Flag-tagged recombinant ICP27 proteins used for EMSA. (C) EMSA with Flag-ICP27 proteins and *in vitro* synthesized viral RNAs. Ten fmol of radiolabeled RNA probes were incubated with 500 fmol or 1000 fmol of either BSA or recombinant Flag-ICP27 WT, N or C proteins. Each mixture was separated and analyzes as in (A). Arrowheads indicate the probe-Flag-ICP27 (N) protein complexes.

that both R2 domain and KH domains are necessary for binding activity of ICP27 to poly U ribohomopolymer. It is highly likely that both R2 and KH domains are required for specific and efficient binding of ICP27 to RNAs.

Identification of ICP27-bound RNAs by CLIP under HSV-2 infection

With the results described above, ICP27 was shown to specifically bind to PML pre-mRNA and poly G and U. Since ICP27 likely binds to viral RNAs to regulate their processing, we tried to identify ICP27-bound RNAs comprehensively including HSV-2 RNAs during HSV-2 infection. For this purpose, we employed a Cross-Linking and Immuno-Precipitation (CLIP)-PCR experiment using HSV-2 infected cells [23,24]. Extracts were prepared from UV-treated HSV-2 infected HeLa cells (6hpi) and treated with RNase A ($4x10^{-5}$U/ul) to partially degrade cross-linked RNA molecules. ICP27 protein was immunoprecipitated from extract under stringent conditions. The complexes were resolved on 10% Novex NuPage gels and transferred to nitrocellulose membrane. The molecular weight of ICP27 in NuPAGE is about 57kDa (Figure 4A). The RNA-ICP27 complexes immunoprecipitated by the anti-ICP27 antibody were separated by NuPAGE and shown in Figure 4A. The ICP27 proteins bound to RNA showed larger molecular weights than free ICP27 protein.

We next recovered RNAs from the radioactively labeled RNA from smeared bands (Figure 4A) of sample immunoprecipitated by anti-ICP27 antibody (Figure 4A, lane 3) or nonspecific mouse IgG (Figure 4A, lane 2), and those RNAs were separated by denaturing PAGE (Figure 4A). The length of RNA purified from complexes was about 35-40nt (data not shown). We then ligated a 5'RNA linker to the recovered RNAs and amplified by RT-PCR. Amplicons were cloned into TOPO vector and sequenced. We could obtain 265 CLIP tag sequences as putative ICP27 RNA targets and analyzed by BLAST (http://blast.ddbj.nig.ac.jp/top-j.html) search to align sequences to the human and HSV-2 genome. Thirty-nine CLIP tags were mapped to 39 positions in 28 HSV-2 genes (Figure 4B). As expected, many of CLIP tag contain G and U rich sequences.

Since we could obtain many of HSV2 RNA as CLIP tags, EMSAs were performed with the 100nt RNAs that contain CLIP tags as probes to confirm ICP27 directly binds to those RNAs. As shown in Figure 5A, all CLIP tag RNAs tested by EMSA bound to ICP27 with different binding efficiencies. These results indicate that ICP27 directly binds to CLIP tag RNAs derived from HSV2 genome. Next we tested whether C-terminal region of ICP27 contributes to binding of ICP27 to viral RNAs by performing EMSAs with ICP27 WT, ICP27 N, ICP27 C (Figure 5B) and the 100nt RNAs of UL27 and UL42 that bound to ICP27 WT efficiently as in Figure 5A. The results in Figure 5C showed that Flag-ICP27 WT was able to bind viral RNAs in a dose dependent manner. Flag-ICP27 N and C also bound viral RNAs, but less efficiently than a full-length protein (Figure 5C). The shift of both RNAs with Flag-ICP27 N was much smaller than that of WT or C, which is likely due to the small size of Flag-ICP27 N protein (Figure 5C, lanes 7 and 16). These results strongly suggest that not only RGG box but also R2 domain and KH domains bind HSV-2 RNAs and all domains are required for efficient binding to its target RNAs.

Discussion

In this study, we found that C-terminus region including from R2 to KH1, KH2 and KH3 domains of HSV-2 ICP27 harbors RNA binding activity. Previously, it was assumed that RGG box of ICP27 confers RNA binding activity [30,31]. To our surprise, at least in *in vitro* binding assay to PML pre-mRNA, C-terminal region of ICP27, not N-terminal portion, binds to PML-pre-mRNA (Figure 1). In addition, RGG box is not required for splicing modulation for PML pre-mRNA (Figure 2). These results strongly suggest that R2 and KH domains of ICP27 are involved in pre-mRNA splicing regulation via RNA binding activity.

The ICP27 was shown to recruit predominantly cytoplasmic splicing factor kinase SRPK1 into the nucleus through binding with RGG box. This results in the aberrant phosphorylation of a family of essential splicing factors termed SR proteins, which are specifically phosphorylated by SRPK1 [8]. The inappropriate phosphorylation of SR proteins prevents their participation in spliceosome assembly, which causes splicing complex formation to stall, and host cell pre-mRNA splicing is impaired. Since RGG box of ICP27 is not necessary for PML splicing regulation (Figure 2), inhibition of splicing by ICP 27 is likely mediated by a different mechanism. We have already established *in vitro* splicing system using Flag-ICP27 recombinant protein and PML pre-mRNA [10]. Further experiments with this system will shed light on the molecular mechanism how ICP27 inhibits splicing.

The C-terminal region of ICP27 consists of two kinds of motifs, R2 and K-homology (KH) domains. This region contains three predicted KH domains. The C-terminal region of ICP27 contains three predicted K-homology (KH) domains. The KH domain was first identified in the human hnRNP K protein [32]. KH domains are often found in multiple copies in RNA binding proteins. For example, fragile X mental retardation protein (FMRP) has two KH

domains [32], and hnRNP K [28] and Nova-1, 2 [33,34], Mer1p [35] proteins have three KH domains. Although it had been implicated that KH domains of ICP27 bind to RNA, RNA or ssDNA binding activity was not demonstrated. Rather, it was assumed that KH3 domain of HSV-1 interacts with splicing regulators and mRNA export factors, such as SR proteins, SF3B2 (SAP145), Aly/REF and NXF1 [8,15,16,36]. With ribohomopolymer binding assays, C-terminal region of ICP27 binds to poly G, U and C (Figure 4). When either of the motif is deleted, the RNA activity of this domain is greatly reduced, and it shows binding activity only for poly G ribohomopolymers. This indicates that the combination of R2 and KH domains is required for poly U binding. Since the C-terminal domain of ICP27 could bind various ribohomopolymers than RGG box did (Figure 3B), it may recognize more specific sequences than RGG box. Our CLIP-PCR experiment identified many of viral RNAs associated with ICP27 (Figure 4C). EMSA demonstrated its specific binding to those RNAs (Figure 5A). Although both N- and C-terminal regions of ICP27 were able to bind viral RNAs, the efficiency was lower than that of the full-length protein (Figure 5C). It is likely that N- and C-terminal regions cooperatively recognize and bind to target RNAs of ICP27. It is of interest to investigate binding RNA sequences with an ICP27 full-length, N- and C-terminal proteins by *in vitro* selection and compare the sequences obtained with each protein.

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Conflicting Interests

The authors have declared that no conflict of interests exist.

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Supporting Information

Table S1. PCR primers used for plasmid construction.

ICP27(2)-CACC-F	CACCATGGCTACCGACATTG
ICP27(2)-717STOP-R	CTACGCCTTTCGCTCCGGGA
ICP27(2)-718-CACC-F	CACCCCCTCTGCCGACACCATC
ICP27(2)-STOP-R	CTAAAATAGGGAGTTGC
ICP27-169STOP	CCGGCGCGTCTCCAGATAGGCCCACAAC
ICP27-169STOP(-)	GTTGTGGGCCTATCTGGAGACGCGCCGG
ICP27-153STOP	GGGGTCGATACGGCTAGGGCGGCGCCGACTC
ICP27-153STOP(-)	GAGTCGGCGCCGCCCTAGCCGTATCGACCCC
ICP27-138STOP	CACCGCATCCCTGAGGCGGGCGG
ICP27-138STOP(-)	CCGCCCGCCTCAGGGATGCGGTG
ICP27-d1-152	GATGACAAGAAGCTTGGATCCCCCGGCGGCGCCGACTCCAC
ICP27-d1-152(-)	GTGGAGTCGGCGCCGCCGGGGGATCCAAGCTTCTTGTCATC
ICP27-d1-168	GATGACAAGAAGCTTGGATCCAACGCCCACAACCAAGGGGG
ICP27-d1-168(-)	CCCCCTTGGTTGTGGGCGTTGGATCCAAGCTTCTTGTCATC
ICP27-d1-109	GATGACAAGAAGCTTGGATCCACCAGGCGGTCGGCTTCCCCC
ICP27-d1-109(-)	GGGGGAAGCCGACCGCCTGGTGGATCCAAGCTTCTTGTCATC
ICP27-450STOP	TGTCGGAGATCGACTACACGTAGAATTCCCGGGTCGACTC
ICP27-450STOP(-)	GAGTCGACCCGGGAATTCTACGTGTAGTCGATCTCCGACA
ICP27-368STOP	GGTGCAAGATGTGCATTTAGCACAATCTGCCGCTCC
ICP27-368STOP(-)	GGAGCGGCAGATTGTGCTAAATGCACATCTTGCACC
ICP27-282STOP	CAAGACCCCTTTGGCTAGATGCCGTTTCCCGC
ICP27-282STOP(-)	GCGGGAAACGGCATCTAGCCAAAGGGGTCTTG

Table S2. PCR primers used for EMSA probe preparation.

T7-UL18-335F	TAATACGACTCACTATAGGGATCCCGTCAGCCTCGTC
UL18-434R	GTTGGGAGCGGGACCGGAAACC
T7-UL27-2480F	TAATACGACTCACTATAGGGAGGAAGGCGCGGAGG
UL27-2579R	TCCGTGCGCTCCATGGCCGAC
T7-UL29-2490F	TAATACGACTCACTATAGGGCAAGCCCCCGGGGGTCGAAC
UL29-2589R	CTCTATGTCCTCGCGCGACAAGAG
T7-US10-629F	TAATACGACTCACTATAGGGTTCATACCCAATGGCTTCGGGCC
US10-728R	TGGACGCAGGGCGCAGCCG
T7-UL39-1976F	TAATACGACTCACTATAGGGCGTGCGTGTACCTGGAACC
UL39-2075R	AAGATGTTGTCGCAGCGCTGGGCC
T7-UL21-290F	TAATACGACTCACTATAGGGACCCGAACGTGAGCTCCGAGC
UL21-389R	GGGCTGGTTCGCAGCGAGGTG
T7-RL2-2171F	TAATACGACTCACTATAGGGCCTCTTCCTCCGCCGCCCC
RL2-2270R	CGGTCGCCCGAGTCCGAGTCC
T7-US4-1798F	TAATACGACTCACTATAGGGATCCGCCCCACGCTCCCGCC
US4-1897R	TGGGGCCCGAGGGCATGTCCTTAG
T7-UL42-516F	TAATACGACTCACTATAGGGCGACCCCGACGTCCAG
UL42-615R	GCCGAGCTCGAACGTGGTGGGTTTG
T7-UL26-1559F	TAATACGACTCACTATAGGGACACGGAGACCCCCGCCCAAC
UL26-1658R	ACCGCCCCGGATAGAGGAGGC
T7-UL27-2379F	TAATACGACTCACTATAGGGCTACGTCCTGCAACTGCAACG
UL27-2678R	TGGAGCGGAGAGTACCTGGC
T7- UL42-417F	TAATACGACTCACTATAGGGCATATGGACGACCGCGTCCG
UL42-716R	AGAATCTGGACCTGGGCGCTG
T7-US12-1F	TAATACGACTCACTATAGGGATGTCTTGGGCCCTGAAAACGAC
US12-261R	TCAAGGGGCCAGCACGCGATCC