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Sung, Po-Yu; Vaughan, Robert; Rahman, Shah Kamranur; Yi, Guanghui;  
Kerviel, Adeline; Kao, C Cheng; Roy, Polly (2018) Bluetongue virus  
VP6 and genomic RNA interaction is essential for genome packaging.  
Journal of virology. ISSN 0022-538X DOI: <https://doi.org/10.1128/jvi.02023-18>

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DOI: [10.1128/jvi.02023-18](https://doi.org/10.1128/jvi.02023-18)

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2 **Bluetongue virus VP6 and genomic RNA interaction is essential for**  
3 **genome packaging**

4

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22 **Running Title: Bluetongue virus VP6 packages RNA**

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26 **Word Count: Abstract 210**

27 **Word Count: Text 5473**

28 **ABSTRACT**

29 The genomes of the *Reoviridae*, including the animal pathogen Bluetongue virus (BTV), are  
30 multi-segmented double-stranded (ds) RNA. During replication, single-stranded (ss) positive-  
31 sense RNA segments are packaged into the assembling virus capsid, triggering genomic  
32 dsRNA synthesis. However, exactly how this packaging event occurs is not clear. A minor  
33 capsid protein VP6, unique for the orbiviruses, has been proposed to be involved in the RNA  
34 packaging process. In this study, we sought to characterize the RNA binding activity of VP6  
35 and its functional relevance. A novel proteomic approach was utilized to map the ss/dsRNA  
36 binding sites of a purified recombinant protein and the genomic dsRNA binding sites of the  
37 capsid-associated VP6. The data revealed each VP6 has multiple distinct RNA binding  
38 regions and only one region is shared between recombinant and capsid-associated VP6. A  
39 combination of targeted mutagenesis and reverse genetics identified the RNA-binding region  
40 that is essential for virus replication. Using an *in vitro* RNA-binding competition assay, a  
41 unique *cell-free* assembly assay and an *in vivo* single cycle replication assay, it was possible  
42 to identify a motif within the shared binding region that binds BTV ssRNA preferentially  
43 consistent with specific RNA recruitment during capsid assembly. These data highlight the  
44 critical roles this unique protein plays in orbivirus genome packaging and replication.

45

46 **Importance**

47 Genome packaging is a critical stage during virus replication. For virus with segmented  
48 genome, the genome segments need to be correctly packaged into a newly formed capsid.  
49 However, the detailed mechanism of this packaging is unclear. Here we focus on VP6, a  
50 minor viral protein of Bluetongue virus, which is critical for genome packaging. We use  
51 multiple approaches including a robust RNA-protein finger-printing assay, which map the  
52 ssRNA binding sites of recombinant VP6 and the genomic dsRNA binding sites of the  
53 capsid-associated VP6. Together with virological and biochemical methods, within VP6, we  
54 for the first time identify the viral RNA packaging motif of a segmented dsRNA virus.

55

56 **Keywords: Genome packaging/RNA-protein interaction/dsRNA virus**

57

58 **INTRODUCTION**

59 Bluetongue virus (BTV) is an important animal pathogen and the prototype of the *Orbivirus*,  
60 a genus of the *Reoviridae* family. The BTV particle has two capsids, an outer capsid and an  
61 inner capsid, the latter of which is also called the core. The outer capsid contains proteins  
62 VP2 and VP5 to facilitate virus entry through the cellular membrane and the release of the  
63 core into the cytoplasm. The icosahedral-shaped core is principally comprised of two  
64 proteins, VP7 and VP3, which are arranged in two layers. The VP3 encloses the viral  
65 genome of ten double-stranded RNA (dsRNA) segments (S1-S10). In addition, the core  
66 contains three minor proteins: the polymerase (VP1), the capping enzyme (VP4), and VP6,  
67 an essential structural protein of 36 kDa with RNA binding and ATP binding activity. VP6 is  
68 unique among the *Orbivirus* genus within the *Reoviridae* family.

69

70 Upon entry, core particles become transcriptionally active, producing and extruding single-  
71 stranded positive-sense RNAs (ssRNA) through the local channels at the five-fold axis,  
72 without further disassembly. These ssRNAs then act both as mRNAs for viral protein  
73 synthesis, and as templates for nascent genomic RNA synthesis. Our current understanding  
74 is that the newly synthesised 10 ssRNA segments are first combined via specific  
75 intersegment RNA-RNA interactions to form RNA complexes of all 10 segments. The RNA  
76 complexes of 10 segments are then packaged together with VP1, VP4 and VP6, into the  
77 assembling VP3 capsid layer (1-4). Genomic dsRNA molecules are subsequently  
78 synthesized within this assembled particle (known as the 'subcore'), prior to encapsidation  
79 by the VP7 layer, leading to a robust core particle formation (5).

80

81 VP1 polymerase and capping enzyme VP4 are likely to be located beneath the VP3 layer at  
82 or near the five-fold axis of icosahedral symmetry to facilitate the release of newly  
83 synthesised transcripts (6, 7). However, the exact location of VP6 is not yet clear, although  
84 VP6 has specific binding affinity for VP3 and this interaction has been shown to be important  
85 for viral ssRNA packaging and replication (8). Using reverse genetics (RG), we have shown  
86 that VP6 is essential for BTV replication and that modified BTV strains lacking VP6 do not  
87 replicate in normal cells but only in a VP6 helper cell line (9). Further, when VP6-deficient  
88 viruses were grown in VP6 helper cells and used for infection of normal cells, viral proteins  
89 are synthesized and assemble as empty particles without the viral genome. This data  
90 suggests that VP6 may be responsible for genome packaging (10, 11).

91

92 The smallest core associated protein, VP6 (328 aa), has high binding affinity for both ssRNA  
93 and dsRNA species, suggesting that it is closely associated with the viral genome (12, 13).  
94 VP6 was previously suggested to be an RNA helicase, despite poor homology with known

95 helicases (14). Current hypothesis is that VP6 assists in ssRNA packaging into the viral core  
96 through the interaction with VP3 (1, 8, 15). However, questions concerning the definition of  
97 the sites that bind viral ssRNAs, whether this is specific over cellular RNAs and how VP6  
98 interacts with genomic dsRNA, remain to be addressed.

99

100 In this study, we used RNA crosslinking and peptide fingerprinting (RCAP) to identify the  
101 RNA binding sites of VP6 using both a recombinant VP6 protein (reVP6) and in purified viral  
102 cores. The data demonstrate that multiple regions of reVP6 and core associated VP6  
103 interact with both ssRNA and dsRNA but that each source of VP6 had a largely unique RNA  
104 binding profile with only one region in common. Mutagenesis of residues within the mapped  
105 RNA-binding regions followed by virus recovery using the RG system demonstrated that the  
106 VP6-RNA binding regions of the core associated VP6 were essential for BTV replication  
107 while those associated with reVP6 were dispensable. Within the essential binding sites,  
108 residues that recognize BTV RNA preferentially, possibly necessary for genome recruitment  
109 and packaging were identified. This study highlights the essential role of the orbivirus protein  
110 VP6 in genome packaging and replication.

111

112

113

**114 MATERIAL AND METHODS****115 Virus, plasmids, mutagenesis and RNA transcripts synthesis**

116 BTV-10 VP6 was used for the mutational analysis and reverse genetics (14, 16). BTV-1  
117 (Genbank accession numbers FJ969719-FJ969728), which VP6 is fully exchangeable with  
118 BTV-10, was used in RCAP for viral capsids. All mutations of VP6 were generated by site-  
119 directed mutagenesis, sequences of these mutations and primers are available upon  
120 request. Transcripts for reverse genetic analyses were prepared using mMACHINE T7  
121 transcription kit (Thermo); RNA transcripts for gel shifting and competition assay were  
122 prepared using T7 polymerase (Thermo), following manufacturers' instructions.

123

**124 Recombinant VP6 (reVP6) expression and purification**

125 The expression and purification of BTV-10 reVP6 using baculovirus in the Sf9 cell line was  
126 described previously (14). Additionally, His tagged wild-type (WT) and mutant reVP6  
127 proteins were expressed in the *E. coli* strain BL21 (DE3) pLysS. The His-tagged reVP6  
128 proteins were purified using Ni-NTA nickel affinity purification eluting the purified protein with  
129 buffer comprising 20 mM Tris-HCl, 200 mM NaCl, pH 7.4, and 250 mM imidazole. Imidazole  
130 was then removed by buffer exchange Sephadex G-75 columns (GE Healthcare).

131

**132 RCAP**

133 The RCAP assay was done as described previously (17, 18). For analysis of recombinant  
134 VP6 binding to RNA, one mole of RNA was added to two moles of recombinant protein. The  
135 molar ratio of RNA was kept low to decrease non-specific protein crosslinking to RNA.  
136 Formaldehyde was then added to a final concentration of 0.1% and incubated for 10 min at  
137 room temperature. Glycine was added to a concentration of 0.2 M for 10 min to quench  
138 additional crosslinking. The crosslinked protein-RNA complexes were digested using  
139 sequencing-grade trypsin (Trypsin Gold, Promega) for 16 h using a 1:20 (w/w) ratio of trypsin  
140 to capsid. RNA-peptide complexes were then selectively precipitated using a final  
141 concentration of 3 M lithium chloride and centrifugation at 16,000 x g. The peptide-RNA  
142 conjugates were reversed by a 1 h incubation at 70°C. Parallel control reactions to assess  
143 background signals were performed without the addition formaldehyde or, in the case of the  
144 recombinant protein, without RNA. RCAP peptides were analysed using an Orbitrap Elite  
145 hybrid ion trap mass spectrometer equipped with an electrospray ionization source  
146 (ThermoFisher Scientific). The peptides were resolved using a Dionex UltiMate 3000 HPLC  
147 with a 1 x 150 mm Zorbax 300SB-C18 column (Agilent) and eluted using a linear gradient of  
148 2–45% acetonitrile in water with 0.1% formic acid over 90 minutes with a flow rate of 50

149  $\mu\text{l}/\text{min}$ . Tandem mass spectra were obtained using collision-induced dissociation in a data-  
150 dependent manner. Raw mass spectral data files were converted to mascot generic format  
151 and analysed using SearchGUI (19). Spectra were searched against a database of BTV  
152 proteins concatenated with the cRAP database (20). Unspecific enzyme cleavage and a  
153 mass tolerance of 10 ppm were used. Search results were compiled and visualized using  
154 PeptideShaker (21), and results were exported as a CSV file for automated processing with  
155 custom KNIME workflows (22).

156

### 157 **Immunofluorescence staining and confocal microscopy**

158 BSR cells transfected with 800 ng of VP6 capped S9 RNA containing wild type and mutant  
159 VP6 together with 800 ng of NS2 encoding capped S8 RNA by using Endofectin  
160 (GeneCopoeia), according to the manufacturer's instructions. 24 h post transfection cells  
161 were fixed with 4% paraformaldehyde (Sigma) solution, permeabilized with 0.5 % Triton  
162 X100 (Sigma), blocked with 1% BSA (Bovine Serum Albumin, Sigma), and subsequently  
163 stained using rabbit anti-NS2 and guinea pig anti-VP6 homemade primary antibodies, and  
164 anti-rabbit Alexa 546 and anti-guinea pig Alexa 488 coupled secondary antibodies (Thermo  
165 Fisher Scientific). Nuclei were stained using Hoechst 33342 (Thermo Fisher Scientific).  
166 Images were acquired using an x100 oil objective and a Zeiss Axiovert LSM510 confocal  
167 microscope supplied with the LSM510 software.

168

### 169 **Circular dichroism (CD) spectra of reVP6**

170 The CD spectra of reVP6 were recorded in CD buffer (20 mM  $\text{Na}_2\text{HPO}_4$ , 100mM NaCl, pH  
171 7.4) at 20 °C. The far UV CD Spectral data were collected from 260–195 nm with a 0.5 mm  
172 rectangular cell path length at 20 °C on the Applied Photophysics Chirascan & Chirascan  
173 Plus spectrometers (Leatherhead, UK) attached to a Peltier unit (Quantum NorthWest  
174 TC125). The UV & CD spectra were smoothed (window factor of 4, Savitzky-Golay method)  
175 and analysed using Origin V6 and APL Prodata Viewer v4.2.15. The estimate of percentage  
176 secondary structural units (alpha helix and beta strand) of protein from the experimental CD  
177 spectra was calculated using K2D3 program as previously described (23).

178

### 179 **Electrophoretic mobility shift assay (EMSA) using $^{32}\text{P}$ -labelled RNA**

180 RNA labelling and the EMSA were performed as described previously (24). A typical binding  
181 assay of 20  $\mu\text{L}$  contains 3  $\mu\text{g}$  of reVP6 in the RNA binding buffer (2 mM  $\text{MgCl}_2$ , 60 mM KCl,  
182 100 mM NaCl, 20 mM Hepes, pH 7.5, 10% glycerol, 1 unit of RNasin (Promega)).  
183 Radiolabelled BTV S10 was heated to 65°C for 2 min, the mixture of VP6 in binding buffer  
184 was added and incubated for 30 min at 4°C. Samples were then analysed on 0.8% agarose

185 gels in TBE (Tris borate-EDTA; 89 mM Tris, pH 7.4, 89 mM boric acid, 2.5 mM EDTA). The  
186 gels were dried and analysed by autoradiography or using Amersham Biosciences  
187 PhosphorImager. For competition assay, 18 nM of radiolabelled S10 RNA was incubated  
188 with 4  $\mu$ M of reVP6 in presence of 1, 3, or 6  $\mu$ g (85, 255, or 510 nM) of non-specific *Xenopus*  
189 elongation factor mRNA (Thermo Scientific). The mixture was analyzed as described.

190

#### 191 **Reverse genetics**

192 Mutations in the cDNA of S9 RNA that encodes VP6 were generated using site-directed  
193 mutagenesis mutations (sequences available upon request), together with the other 9 BTV  
194 genome segments that were used to transfect BSR cells or a BSR cell line that stably  
195 expresses VP6 (BSR-VP6), as described by Boyce et al. (25). CPE was monitored after 3  
196 days and the mutations in S9 in the recovered viruses was confirmed by RT-PCR and  
197 sequencing.

198

#### 199 **Plaque assay**

200 WT and mutant viruses were diluted and applied to BSR cell monolayers at 0.01 to 0.1 MOI,  
201 covered by Avicel overlays as described by Matrosovich et al. (26). Cells were fixed with  
202 formaldehyde and the plaque size monitored after 3 days.

203

#### 204 **Single cycle replication assay**

205 WT BTV, VP6 truncated virus, and VP6 mutant viruses, were used to infect BSR cells or  
206 BSR cells that stably express VP6 (BSR-VP6). The cells were harvested at 12 hr post  
207 infection (hpi) and viral particles purified as described previously by Matsuo et al. (11). The  
208 purified virus was treated with RNaseA to remove non-packaged RNA. The RNA was  
209 extracted using a viral RNA purification kit (Thermo Fisher Scientific). The positive and  
210 negative-sense RNA, representing genomic ssRNA and dsRNA respectively, were  
211 measured by qRT-PCR using viral RNA-specific primers (sequences available upon request)  
212 (27). To measure dsRNA synthesis, cell lysate at different time points were collected,  
213 immediately frozen in -80°C, and the quantity of ssRNA and dsRNA in the cell lysate were  
214 similarly measured with qRT-PCR.

215

#### 216 ***In vitro* cell-free assembly (CFA) assay**

217 The BTV *in vitro* assembly assay has been described previously (5). Briefly, the ten  
218 segments of ssRNA were incubated with VP1, VP4 and VP6, for 30 min, followed by adding  
219 VP3 and VP7 sequentially with a 1.5 hr incubation after each addition. As controls, the VP6  
220 protein was either left out of the assembly reaction, or added after VP3. To avoid the VP6



221 protein being translated from the S9 of the ten ssRNAs, we used a vaccine strain S9 RNA,  
222 which does not translate VP6. The samples were then subjected to a 15%-65% sucrose  
223 gradient and the packaged RNA quantified with qRT-PCR.

224

#### 225 **RNA binding assay**

226 5 µg of purified WT or mutant His-tag VP6 were bound with 30 µl of Ni-NTA agarose beads  
227 (Thermo) in a buffer of 150 mM NaCl and 40 mM Tris-HCl pH 8.0. The protein-coated beads  
228 were then incubated with 0.3 µg (1.1 pmol) of BTV S10 RNA in the presence or absence of  
229 30 µg (1 nmol) of yeast tRNA. After extensive washes, the bound RNA was eluted by  
230 heating and quantified by qRT-PCR.

231

#### 232 **qRT-PCR**

233 The extracted viral RNA from the single cycle replication and CFA assay was subjected to  
234 RT reaction, using specific BTV S6 forward or reverse primers, followed by qPCR, using  
235 SYBR-Green master mix (Labtech) and S6 primers (27). RNA eluted from VP6-RNA beads  
236 binding assay was quantified using specific BTV S10 primers.

237

238 **RESULTS**239 **Genomic ssRNA is not packaged in the viral capsid in the absence of VP6**

240 During BTV replication, newly synthesised ssRNA segments are first packaged into the  
241 assembling viral cores, which in turn serve as templates for the synthesis of genomic dsRNA  
242 segments (5). VP6 has been shown to be an essential component of the primary replication  
243 complex and has been hypothesized to function in recruiting and packaging BTV RNA (9,  
244 10, 13). Rescued BTV carrying a truncated VP6 was only possible in a VP6 complementary  
245 cell line and although these rescued VP6-defective viruses could express BTV proteins on  
246 infection, they assembled only a low level of particles, which lacked the viral genome as  
247 visualized by electron microscopy (11). To confirm correlation between a functional VP6 and  
248 RNA packaging, an available BTV strain with a truncated VP6 (triple stop codons introduced  
249 at residues 87-89 (28)) was grown in a VP6-complementing BSR-derived cell line (BSR-  
250 VP6) and, after 3 days, the recovered virion particles were used to infect both parental BSR  
251 cells and BSR-VP6 cells. Although these particles lacked the S9 RNA segment that encodes  
252 VP6, they still contained VP6 protein incorporated from BSR-VP6 cells used to recover the  
253 virus and thus were capable of synthesizing first round ssRNAs, although not capable of  
254 completion of replication or second round transcription following infection of WT BSR cells.  
255 Newly synthesised viral particles were harvested after 12 hours, the result of only one  
256 replication cycle. The packaged RNA was quantified by qRT-PCR, assaying for both positive  
257 and negative strands of S6 RNA with a S6 RNA segment-specific probe. While both the  
258 positive and negative strands of RNA molecules were present in the particles recovered from  
259 BSR-VP6 cells, particles from normal cells had less than 3% of the amount observed in the  
260 BSR-VP6 cells grown particles (Figure 1A). This low-level signal is most likely residual RNA  
261 from the inoculum. These data confirm that in the absence of functional VP6 newly  
262 synthesized viral RNA segments are not packaged, consistent with a role in the packaging of  
263 viral RNA. As a control, we measured the BTV ssRNA in the cell lysate from BSR-VP6 or  
264 BSR cells infected with VP6-truncated particles (Figure 1B). BTV ssRNA transcripts were  
265 synthesized abundantly in both cell lines, slightly more so in BSR-VP6 cells than the WT  
266 BSR cells.

267

268 To investigate at which stage VP6 can affect virus assembly, we used the established *in*  
269 *vitro* cell-free assembly (CFA) assay (5). In this assay, the ten ssRNA segments of BTV are  
270 first incubated with replicase complex proteins VP1, VP4 and VP6, followed by the  
271 incubation with the inner capsid proteins VP3 and VP7, to assemble a core particle. We  
272 modified this assay for our study by either excluding VP6, or by including it after the addition  
273 of VP3, so that it should not be incorporated into the core particle. The amount of RNA  
274 encapsidated was then quantified using RT-PCR as before. In the absence of VP6, ssRNA

275 packaging was reduced to less than 10% of the control (Figure 1C). Further, the addition of  
276 VP6 after VP3, which forms the inner layer of the core, failed to rescue RNA packaging.  
277 These data further suggest that VP6 plays a role in the early stage of genomic ssRNA  
278 packaging prior to inner core assembly.

279

#### 280 **Identification of the RNA binding sites of VP6 and their impact on virus replication**

281 VP6 has a high number of charged residues and readily binds non-specific ssRNA and  
282 dsRNA *in vitro* (16, 29). We sought to identify the RNA binding regions in VP6 using a  
283 proteomic-based RCAP method. For the RCAP analysis, we used S10, the smallest of the  
284 BTV RNA segments and a recombinant VP6 (reVP6) expressed in the baculovirus  
285 expression system. Two independent experiments, each with independent samples  
286 replicated in triplicate, identified very similar RNA binding regions within reVP6 (Figure 2),  
287 indicating that such an approach can reproducibly identify residues that contact RNA. All of  
288 the peptides were not present in reactions lacking RNA and the majority are also absent in  
289 control reactions that were not crosslinked with formaldehyde (Table 1). Three regions of  
290 VP6 were strongly associated with binding ssRNA: aa2-15 (Re1), aa110-141 (Re2) and  
291 aa220-284 (Re3) (Figure 2). To assess whether these *in vitro* RNA-binding sites are  
292 important for virus replication, six positively-charged sites within VP6 (KR<sub>14-5</sub>, K<sub>110</sub>, K<sub>131</sub>, K<sub>141</sub>,  
293 KK<sub>246-7</sub>, RK<sub>257-8</sub>) were selected for substitution mutagenesis. To perturb the potential RNA  
294 binding affinity, each residue was substituted for glutamic acid (Glu, E), either individually  
295 (K<sub>110</sub>, R<sub>131</sub>, R<sub>141</sub>) or as double substitution mutations (KR<sub>14-5</sub>, KK<sub>246-7</sub>, RK<sub>257-8</sub>). Each of these  
296 mutant segments, together with the other nine WT BTV RNA segments, were then  
297 transfected into BSR cells for virus recovery.

298

299 Mutations in Re1 (KR<sub>14-5</sub>EE) or Re2 (K<sub>110</sub>E, R<sub>131</sub>E, and R<sub>141</sub>E) did not affect virus recovery;  
300 recovered mutant viruses produced plaques with a similar size to the parental virus (Figure  
301 3A). DNA sequencing confirmed the presence of the mutations within S9 for all recovered  
302 viruses and the absence of compensatory mutations elsewhere. In contrast, the double  
303 mutations located within the RNA binding region Re3 (KK<sub>246-7</sub>EE and RK<sub>257-8</sub>EE) prevented  
304 virus recovery despite several independent attempts. To examine this further, two pairs of  
305 negatively-charged residues from a conserved Glu rich motif (EE-K-XX-EE) in the same Re3  
306 region were also mutated to neutral charged glutamine (EE<sub>225-6</sub>QQ and EE<sub>230-1</sub>QQ). Both  
307 mutant constructs were then tested for their impact on virus recovery by RG. In both cases,  
308 the virus was successfully recovered, suggesting that positively-charged residues in region  
309 Re3, but not the negatively-charged residues, are important for viral infection. The positively-  
310 charged residues located in RNA binding region Re3 are thus essential for virus replication.

311

312 Although VP6 binds both ssRNA and dsRNA (16) it is not known if the sites concerned are  
313 the same. To investigate this, BTV genomic dsRNA segments were isolated from BTV  
314 infected cells and the RCAP analysis with reVP6 was repeated using dsRNA. The dsRNA-  
315 binding regions identified were similar with those that bind ssRNA (Figure 2, Table 1),  
316 suggesting that, in the absence of other viral proteins, VP6 binds ssRNA and dsRNA in a  
317 similar manner.

318

### 319 **Identification of VP6-RNA binding regions within the viral capsid and their importance** 320 **on virus replication**

321 Recombinant VP6 has multiple regions that contacts ssRNA and dsRNA. However, VP6  
322 found in the virus cores are in contact with capsid protein and this could impact RNA binding.  
323 To examine this, we purified mature cores and subjected them to RCAP analysis (Figure 2).  
324 Peptides from several core-associated proteins were identified and those from VP6 were  
325 assigned based on collision-induced fragmentation of each peptide. Within VP6, four  
326 regions were found to contact the encapsidated genomic dsRNA: residues aa151-177 (Ca1),  
327 aa246-257 (Ca2), aa281-300 (Ca3) and aa305-328 (Ca4). Notably, only Ca2 overlaps with  
328 the RNA-binding region 3 found when the analysis was done with reVP6. Thus, core-  
329 associated VP6 has a different binding profile to recombinant VP6. To map the essential  
330 binding sites a number of positively-charged residues in the identified Ca regions were  
331 targeted for site-directed mutagenesis and virus recovery using RG system. Five site-  
332 specific mutations were introduced, R<sub>167</sub>E and R<sub>177</sub>E, located in region Ca1, K<sub>296</sub>E in region  
333 Ca3, KRR<sub>303-5</sub>EEE and K<sub>318</sub>E in region Ca4. Among the five mutations introduced into VP6,  
334 only K<sub>318</sub>E, permitted recovery of viruses with normal plaque size (Figure 3B). All other  
335 mutations abrogated virus recovery. Consistent with previous data, the two positively-  
336 charged sites (KK<sub>246-7</sub>EE and RK<sub>257-8</sub>EE) in the Ca2/Re3 shared region (Ca2/Re3) were  
337 lethal confirming these regions as critical for virus replication. Residues RK<sub>208-209</sub> of VP6  
338 that were previously proposed to contact BTV RNA by bioinformatics analysis, were not  
339 identified by RCAP (14). In contrast to the Ca mutants, a RK<sub>208-209</sub>EE VP6 mutant did not  
340 perturb virus recovery, demonstrating that RCAP is more precise method for recognizing  
341 RNA-contacting residues.

342

343 Previous data has suggested that certain RNA sequences in the BTV genome, especially in  
344 the smaller RNA segments, act as packaging signals that mediate RNA assembly through  
345 RNA-RNA interaction (1, 15). To ensure that the mutations introduced into the S9 RNA  
346 sequence encoding VP6 did not affect RNA packaging, the lethal mutations described above  
347 were also rescued using a RG system in the VP6-complementary BSR-VP6 cells (9).  
348 Viruses with mutations in all six Ca regions produced the virus in BSR-VP6 cells.

349 Furthermore, the rescued virus failed to grow when passaged on normal BSR cells. These  
350 results confirmed that the lethal effect of these mutations was due to changes in VP6  
351 protein, not due to a change in the packaging signals in S9 RNA.

352

353 To exclude the concern that substitutions of negatively-charged residues influences protein  
354 conformation or isoelectric point, five lethal mutations were also redesigned to change the  
355 positively-charged R or K into alanine (Ala, A) (R<sub>167</sub>A, R<sub>177</sub>A, KK<sub>246-7</sub>AA, RK<sub>257-8</sub>AA, and  
356 K<sub>296</sub>A). None of these mutants was recovered following RG transfection in the normal BSR  
357 cells (data not shown). However, when S9 RNA carrying critical Ca2/Re3 region mutations  
358 (KK<sub>246-7</sub>EE, RK<sub>257-8</sub>EE, KK<sub>246-7</sub>AA and RK<sub>257-8</sub>AA) were transfected into BSR cells, all the  
359 mutant VP6 expressed in the cells and the localization was not different from WT VP6  
360 (Figure 4).

361

362 To ensure no gross conformational change in the mutation mapped as critical, several  
363 mutant VP6 proteins were expressed in *E. coli*, purified and analysed by gel electrophoresis  
364 and for secondary structure. Each mutant VP6 exhibited the same mobility as the WT VP6  
365 (Figure 5A) and when analysed by circular dichroism (CD) spectroscopy, all showed similar  
366 spectra except a slight difference with RK<sub>208-9</sub>EE and KRR<sub>303-5</sub>EEE compared to the WT.  
367 However, relative values of alpha helix and beta sheet did not vary beyond the variance of  
368 the assay (Figure 5B and Table 2). We calculated the secondary structure elements using  
369 tool K2D3. The change in % of individual secondary structure components clearly showed it  
370 is not significant.

371

372 Taken together, these data suggest that mutations introduced into VP6 to probe its RNA  
373 binding function studied here did not induce major conformational change in any of the  
374 mutants described.

375

### 376 **The lethal VP6 mutant lacks preferential binding affinity for viral RNA**

377 BTV S10 ssRNA was previously found to be critical for efficient RNA packaging (1, 15, 30)  
378 and was therefore used as the source of RNA to investigate the preference of the RNA  
379 binding by VP6. VP6 binding to S10 RNA was examined using <sup>32</sup>P-labelled S10 RNA by a  
380 gel electrophoretic mobility shift assay (EMSA). Three recombinant VP6 proteins were  
381 tested, a replication competent mutant, RK<sub>208-9</sub>EE, a replication incompetent mutant, RK<sub>257-8</sub>  
382 EE, which is located within the Ca2/Re3 region and WT reVP6. In the EMSA, all three  
383 proteins exhibited strong band retardation, plausibly a measure of the non-specific RNA  
384 binding function described above (Figure 6A). To investigate preferential binding by VP6 an  
385 alternate, competition method of RNA-protein interaction was used. S10 ssRNA was

386 exposed to Ni-NTA agarose beads coated with His-tagged WT reVP6 or RK<sub>257-8</sub>EE mutant in  
387 the presence or absence of an excess of yeast tRNA and, after pull-down, the bound RNA  
388 was quantified by qRT-PCR. The amount of RNA bound was not significantly affected in the  
389 presence of excess yeast tRNA for WT reVP6 while for the RK<sub>257-8</sub>EE mutant the presence  
390 of excess tRNA significantly reduced the level of S10 RNA bound (Figure 6B).

391

392 To confirm this in a quantitative manner, we performed competition assay in presence of  
393 different quantities of non-specific RNA (Figure 7). To exclude the possibility that the  
394 difference in binding was due to small size of the competitor tRNA, we used a longer ssRNA  
395 encoding *Xenopus* elongation factor 1 $\alpha$  as a non-specific RNA. Further, to ensure the RNA  
396 binding was not influenced by changes in charge, we expressed the mutant RK<sub>257-8</sub>AA in *E*  
397 *coli*, purified and utilized for EMSA. The data showed that RK<sub>257-8</sub>AA reVP6 had much lower  
398 preferential binding with BTV RNA compared to WT reVP6 (Figure 7B). Moreover, when an  
399 additional positively-charged site RK<sub>246-7</sub> within the same Ca2/Re3 region, was mutated to  
400 Ala, it also exhibited a significant effect on the preference of VP6 for BTV RNA (Figure 7C).  
401 However, the replication competent mutant, RK<sub>208-9</sub>EE, exhibited gel shifting patterns similar  
402 to the WT reVP6 (Figure 7D). These results indicate that the Re3/Ca2 region in VP6 is part  
403 of a preferential BTV RNA binding site.

404

#### 405 **Mutant VP6 is unable to package viral RNAs**

406 The Ca2/Re3 region binds both ssRNA *in vitro* and dsRNA in the viral capsid. Further, it  
407 exhibits preference for BTV RNA, consistent with a key role in the recruitment of ssRNA into  
408 assembling capsids. To confirm this, we employed the *in vitro* CFA assay, which had already  
409 demonstrated that RNA packaging was dependent on VP6. Making use of the RK<sub>257-8</sub>EE  
410 VP6 mutant, WT VP6 (positive control) or no VP6 (negative control) the level of incorporated  
411 genomic RNAs in the *in vitro* assembled cores was measured by qRT-PCR. RNA packaging  
412 was significantly reduced compared to WT when the RK<sub>257-8</sub> mutant protein was present,  
413 almost to the background level of incorporation observed in the absence of VP6 (Figure 8).  
414 To assess if a similar effect occurs in infected cells, RK<sub>257-8</sub>EE mutant virus grown in the  
415 BSR-VP6 helper cell line was used to infect the parental BSR cell line for 6 hrs and total  
416 RNA was extracted from 0 to 6 hpi. As control, BSR-VP6 cells, which are permissive for the  
417 growth of the mutant, were also infected with the same virus preparation and RNA extracted.  
418 All RNA samples were analysed by qRT-PCR using primers specific for the detection of  
419 positive or negative-sense RNA, as previously described. In this analysis quantification of  
420 the negative strand act as a marker for dsRNA synthesis as previously described (5, 11).  
421 During the infected period, a similar quantity of positive-sense BTV-RNA was produced by  
422 the mutant virus in both cell lines. However, in the parental BSR cells negative-sense RNA

423 synthesis was reduced to ~1000 folds at 6 hpi, when compared to the parental virus (Figure  
424 9). These data are consistent with VP6 acting in the packaging of BTV RNA into the core via  
425 preferential recognition by the Ca2/Re3 region.

426

427



428

429 **DISCUSSION**

430 Previous reports have implicated VP6, a protein with clusters of charged residues, in the  
431 packaging of the viral genomic RNA, but precise mapping of the binding sites and their  
432 contribution to virus replication was not reported (8, 11, 13, 14, 16). We used a robust  
433 peptide mapping method, RCAP, which could identify interactions between VP6 and the viral  
434 genome in the natural state leading to the identification of four (Ca) VP6-BTV RNA binding  
435 sites. When compared to binding sites mapped by the binding of recombinant (Re) VP6 to  
436 RNA *in vitro*, several regions were distinct suggesting that the RNA binding by VP6 contains  
437 both specific and non-specific elements. Mutations in the Ca RNA binding sites targeting  
438 positively-charged residues (R<sub>167</sub>, R<sub>177</sub>, KK<sub>246-7</sub>, RK<sub>257-8</sub>, K<sub>296</sub>, and KRR<sub>303-5</sub>) prevented virus  
439 recovery irrespective of the charge status of the mutated residues. These six sites are highly  
440 conserved among the VP6 of different BTV serotypes and the related orbiviruses EHDV and  
441 AHSV. This suggests the interaction between VP6 and RNA within the core is invariant  
442 consistent with a key functional role.

443

444 More recently, a specific site of VP6 (aa276-287) was reported to interact with the inner layer  
445 capsid protein VP3, an interaction that was essential for virus assembly (8). This interaction  
446 site is distinct from the RNA interaction sites reported here suggesting it may act to bridge  
447 the captured genomic RNA with the assembling capsid. Some viruses, such as  
448 bacteriophage MS2 use their coat protein to package genomic RNA through specific signals  
449 (31, 32) but for complex, multi-layered capsid viruses, such as orbiviruses, genome  
450 packaging is likely to engage more than one viral protein. The two different kinds of  
451 interactions that are seen in BTV, VP6-VP3 and VP6-RNA, are consistent with this  
452 hypothesis.

453

454 In contrast, RCAP analysis of the *in vitro* RNA-binding regions of reVP6, were largely not  
455 specific, a conclusion supported by the EMSA data. The amino terminal region (Re1) of  
456 reVP6 is also conserved among the Orbiviruses but changing the only positively-charged  
457 residues in this region, KR<sub>14-5</sub>, did not influence virus replication suggesting this conservation  
458 of amino acid sequence is due to other functional reasons. The disorder prediction of VP6  
459 structure showed that the N-terminal part of VP6 was more disordered than the C-terminal  
460 part, suggesting the N-terminus is highly mobile with non-specific binding while the C-  
461 terminus with a conserved function, which was consistent with our findings (33). Previous  
462 study suggested that only hexameric form of VP6 could perform helicase activity *in vitro*,  
463 although monomeric VP6 could still bind RNA (14, 16). However, it is still not known what  
464 form of VP6 exists within the viral capsid and in cells when it functions as an RNA binding



465 protein. The relationship between VP6 oligomerization and its different functions required  
466 further investigation.

467

468 One region revealed by the comparative RCAP analysis (aa246-257; amino acid sequence:  
469 KKLLSMIGGVERK) was associated with RNA binding both *in vitro* and in the viral capsid.  
470 This region has two sites, each with two positively-charged residues, KK<sub>246-7</sub> and RK<sub>257-8</sub> and  
471 both were shown to be essential for viral replication. The same mutations led to VP6 losing  
472 its preference for BTV RNA in completion binding assays suggesting the region is part of  
473 discriminatory mechanism which selects BTV RNA over the cellular pool. This observation of  
474 preferential binding could be due to the region of VP6 recognizing certain sequences or  
475 secondary structures in the viral RNA segments. However, the precise sequence(s) that VP6  
476 binds during packaging and if VP6 plays an active role in the RNA complex formation remain  
477 to be investigated.

478

479 In an earlier report, a cell-free assembly assay was established whereby the sequential  
480 mixing of replicase proteins VP1, VP4, and VP6, together with BTV ssRNAs, followed by the  
481 sequential addition of the inner capsid proteins VP3 and VP7, resulted in the successful  
482 packaging of ssRNAs into a core structure (5). In the absence of VP6 during sequential  
483 assembly packaging of ssRNAs is not observed, again consistent with a role for VP6 in the  
484 packaging of ssRNA into the BTV core structure. When a single cycle infection assay was  
485 performed with the RK<sub>257-8</sub> mutant virus, amplified in a helper cell line, despite still being able  
486 to synthesize positive-sense viral ssRNAs, only low levels of negative-sense RNAs were  
487 detected, insufficient to support viral rescue. These data may indicate a marginal VP6  
488 activity, below the threshold required for growth or the residual activity of the VP6  
489 incorporated from the BSR-VP6 cell used for mutant virus recovery. These data clearly  
490 indicate that VP6 is critical in an early function, such as selectively binding to BTV RNA, and  
491 that the region encompassing aa246-257 is a required motif.

492

493 Viral protein helps RNA virus to package correctly its genome through diverted mechanism.  
494 Besides the bacteriophage coat protein mentioned above, dsRNA bacteriophages of the  
495 Cystoviridae family contains a hexameric ATPase, P4, serves as RNA packaging motor (34,  
496 35). Recently, alphatetravirus was found to have a small encapsidated protein P17 that  
497 specifically binds to viral RNA and assist RNA packaging (36). Orbivirus VP6, despite some  
498 similarities with other packaging proteins, appears to have its unique mechanism.

499

500 Previous study have suggested ATPase motif and helicase motif of VP6 based on *in vitro*  
501 functional analysis (14). K<sub>110</sub>, the previously reported ATPase motif, was in the RCAP

502 identified RNA binding region (Re2). However, changing this site also did not influence virus  
503 replication. E<sub>157</sub>, a site in the previously suggested helicase (DEAD box) also did not show  
504 any influence on virus grow (unpublished observation). Both these two sites are conserved  
505 within BTV but not shared with EHDV or AHSV. Studies have shown that ATP-driven duplex  
506 unwinding function is not necessarily the primary mechanism of helicase proteins (37, 38).  
507 Helicase protein can serve as translocator without unwinding the duplex (39-42). Further  
508 investigation is required to clarify the role of ATPase and helicase activity of VP6 during  
509 genome packaging.

510 It is highly likely that RNA segments interact with each other in a sequential manner prior to  
511 the RNA complex packaging into capsid (1, 5, 15). In our recent report we showed that the  
512 RNA segments assortment is mainly based on RNA-RNA interactions via specific sequences  
513 (43). It is possible that VP6 may also be actively involved in assortment, but our current data  
514 does not support this notion.

515

516 Structural analysis showed that VP1 and VP4 could be encapsidated within the core-like  
517 particles of VP3 and VP7, whilst VP6 alone could not be encapsidated without viral RNA (7).  
518 It can be hypothesized that not only is VP6 essential for genome packaging, but also,  
519 perhaps reciprocally, that genomic RNA plays a role in VP6 encapsidation. More detailed  
520 structural and functional studies are required to elucidate further the protein-RNA  
521 arrangements in the viral capsid, and precisely how these interactions promote genome  
522 packaging during capsid assembly.

523

524

#### 525 **ACKNOWLEDGEMENT**

526 We thank C.C. Celma for providing BTV core sample, T. Fajardo for helping in the RG  
527 experiment and D. Villani for helping the mutant constructs cloning. We also thank Tam Bui  
528 of the Bimolecular Spectroscopy Centre, King's College London who helped with collection  
529 of CD data.

530

#### 531 **FUNDING**

532 This work was supported by the US National Institutes of Health (R01AI045000), and partly  
533 by the UK Biotechnology and Biological Sciences Research Council (BB/P00542X) and  
534 Wellcome Trust, UK (100218, Investigator Award to PR). Funding for open access charge:  
535 National Institutes of Health (R01AI045000).

536

#### 537 **CONFLICT OF INTEREST**

538 The authors declare no conflict of interest.



540 **FIGURE LEGENDS**

541

542 **Figure 1. RNA packaging in viral particles relied on VP6.** (A) VP6 truncated virus was  
543 used to infect BSR or BSR-VP6 cells, 12 hours later the viral particles were harvested and  
544 purified, and the positive (+) and negative (-) strand, representing genomic ssRNA and  
545 dsRNA respectively, were measured by qRT-PCR. (B) BTV ssRNAs in the cell lysate from  
546 (A) were measured by qRT-PCR (C) CFA assay was performed in original method (control),  
547 in absence of VP6 (no VP6), or adding VP6 at later time point (late VP6 adding). The  
548 assembled complex was purified and packaged and viral RNA measured by qRT-PCR. The  
549 mean values  $\pm$ SD are shown (n=3).

550

551 **Figure 2. Regions of the BTV VP6 that contacts RNA.** The grey bar represents the VP6  
552 protein, with the vertical dashes denoting the positions of positively and negatively-charged  
553 amino acids. The thick black lines represent peptides identified in the RCAP analyses  
554 performed with recombinant VP6 protein (reVP6) and either single-stranded or double-  
555 stranded RNAs and from purified cores. Details of the peptides are presented in Table 1.  
556 The three regions in reVP6 contact RNAs are named Re1-3. Regions in the core-associated  
557 VP6 that contact packaged genomic RNAs are named Ca1-4. Mutations were introduced on  
558 13 specific charged sites (number 1-13) and the sites that mutations had lethal effect  
559 indicated (\*).

560

561 **Figure 3. Plaque formations of WT and mutant viruses.**

562

563 **Figure 4. Mutant VP6 express similar to WT VP6.** Four mutants (RK<sub>246-7</sub>AA, RK<sub>246-7</sub>EE,  
564 RK<sub>257-8</sub>AA and RK<sub>257-8</sub>EE) and wild type (WT) VP6 encoding S9 RNAs were used to transfect  
565 BSR cells. Protein expression and localization were monitored by immunofluorescence  
566 staining and confocal microscopy. Green: VP6; blue: Hoechst staining.

567

568 **Figure 5. Conformational analysis of mutant reVP6.** (A) Five mutant reVP6 protein were  
569 expressed in *E. coli* and analyzed by SDS-PAGE together with wild type (WT) VP6, followed  
570 by coomassie blue staining. The sizes of protein ladder (M) are indicated. (B) The figure  
571 shows CD spectra of VP6 mutants normalized to wild type VP6. Each mutant and wild-type  
572 VP6 are indicated with different colours.

573

574 **Figure 6. VP6 shows BTV RNA binding preference.** (A) Two mutant VP6 protein, RK<sub>208-</sub>  
575 <sub>9</sub>EE and RK<sub>257-8</sub>EE, together with WT VP6, were incubated with <sup>32</sup>P-labelled BTV S10

576 ssRNA for interaction. The shifting of the VP6-RNA complex was subjected to EMSA using  
577 0.8% agarose gel and TBE buffer before being analysed with phosphorimager. (B) WT or  
578 RK<sub>257-8</sub>EE reVP6 were bound to Ni-beads and incubated with BTV ssRNA S10, in absence  
579 or presence of 100 folds quantity of tRNA (+tRNA). The bound RNA was then eluted and  
580 quantified with qRT-PCR. The mean values  $\pm$ SD are shown (n=3).

581

582 **Figure 7. Mutations in Ca2/Re3 region destroys BTV RNA preferential binding.**

583 Competition assay was performed using 1 $\mu$ g of WT (A), RK<sub>257-8</sub>AA (B), KK<sub>246-7</sub>AA (C), or  
584 RK<sub>208-9</sub>EE reVP6 (D) and 0.1 $\mu$ g of <sup>32</sup>P-labelled BTV S10. Xenopus elongation factor (Xef)  
585 mRNA was added in quantities as indicated. The free S10 RNA size is indicated.

586

587 **Figure 8. Mutation on VP6 prohibits genomic RNA packaging.** CFA assay was

588 performed in the presence of WT VP6 (control), RK<sub>257-8</sub>EE VP6, or in absence of VP6 (no  
589 VP6). The assembled complex was purified and the packaged viral RNA measured by qRT-  
590 PCR. The mean values  $\pm$ SD are shown (n=3).

591

592 **Figure 9. VP6 mutant virus is deficient in producing dsRNA genome.** The VP6 RK<sub>257-</sub>

593 <sub>8</sub>EE mutant virus was used to infect BSR cells (RK<sub>257-8</sub>EE, red line) or BSR-VP6 cells (WT,  
594 blue line). Total cytoplasmic RNA was harvested at 0, 2, 4, and 6 hpi. The positive (+) and  
595 negative (-) strand RNA representing genomic ssRNA (left panel) and dsRNA (right panel)  
596 respectively, were measured by qRT-PCR. The mean values  $\pm$ SD are shown (n=3).

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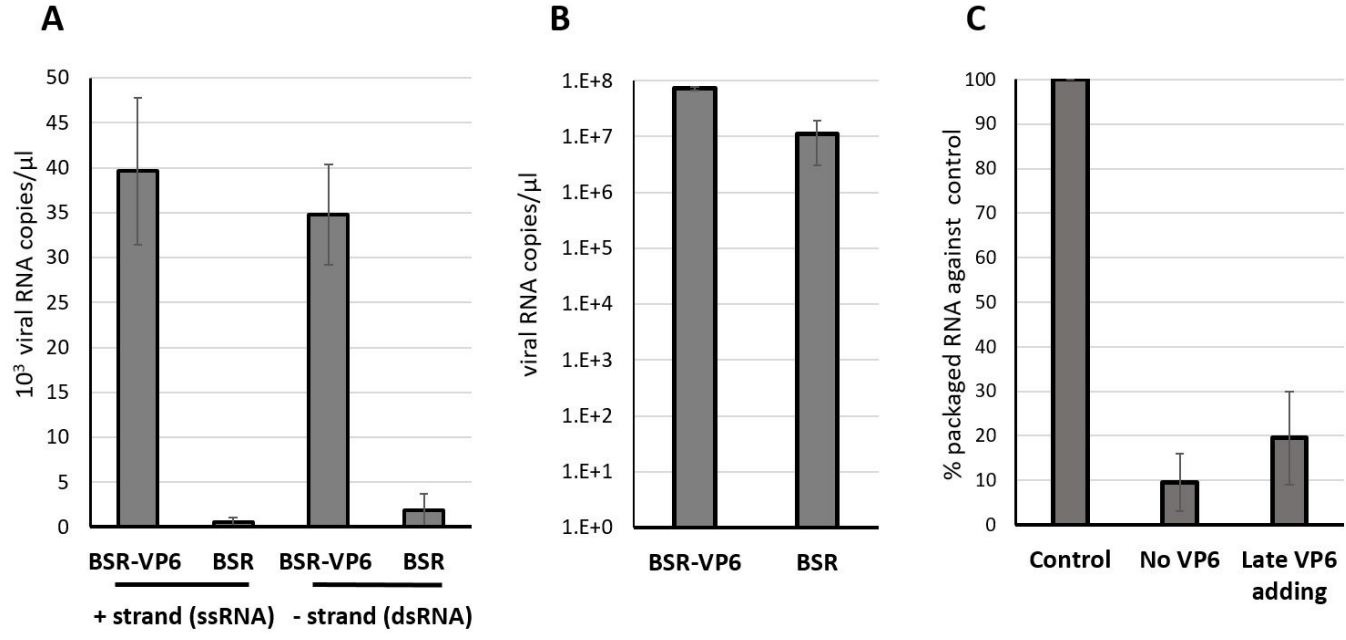
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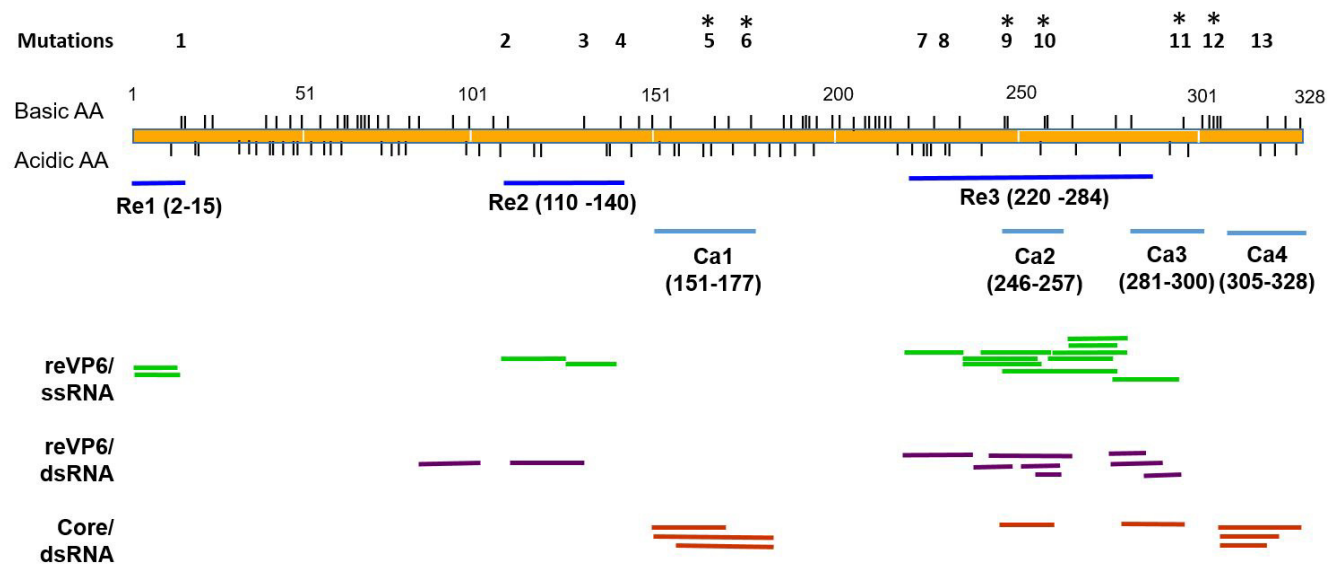


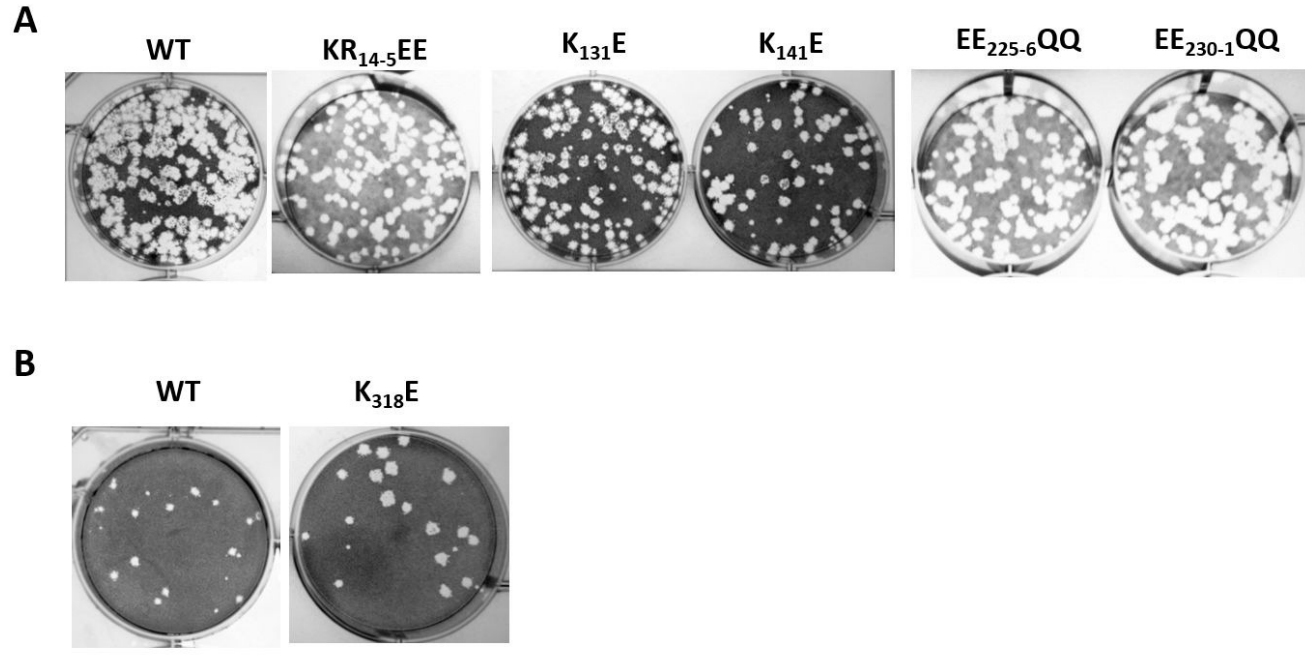
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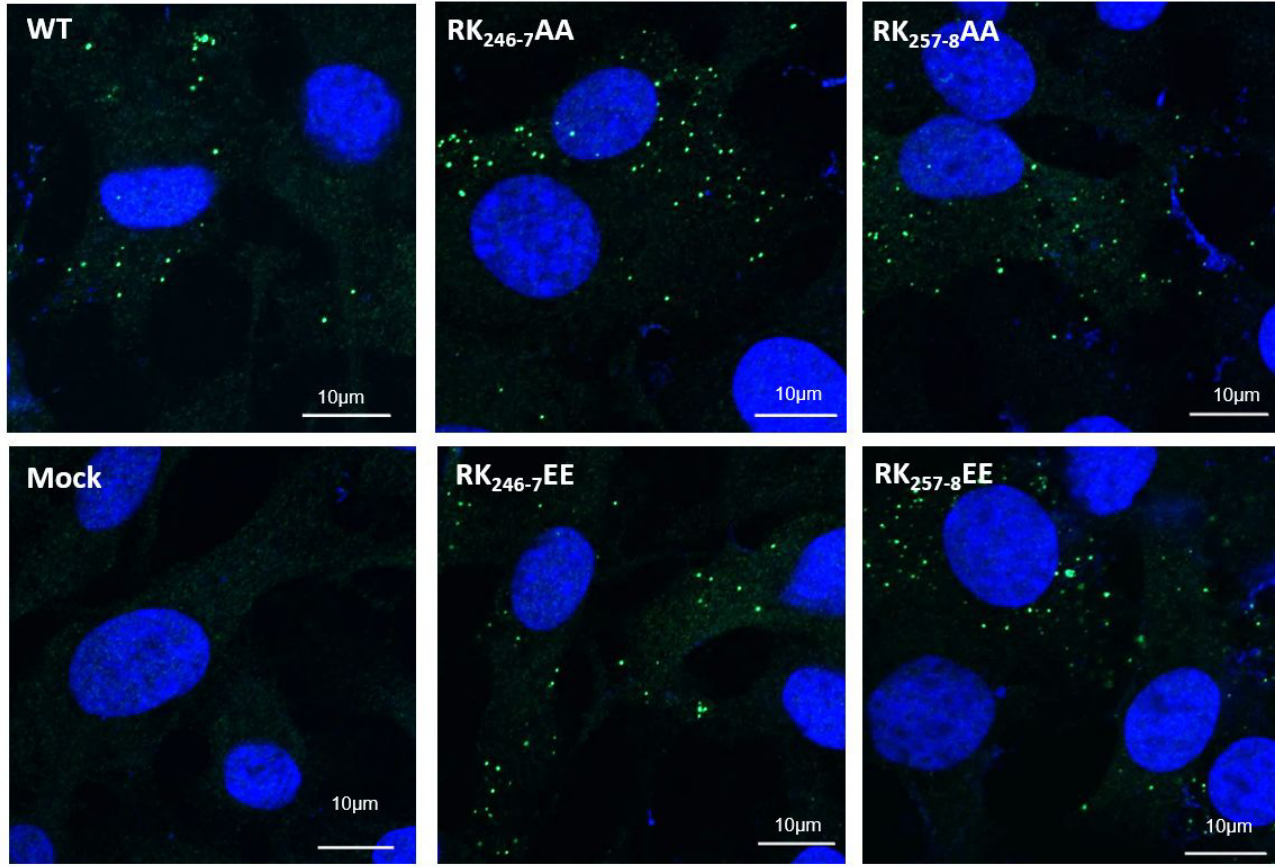
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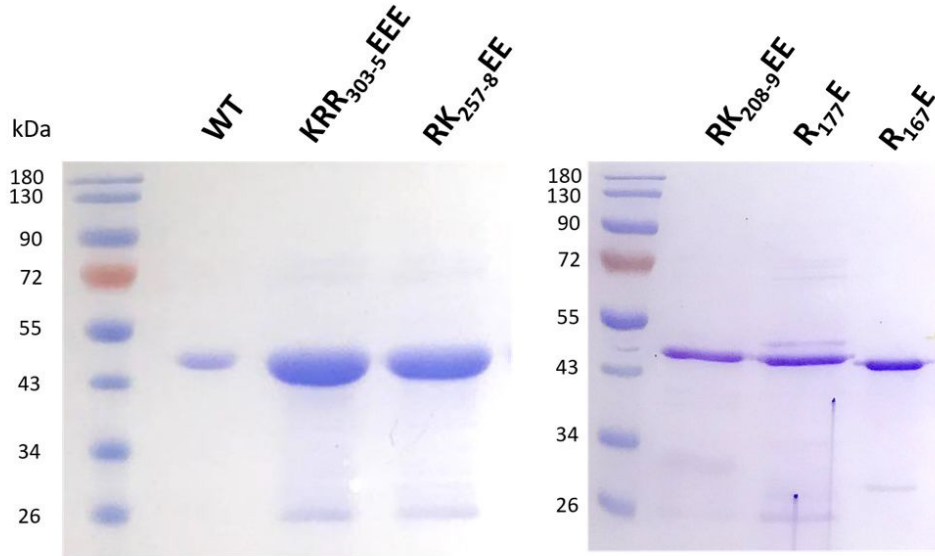
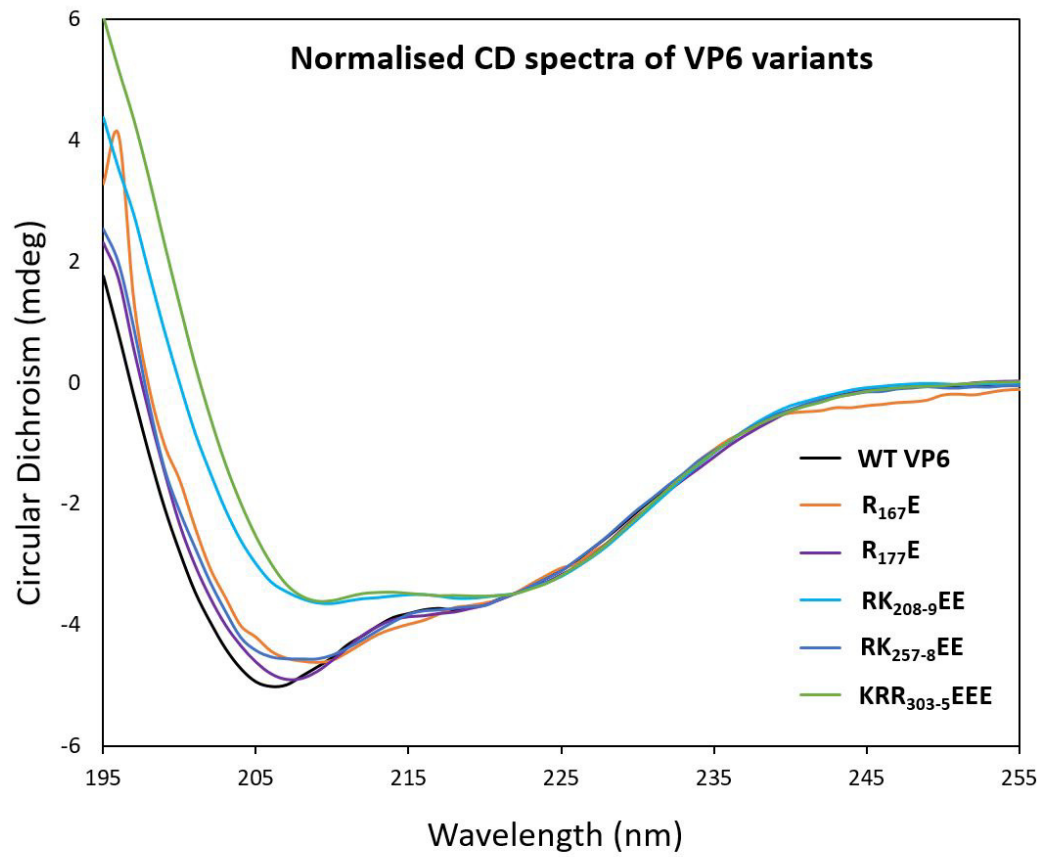


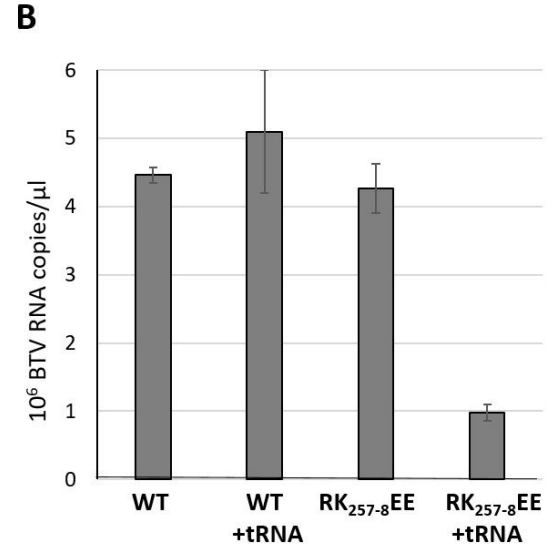
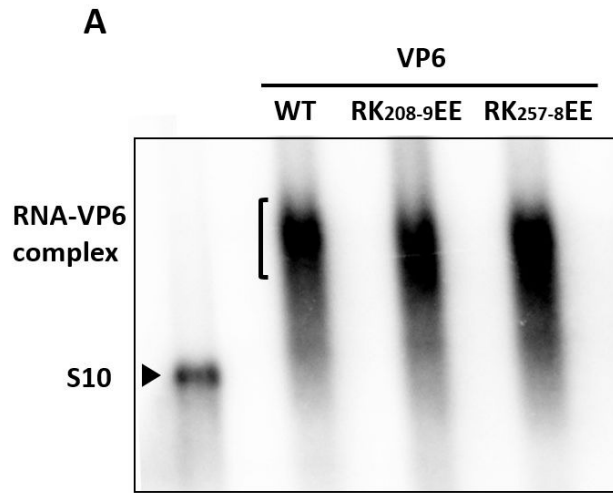


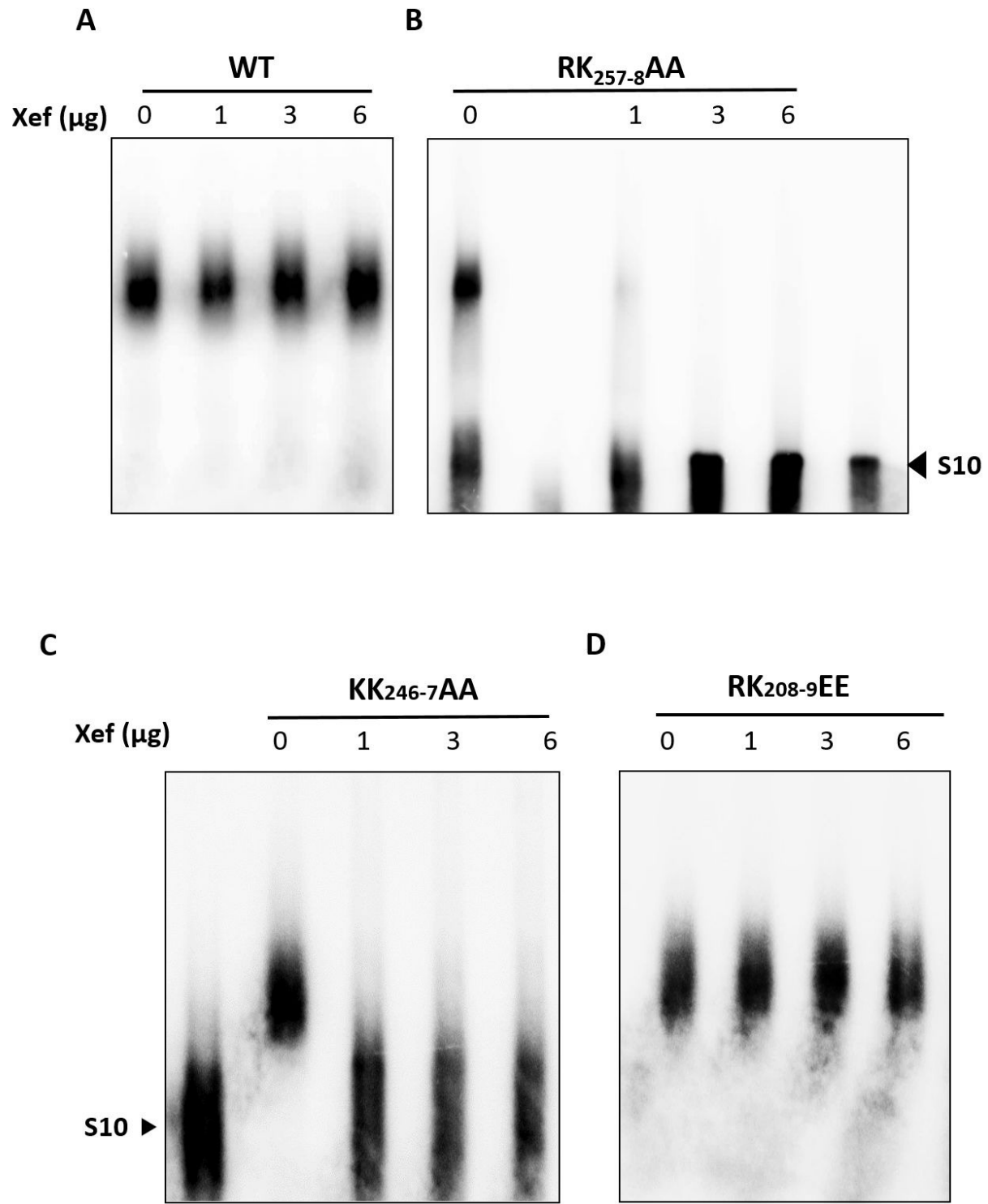


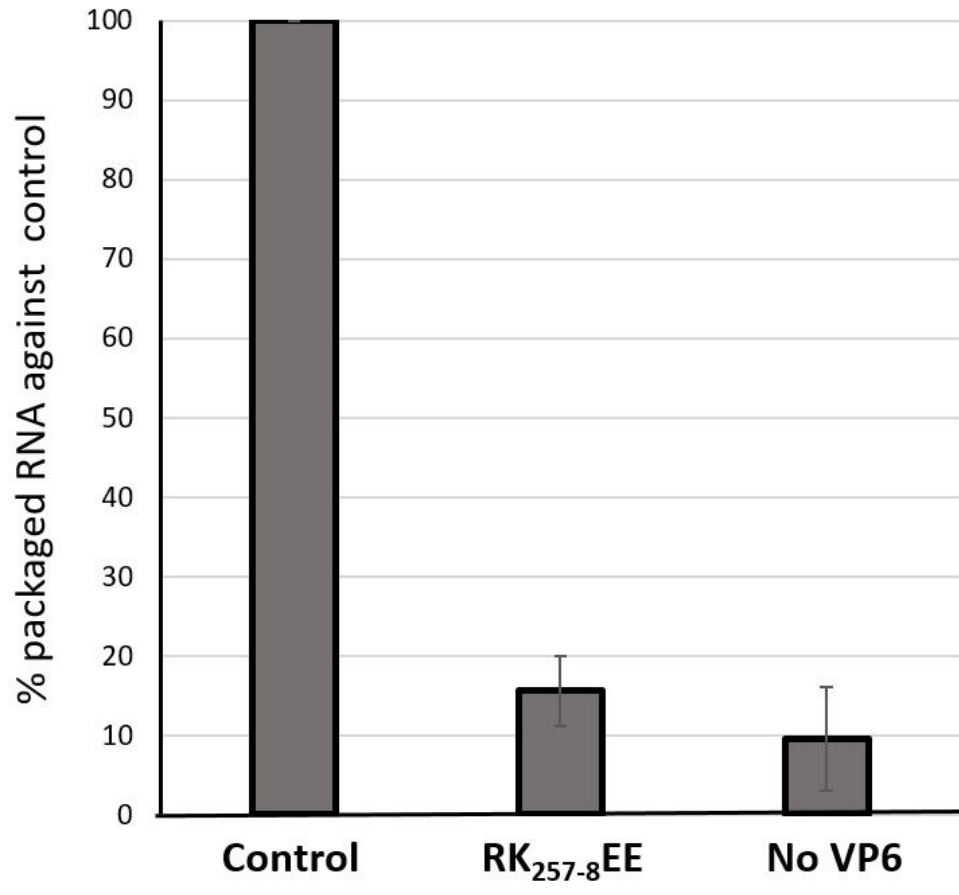




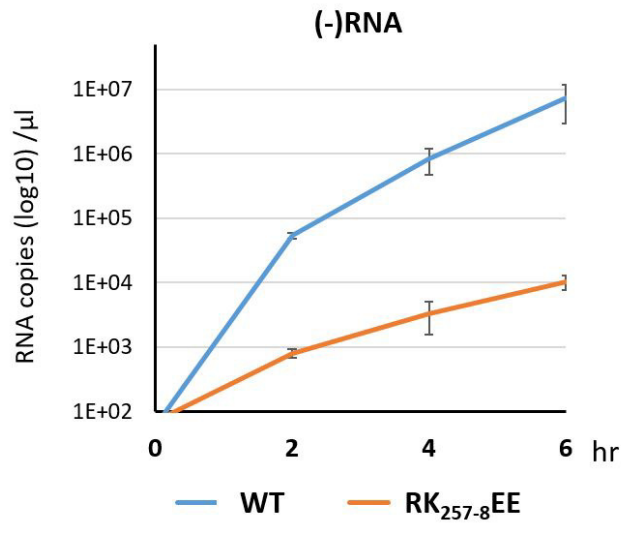
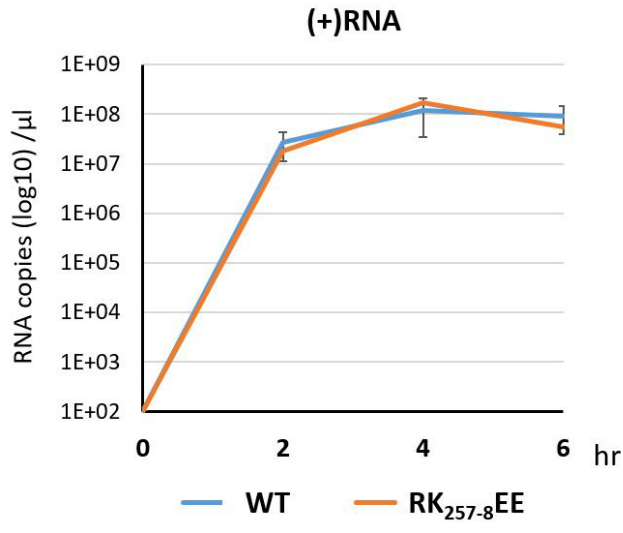
**A****B**











**Table 1. Characteristics of the VP6 peptides assigned to contact RNA.**

Sample	M/Z	Error (ppm)	Assign.		Peptide sequence	AA #	Modifications	Fold above no HCHO
			Confid. (%)					
reVP6/ssRNA	655.39	4.7	97.8 <sup>a</sup>		SAAILLAPGDVIK	2-14	Acetylation, N-term	>10*
reVP6/ssRNA	733.44	4.6	100		SAAILLAPGDVIKR	2-15	Acetylation, N-term	>10
reVP6/dsRNA	557.80	4.4	100		IHTAVGSGSGTK	83-94	None	>10
reVP6/ssRNA & reVP6/dsRNA	851.90	5.3	100		VGGGGGDADAGVGATGTNGGR	111-131	None	>10, 5.6
reVP6/ssRNA	608.34	0.5	100		WVVLTEEIAR	132-141	None	>10
Core/dsRNA	682.03	0.4	100		IDVYRDEVPAQIIEVER	151-167	None	>10
Core/dsRNA	789.68	2.4	100		IDVYRDEVPAQIIEVERSLQKELGISR	151-177	None	>10
Core/dsRNA	699.37	0.4	100		DEVPAQIIEVERSLQKELGISR	156-177	None	>10
reVP6/ssRNA & dsRNA	795.37	-3.6	100		EGVEEEKTSEEPAR	221-234	None	>10
reVP6/ssRNA & dsRNA	646.35	3.5	100		IGITIEGVMSQK	235-246	Oxidation of M9	>10
reVP6/ssRNA & dsRNA	545.80	1.6	100		GVMSQKLLSMIGGVERKMA	241-260	Oxidation of M11	>10
reVP6/ssRNA, dsRNA, & Core	609.85	1.7	100		KLLSMIGGVER	247-257	Oxidation of M5	>10
reVP6/ssRNA & dsRNA	545.80	2.2	100		LLSMIGGVER	248-257	Oxidation of M4	5.2, 5.2
reVP6/ssRNA & dsRNA	647.34	1.3	100		ESAVMLVNSIK	266-277	Oxidation of M5	>10
reVP6/ssRNA & dsRNA	588.32	1.5	100		ESAVMLVNSIKDVVR	266-281	Oxidation of M5	>10
reVP6/ssRNA & dsRNA	554.94	-0.1	100		ATAYFTAPTGDPHWK	282-296	None	>10
Core/dsRNA	706.69	-1.6	83.9		ATAYFTAPTGDPHWKEVAR	282-300	None	>10
Core/dsRNA	669.85	0.1	100		NILAYTSTGGDVK	307-319	None	>10
Core/dsRNA	784.89	-0.8	100		NILAYTSTGGDVKTE	307-321	None	>10
Core/dsRNA	853.12	1.1	100		NILAYTSTGGDVKTEFLHLIDHL	307-329	None	>10
reVP6/ssRNA	473.94	4.5	100		IGITIEGVMSQKK	235-247	None	>10
reVP6/ssRNA	609.85	-2.6	82.9		LLSMIGGVERK	248-258	None	>10
reVP6/ssRNA	558.82	2.9	93.5		VSNSIKDVVR	272-281	None	>10

<sup>a</sup>Confidence of peptide assignment from collision-induced peptide fragmentation.

\*The area of the peptide peak relative to the area in the reaction performed without formaldehyde.

**Table 2. Conformational analysis of mutant reVP6.**

VP6	Helix /loop (%) ( $\alpha$ , $\pi$ , 3,10 helix, loop)	Beta strands (%)	Turn (%)	RMSD
WT	48.0	36.6	15.5	0.0467
R <sub>167</sub> E	47.7	37.0	15.3	0.0499
R <sub>177</sub> E	47.3	37.1	15.5	0.0506
RK <sub>208</sub> <sup>9</sup> EE	47.4	37.2	15.5	0.0493
RK <sub>257</sub> <sup>8</sup> EE	47.8	36.8	15.4	0.0478
KRR <sub>303</sub> <sup>5</sup> EEE	47.3	37.3	15.4	0.0486