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Co-evolution in a putative bundling signal of bluetongue and epizootic hemorrhagic disease viruses

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Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) possess a genome of 10 segmented RNAs (S1–S10), one copy of each of which is considered to be packaged in a virion. This selective packaging is thought to be mediated by supramolecular complex formation of the 10 RNAs, through intermolecular base pairing of complementary nucleotide sequences termed the bundling signal. Here, the whole genomic sequences of BTV and EHDV isolates were analyzed to identify co-evolving pairs of complementary nucleotide sequences within and between genomic segments. One co-evolving pair was identified within S5 and another between S5 and S10. The co-evolving pair between S5 and S10, consisting of six bases in each segment, was a candidate for a bundling signal and was identical to one of two putative bundling signals reported in a previous experimental study, validating the effectiveness of the method used in the present study. The six bases in S10 were confirmed to be located in a loop at the end of a stable stem. Although the six bases in S5 were located in a loop at the end of a stem of only four bases long, the complementary nucleotide sequences constituting this stem were, remarkably, the co-evolving pair within S5. These results highlight the importance not only of loops but also of stems in the intermolecular base pairing of bundling signals.

Key words: bluetongue virus, bundling signal, co-evolution, complementarity, epizootic hemorrhagic disease virus, stem-loop

Among the viruses that infect humans and livestock, those belonging to the families *Orthomyxoviridae* and *Reoviridae* possess RNA genomes that are highly divided into 6–8 and 9–12 segments, respectively (Carstens, 2012; Condit, 2013). Bluetongue virus (BTV) is the type species of the genus *Orbivirus* in *Reoviridae*, and epizootic hemorrhagic disease virus (EHDV) is the species most closely related to BTV in *Orbivirus*, in which species are defined as a unit of reassortment (Belaganahalli et al., 2014). The genomes of BTV and EHDV consist of 10 double-stranded RNA (dsRNA) segments designated S1–S10, each of which encodes one or two proteins (Attoui et al., 2012; Roy, 2013). These viruses are considered to undergo selective packaging, in which one copy each of the 10 genomic segments is packaged in a virion (Lourenco and Roy, 2011; Sung and Roy, 2014; Boyce et al., 2016). The selective packaging is thought to be mediated by supramolecular complex formation of 10 positive-sense RNAs (+RNAs) (Sung and Roy, 2014; Fajardo et al., 2015), which are encapsidated in the core prior to the synthesis of negative-sense RNAs (−RNAs) to generate dsRNAs (Lourenco and Roy, 2011; Sung and Roy, 2014). The supramolecular complex of +RNAs is thought to be formed by intermolecular base pairing of complementary nucleotide sequences termed the bundling signal (Goto et al., 2013). Intramolecular secondary structures within +RNAs, particularly stem-loop structures, are thought to facilitate intermolecular base pairing (Burkhardt et al., 2014; Sung and Roy, 2014; Boyce and McCrae, 2015).

It has been reported that a pair of nucleotide sequences consisting of 6–11 bases located in the 5' end of the open reading frame (ORF) of S1 and in the 3' untranslated region (UTR) of S7, and also a pair of nucleotide sequences consisting of 6–8 bases located in the 5' UTR of S5 and in the 3' UTR of S10, were both complementary in each of 15 *Orbivirus* species, although the length and the absolute sequence of these complementary nucleotide sites varied among the species (Boyce et al., 2016). By predicting the intramolecular secondary structures for +RNAs of these segments in each species, the sites identified in S7 and S10 were inferred to be located in loops at the end of stable stems, whereas those identified in S1 and S5 were inferred to be located in regions of low base pairing (Boyce and McCrae, 2015; Boyce et al., 2016). In addition, the experi-

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mental studies using BTV demonstrated that the complementarities between S1 and S7 and between S5 and S10 were critical not only for interaction of +RNAs *in vitro* but also for production of infectious progeny virions *in vivo* (Boyce and McCrae, 2015; Boyce et al., 2016). These observations suggest that the two pairs of complementary nucleotide sequences are putative bundling signals of *Orbivirus*.

Assuming that a bundling signal comprises contiguous complementary nucleotide sequences between different segments and that the genomic location of the bundling signal has been maintained during evolution, complementary nucleotide sequences constituting a bundling signal may have been allowed to evolve only simultaneously through compensatory nucleotide substitutions at complementary nucleotide positions (Kimura, 1983, 1985). Such a co-evolution in bundling signals may suppress reassortment of wild-type and mutant genomic segments, which may eventually promote speciation (Hutchinson et al., 2010; McDonald and Patton, 2011). In *Orbivirus*, reassortment takes place within species but not between species, suggesting that bundling signals may be represented as pairs of complementary nucleotide sequences that are conserved within species but are variable between species. Therefore, such a co-evolving pair of complementary nucleotide sequences located on different genomic segments may be a candidate for a bundling signal. The purpose of the present study was to detect co-evolving pairs of complementary nucleotide sequences in BTV and EHDV.

As of March 12, 2016, the whole genomic +RNA sequences without ambiguous nucleotides in any of the 10 segments were available for 103 BTV and 15 EHDV isolates in the International Nucleotide Sequence Database (INSD) (Supplementary Table S1). Multiple alignment of these 118 nucleotide sequences was made for each genomic segment using the computer program MAFFT (Katoh et al., 2002). The 10 multiple alignments thus obtained were analyzed to identify every pair of single-nucleotide sites that were complementary in each of the BTV and EHDV isolates within and between genomic segments (Suzuki, 2014, 2015). Sites containing gaps were eliminated from the analysis. Only the Watson-Crick base pairs of U and A, and C and G were regarded as complementary, even though U and G may also form a wobble base pair, to reduce false positives in identifying functionally relevant pairs of complementary nucleotide sequences (Boyce et al., 2016). A pair of complementary single-nucleotide sites was considered to have co-evolved between BTV and EHDV when nucleotides at both sites in BTV isolates were all different from those in EHDV isolates. The proportion of co-evolving pairs among all pairs of complementary single-nucleotide sites was designated $p_{\text{co-evolving}}$.

Pairs of complementary nucleotide sequences consisting of two or more contiguous bases were also identified within and between genomic segments. Note that a pair of complementary nucleotide sequences of length X comprised X pairs of complementary single-nucleotide sites, each of which was expected to be a co-evolving pair of complementary single-nucleotide sites with probability $p_{\text{co-evolving}}$ assuming a random distribution for the co-evolving pairs of complementary single-nucleotide sites among all pairs of complementary single-nucleotide sites. For each pair of complementary nucleotide sequences of length X, a statistical test was conducted to examine whether the number of co-evolving pairs of complementary single-nucleotide sites (Y) was significantly greater than expected under the null hypothesis of random distribution as described above. Specifically, the binomial probability for the occurrence of Y or more events in X trials was computed with the parameter value of $p_{\text{co-evolving}}$ and the Bonferroni correction for multiple testing was conducted with the total number of pairs of complementary nucleotide sequences of length X and the family-wise significance level (P) of 0.05 (Sokal and Rohlf, 1995). Pairs of complementary nucleotide sequences with significantly large values of Y were named co-evolving pairs of complementary nucleotide sequences, which were considered to be under the functional constraint of maintaining the complementarity and thus to interact with each other (Kimura, 1983, 1985). The co-evolving pairs of complementary nucleotide sequences within and between genomic segments may contribute to the formation of intramolecular secondary structure and intermolecular interaction of +RNAs, respectively, and the latter were candidates for bundling signals.

The length of the pairs of complementary nucleotide sequences identified within and between genomic segments of 103 BTV and 15 EHDV isolates analyzed in the present study (Supplementary Table S1) ranged from one to six (Table 1). Note that pairs of complementary nucleotide sequences listed in Table 1 may overlap; such overlapping pairs may be found in the same column in the same row, in different columns in the same row, in different rows in the same column, and in different columns in different rows, because a pair of length n contained $(n - i + 1)$ pairs of length i $(1 \leq i \leq n)$. The $p_{\text{co-evolving}}$ value was obtained as 0.00737 (= 47,753/6,482,278), and six pairs of complementary nucleotide sequences consisting of four to six bases were judged by the statistical test as co-evolving pairs of complementary nucleotide sequences, which contained significantly large numbers of co-evolving pairs of complementary single-nucleotide sites (Table 1). The six co-evolving pairs of complementary nucleotide sequences are presented in Table 2, in which the positions are numbered according to the prototype isolates South Africa for BTV (Boyce et al., 2008) and USA1955/01 for EHDV (Anthony et al., 2009a, 2009b, 2009c). After eliminating overlaps, two co-evolving pairs remained: one co-evolving

pair comprised complementary nucleotide sequences of four bases long within S5, whereas the other comprised complementary nucleotide sequences of six bases long between S5 and S10, which was a candidate for a bundling signal (Table 2).

The co-evolving pair of complementary nucleotide sequences between S5 and S10 identified in the present study corresponded to one of two putative bundling signals reported in the previous experimental study mentioned above (Boyce et al., 2016). This observation validates the effectiveness of the strategy of identifying co-evolving pairs of complementary nucleotide sequences as candidates for bundling signals in viruses with segmented genomes (Suzuki, 2014, 2015). On the other hand, however, a second putative bundling signal between S1 and S7 reported by Boyce et al. (2016) was not identified in the present study. In fact, this putative bundling signal was identified as a pair of complementary nucleotide sequences consisting of six bases in Table 1. However, this pair was not identified as a candidate for a bundling signal as defined in the present study because the complementary nucleotide sequences were invariable not only within BTV and EHDV but also between these species, without any signature of co-evolution.

Table 1. Numbers of complementary nucleotide sequences of length X containing a particular number of co-evolving pairs of complementary single-nucleotide sites (Y) in the genomes of BTV and EHDV

X[a]	Y[b]			Total
	2	1	0	
6	**1**[c]	0	1[e]	2
5	**2**	0	6	8
4	**3**	5	96	104
3	3	17	1,218	1,238
2	122	1,040	285,352	286,514
1	N.A.[d]	47,753	6,434,525	6,482,278

[a] No complementary nucleotide sequences of length greater than six were identified. For each value of X, the values of Y judged as significantly greater than expected by chance are bold-faced.
[b] No complementary nucleotide sequences containing three or more co-evolving pairs of complementary single-nucleotide sites were observed.
[c] Candidate bundling signal between S5 and S10 identified in the present study, corresponding to one of two putative bundling signals reported by Boyce et al. (2016).
[d] Not applicable.
[e] Putative bundling signal between S1 and S7 reported by Boyce et al. (2016).

In Boyce et al. (2016), structural features of the putative bundling signal between S5 and S10 consisting of six bases were investigated by predicting the intramolecular secondary structures of S5 and S10 +RNAs for BTV and EHDV. The six bases in S10 +RNA were inferred to be located in a loop at the end of a stable stem in both BTV and EHDV (Boyce et al., 2016). Indeed, when the secondary structure of S10 +RNA was predicted for each of the 103 BTV and 15 EHDV isolates in the present study using mfold (Zuker, 2003), the local structure similar to that reported in Boyce et al. (2016) was observed in all isolates (Fig. 1). By contrast, in Boyce et al. (2016), the six bases in S5 +RNA were inferred to be located in a region of low base pairing in both BTV and EHDV. In fact, in Boyce et al. (2016), these bases appeared to form a loop at the end of a stem in the predicted secondary structure of S5 +RNA. However, the stem was only four bases long and thus was not considered to be biologically relevant (Boyce et al., 2016). When the secondary structure of S5 +RNA was predicted for the 103 BTV and 15 EHDV isolates in the present study, a similar local structure to that reported by Boyce et al. (2016) was observed in all isolates except for one (Supplementary Table S1). Importantly, however, the pair of complementary nucleotide

Table 2. Co-evolving pairs of complementary nucleotide sequences in the genomes of BTV and EHDV

X[a]	Y[b]	Genomic segment	Positions in BTV[c]	Sequence in BTV[d]	Positions in EHDV[e]	Sequence in EHDV[d]	Genomic segment	Positions in BTV[c]	Sequence in BTV[d]	Positions in EHDV[e]	Sequence in EHDV[d]
6[f]	*2*	*S5*	*23–28*	*5′C<u>AA</u>CCA3′*	*22–27*	*5′C<u>UG</u>CCA3′*	*S10*	*754–759*	*5′UGG<u>UUG</u>3′*	*740–745*	*5′UGG<u>CAG</u>3′*
5	2	S5	24–28	5′<u>AA</u>CCA3′	23–27	5′<u>UG</u>CCA3′	S10	754–758	5′UGG<u>UU</u>3′	740–744	5′UGG<u>CA</u>3′
5	2	S5	23–27	5′C<u>AA</u>CC3′	22–26	5′C<u>UG</u>CC3′	S10	755–759	5′GG<u>UUG</u>3′	741–745	5′GG<u>CAG</u>3′
4	2	S5	24–27	5′<u>AA</u>CC3′	23–26	5′<u>UG</u>CC3′	S10	755–758	5′GG<u>UU</u>3′	741–744	5′GG<u>CA</u>3′
4	2	S5	23–26	5′C<u>AA</u>C3′	22–25	5′C<u>UG</u>C3′	S10	756–759	5′G<u>UUG</u>3′	742–745	5′G<u>CAG</u>3′
4[f]	*2*	*S5*	*19–22*	*5′<u>UUG</u>G3′*	*18–21*	*5′<u>UCGA</u>3′*	*S5*	*29–32*	*5′<u>CCAA</u>3′*	*28–31*	*5′<u>UCGA</u>3′*

[a] Length of co-evolving pairs of complementary nucleotide sequences.
[b] Number of co-evolving pairs of complementary single-nucleotide sites.
[c] Positions are numbered according to the prototype isolate South Africa for BTV (Boyce et al., 2008).
[d] Co-evolving pairs of complementary single-nucleotide sites are underlined.
[e] Positions are numbered according to the prototype isolate USA1955/01 for EHDV (Anthony et al., 2009a, 2009b, 2009c).
[f] Co-evolving pairs of complementary nucleotide sequences that remained after eliminating overlaps are italicized.

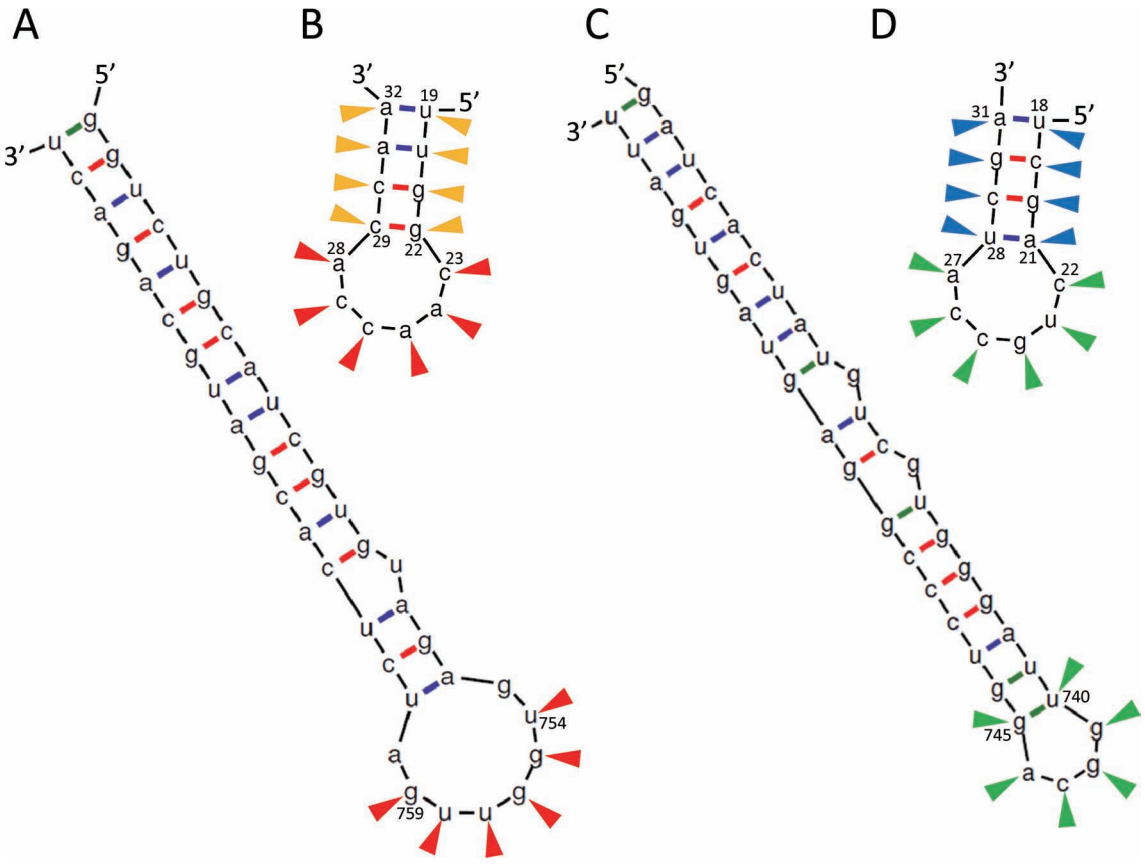

Fig. 1. Local secondary structures of (A) S10 and (B) S5 +RNAs of the prototype isolate South Africa for BTV (Boyce et al., 2008) and (C) S10 and (D) S5 +RNAs of the prototype isolate USA1955/01 for EHDV (Anthony et al., 2009a, 2009b, 2009c). Intramolecular secondary structures were predicted for the entire +RNA sequence of each genomic segment using mfold (Zuker, 2003). Each co-evolving pair of complementary nucleotide sequences, whose ends are labeled with position numbers, is indicated with arrowheads in a distinct color. The base pairings between U and A, C and G, and G and U are indicated with blue, red, and green bars, respectively.

sequences constituting the stem turned out to be the co-evolving pair within S5 identified above (Table 2). Considering the effectiveness of inferring that co-evolving pairs of complementary nucleotide sequences are involved in biologically relevant interactions, as observed above, it is feasible that the stem has been functionally constrained during the evolution of BTV and EHDV. These results indicate that the intermolecular base pairing by the putative bundling signal between S5 and S10 may be facilitated by stem-loop structures within +RNAs of both genomic segments (Burkhardt et al., 2014; Sung and Roy, 2014; Boyce and McCrae, 2015). It is plausible that the loop exposes the complementary nucleotide sequence to make it available for intermolecular interaction, whereas the stem delineates the loop to ensure the specificity of intermolecular interaction.

In the present study, one co-evolving pair of complementary nucleotide sequences was identified as a candidate for a bundling signal in BTV and EHDV. The number of putative bundling signals so far identified in BTV and EHDV is two (Boyce et al., 2016), which is smaller than the minimum number (nine) required for bundling 10 genomic segments. Influenza A virus (IAV) with eight genomic segments (Noda et al., 2006; Chou et al., 2012; Fournier et al., 2012; Giese et al., 2016) and rotavirus A (RVA) with 11 genomic segments (Hundley et al., 1985; Patton, 1990; Joklik and Roner, 1995) are also considered to undergo selective packaging. However, the numbers of putative bundling signals so far identified for IAV and RVA are one (Gavazzi et al., 2013; Gerber et al., 2014) and two (Suzuki, 2014, 2015), respectively, which are again smaller than the minimum numbers (seven and 10, respectively) required for bundling all genomic segments. It is puzzling why only small numbers of bundling signals have been identified for these viruses. In the search for the molecular entity of bundling signals, it has usually been assumed that the bundling signals comprise contiguous complementary nucleotide sequences between different segments and that their genomic locations have not changed during evolution (Gavazzi et al., 2013; Gerber et al., 2014; Suzuki, 2014, 2015; Boyce et al., 2016). Therefore, bundling signals may be overlooked if these assumptions are violated (Gavazzi et al., 2013; Gerber et al., 2014). In particular, it has been reported

for IAV that at least one genomic segment is missing in most virions, which were named semi-infectious (SI) particles (Brooke et al., 2013). The high prevalence of SI particles suggests that IAV is normally transmitted at high multiplicity of infection (MOI), with complementation taking place commonly (Brooke, 2014), and that bundling signals have not been established perfectly to ensure bundling of all genomic segments. It is therefore possible that not only selective packaging but also random packaging (Noda and Kawaoka, 2012) contributes to the packaging process of genomic segments (the semi-selective packaging model), in which the number of bundling signals can be smaller than the minimum number required for bundling all genomic segments. Further studies are required to understand the mechanism of packaging in viruses with segmented genomes.

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DISCLOSURE

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REFERENCES

Anthony, S. J., Maan, S., Maan, N., Kgosana, L., Bachanek-Bankowska, K., Batten, C., Darpel, K. E., Sutton, G., Attoui, H., and Mertens, P. P. (2009a) Genetic and phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic haemorrhagic disease virus (EHDV): comparison of genetic and serological data to characterise the EHDV serogroup. Virus Res. **145**, 200–210.

Anthony, S. J., Maan, N., Maan, S., Sutton, G., Attoui, H., and Mertens, P. P. (2009b) Genetic and phylogenetic analysis of the core proteins VP1, VP3, VP4, VP6 and VP7 of epizootic haemorrhagic disease virus (EHDV). Virus Res. **145**, 187–199.

Anthony, S. J., Maan, N., Maan, S., Sutton, G., Attoui, H., and Mertens, P. P. (2009c) Genetic and phylogenetic analysis of the non-structural proteins NS1, NS2 and NS3 of epizootic haemorrhagic disease virus (EHDV). Virus Res. **145**, 211–219.

Attoui, H., Mertens, P. P. C., Becnel, J., Belaganahalli, S., Bergoin, M., Brussaard, C. P., Chappell, J. D., Ciarlet, M., del Vas, M., Dermody, T. S., et al. (2012) Family Reoviridae. In: Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, (eds.: King, A. M. Q., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J.), pp. 541–637. Elsevier Academic Press, Amsterdam.

Belaganahalli, M. N., Maan, S., Maan, N. S., Pritchard, I., Kirkland, P. D., Brownlie, J., Attoui, H., and Mertens, P. P. C. (2014) Full genome characterization of the *Culicoides*-borne marsupial orbiviruses: Wallal virus, Mudjinbarry virus and Warrego viruses. PLoS ONE **9**, e108379.

Boyce, M., and McCrae, M. A. (2015) Rapid mapping of functional *cis*-acting RNA elements by recovery of virus from a degenerate RNA population: application to genome segment 10 of bluetongue virus. J. Gen. Virol. **96**, 3072–3082.

Boyce, M., Celma, C. C., and Roy, P. (2008) Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. J. Virol. **82**, 8339–8348.

Boyce, M., McCrae, M. A., Boyce, P., and Kim, J. T. (2016) Intersegment complementarity in orbiviruses: a driver for coordinated genome packaging in the *Reoviridae*? J. Gen. Virol. **97**, 1145–1157.

Brooke, C. B. (2014) Biological activities of 'noninfectious' influenza A virus particles. Future Virol. **9**, 41–51.

Brooke, C. B., Ince, W. L., Wrammert, J., Ahmed, R., Wilson, P. C., Bennink, J. R., and Yewdell, J. W. (2013) Most influenza A virions fail to express at least one essential viral protein. J. Virol. **87**, 3155–3162.

Burkhardt, C., Sung, P.-Y., Celma, C. C., and Roy, P. (2014) Structural constraints in the packaging of bluetongue virus genomic segments. J. Gen. Virol. **95**, 2240–2250.

Carstens, E. B. (2012) Introduction to virus taxonomy. In: Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, (eds.: King, A. M. Q., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J.), pp. 3–20. Elsevier Academic Press, Amsterdam.

Chou, Y.-y., Vafabakhsh, R., Doganay, S., Gao, Q., Ha, T., and Palese, P. (2012) One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. Proc. Natl. Acad. Sci. USA **109**, 9101–9106.

Condit, R. C. (2013) Principles of virology. In: Fields Virology, Sixth Edition, (eds.: Knipe, D. M., Howley, P. M., Cohen, J. I., Griffin, D. E., Lamb, R. A., Martin, M. A., and Roizman, B.), pp. 21–51. Lippincott Williams & Wilkins, Philadelphia.

Fajardo, T. Jr., Sung, P.-Y., and Roy, P. (2015) Disruption of specific RNA-RNA interactions in a double-stranded RNA virus inhibits genome packaging and virus infectivity. PLoS Pathog. **11**, e1005321.

Fournier, E., Moules, V., Essere, B., Paillart, J.-C., Sirbat, J.-D., Isel, C., Cavalier, A., Rolland, J.-P., Thomas, D., Lina, B., and Marquet, R. (2012) A supramolecular assembly formed by influenza A virus genomic RNA segments. Nucleic Acids Res. **40**, 2197–2209.

Gavazzi, C., Yver, M., Isel, C., Smyth, R. P., Rosa-Calatrava, M., Lina, B., Moules, V., and Marquet, R. (2013) A functional sequence-specific interaction between influenza A virus genomic RNA segments. Proc. Natl. Acad. Sci. USA **110**, 16604–16609.

Gerber, M., Isel, C., Moules, V., and Marquet, R. (2014) Selective packaging of the influenza A genome and consequences for genetic reassortment. Trends Microbiol. **22**, 446–455.

Giese, S., Bolte, H., and Schwemmle, M. (2016) The feat of packaging eight unique genome segments. Viruses **8**, 165.

Goto, H., Muramoto, Y., Noda, T., and Kawaoka, Y. (2013) The genome-packaging signal of the influenza A virus genome comprises a genome incorporation signal and a genome-bundling signal. J. Virol. **87**, 11316–11322.

Hundley, F., Biryahwaho, B., Gow, M., and Desselberger, U. (1985) Genome rearrangements of bovine rotavirus after serial passage at high multiplicity of infection. Virology **143**, 88–103.

Hutchinson, E. C., von Kirchbach, J. C., Gog, J. R., and Digard, P. (2010) Genome packaging in influenza A virus. J. Gen. Virol. **91**, 313–328.

Joklik, W. K., and Roner, M. R. (1995) What reassorts when reovirus genome segments reassort? J. Biol. Chem. **270**, 4181–4184.

Katoh, K., Misawa, K., Kuma, K.-i., and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res.

30, 3059–3066.
Kimura, M. (1983) The Neutral Theory of Molecular Evolution. Cambridge University Press, Cambridge, New York, Melbourne.
Kimura, M. (1985) The role of compensatory neutral mutations in molecular evolution. J. Genet. **64**, 7–19.
Lourenco, S., and Roy, P. (2011) In vitro reconstitution of Bluetongue virus infectious cores. Proc. Natl. Acad. Sci. USA **108**, 13746–13751.
McDonald, S. M., and Patton, J. T. (2011) Assortment and packaging of the segmented rotavirus genome. Trends Microbiol. **19**, 136–144.
Noda, T., and Kawaoka, Y. (2012) Packaging of influenza virus genome: robustness of selection. Proc. Natl. Acad. Sci. USA **109**, 8797–8798.
Noda, T., Sagara, H., Yen, A., Takada, A., Kida, H., Cheng, R. H., and Kawaoka, Y. (2006) Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature **439**, 490–492.
Patton, J. T. (1990) Evidence for equimolar synthesis of double-stranded RNA and minus-strand RNA in rotavirus-infected cells. Virus Res. **17**, 199–208.
Roy, P. (2013) Orbiviruses. In: Fields Virology, Sixth Edition, (eds.: Knipe, D. M., Howley, P. M., Cohen, J. I., Griffin, D. E., Lamb, R. A., Martin, M. A., and Roizman, B.), pp. 1402–1423. Lippincott Williams & Wilkins, Philadelphia.
Sokal, R. R., and Rohlf, F. J. (1995) Biometry. Third Edition. W.H. Freeman and Company, New York.
Sung, P.-Y., and Roy, P. (2014) Sequential packaging of RNA genomic segments during the assembly of Bluetongue virus. Nucleic Acids Res. **42**, 13824–13838.
Suzuki, Y. (2014) A possible packaging signal in the rotavirus genome. Genes Genet. Syst. **89**, 81–86.
Suzuki, Y. (2015) A candidate packaging signal of human rotavirus differentiating Wa-like and DS-1-like genomic constellations. Microbiol. Immunol. **59**, 567–571.
Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**, 3406–3415.