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NOTE

A candidate packaging signal of human rotavirus differentiating Wa-like and DS-1-like genomic constellations

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ABSTRACT

Rotavirus A **(RVA) possesses a genome of 11 segmented RNAs. In human RVA, two major genomic constellations are represented by prototype strains Wa and DS-1. Here packaging signals differentiating Wa-like and DS-1-like genomic constellations were searched for by analyzing genomic sequences of Wa-like and DS-1-like strains. One pair of 11 nucleotide sites in the coding regions of viral structural protein (VP) 2 and VP6 was found to be complementary specifically among Wa-like strains. These sites tended to be free from base-pairing in secondary structures of genomic segments, suggesting that they may serve as a packaging signal in Wa-like strains.**

Key words complementarity, human rotavirus, packaging signal, secondary structure.

Rotavirus A, a species in genus *Rotavirus*, subfamily *Sedoreovirinae*, family *Reoviridae*, possesses a genome of 11 segmented dsRNA (1). Each genomic segment encodes either VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 or NSP5/NSP6 (2). According to the similarity in the nucleotide sequence, each genomic segment has been classified into genotypes. To date, 27 G, 37 P, 17 I, 9 R, 9 C, 8 M, 18 A, 10 N, 12 T, 15 E and 11 H genotypes have been recognized for the genomic segments encoding VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5/6, respectively. The genomic constellation of RVA strain is annotated as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x denotes a corresponding genotype number (3, 4).

The RVA virion is a non-enveloped, icosahedral, triple-layered particle consisting of a core shell, inner capsid and outer capsid, which are composed of VP2, VP6 and VP4/VP7, respectively (5). Genomic segments are assembled and packaged into the core shell as 5′ capped, 3′ non-polyadenylated, +RNAs, which are subsequently converted to dsRNAs (6). Because of a low particle-to-plaque forming unit ratio and equimolar synthesis of 11 dsRNAs and 11 –RNAs in infected cells, it is believed that one copy of each genomic segment is packaged in an RVA virion (7–9). However, the molecular mechanisms underlying the selective packaging of genomic segments for RVA are not well understood.

In RVA, +RNA of each genomic segment appears to form secondary structures, including a panhandle structure, via 5′ and 3′ terminal long range interaction (10–12). Packaging signals are thought to be located at the 5′ and 3′ termini of each genomic segment and to extend from the untranslated regions to ends of open reading frames, where nucleotide sequences are segment specific and highly conserved (6, 12). In this regard, it is

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List of Abbreviations: dsRNA, double-stranded RNA; NSP, nonstructural protein; p_{Wa}, proportion of Wa-differentiating sites among single Wa-complementary sites; –RNA, negative-strand RNA; +RNA, positive-strand RNA; RVA, *Rotavirus A*; VP, viral structural protein; Wa-complementary site, pair of nucleotide sites complementary in Wa-like strains; Wa-differentiating site, single Wa-complementary site lacking complementarity between Wa-like and DS-1-like strains.

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interesting to note that partial complementarity has been observed between 5′ and 3′ terminal ≈50 nucleotides of +RNAs for NSP2 and NSP3 segments in bovine RVA strain UK (RVA/Bovine-tc/USA/UK/1984/G6P[5]), although its involvement in packaging is unknown (12).

Where reassortment is known to occur within but not between different groups of RVA strains, analyzing genomic sequences may be useful in searching for packaging signals of RVA. Packaging signals may be located at the pairs of nucleotide sites between +RNAs of different genomic segments that are complementary within each group, but not between different groups. Mammalian and avian RVAs appear to have evolved independently without reassortment (13, 14); however, reassortment reportedly occurs within mammalian or avian RVA (15, 16). An analysis of genomic sequences for mammalian and avian RVAs revealed a pair of co-evolving complementary nucleotide sites of length six between the NSP2 and NSP3 segments (17). These sites constitute loops in the secondary structures predicted for +RNAs of NSP2 and NSP3 segments in avian and some mammalian RVA strains and were inferred to be a possible packaging signal.

It has been well documented that there are two major genomic constellations in human RVA; namely, G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 represented by prototype strain Wa (RVA/Human-tc/USA/Wa/1974/G1P[8]) and G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2 represented by prototype strain DS-1 (RVA/Human-tc/USA/DS-1/1976/G2P[4]) (18, 19). Although reassortment within Wa-like or DS-1-like strains has been reported to occur, reassortants between these strains have not become prevalent (20–22), suggesting that packaging signals may be compatible within Wa-like or DS-1-like strains but not between these strains. The purpose of the present study was to search for packaging signals of human RVA differentiating Wa-like and DS-1-like genomic constellations by analyzing genomic sequences for Wa-like and DS-1-like strains.

As of December 26, 2014, the complete genomic sequence, ranging from 5′ terminal 5′-GGC-3′ to 3′ terminal 5′-ACC-3′ without ambiguous nucleotides for 11 +RNAs was available for five Wa-like and seven DS-1-like strains in the International Nucleotide Sequence Database (Supplementary Table S1). Multiple alignment of nucleotide sequences was made for each genomic segment of 12 strains using the computer program MAFFT (23). It has been postulated for *Influenza A virus*, a species in genus *Influenzavirus A*, family *Orthomyxoviridae*, which possesses a genome of eight-segmented –RNAs and shares packaging-related characteristics with RVA (6, 17, 24), that positions of packaging signals may change during evolution (25). In the present study, packaging signals specific to Wa-like and DS-1-like strains were searched for by identifying pairs of nucleotide sites between +RNAs of different genomic segments that were complementary within Wa-like or DS-1-like strains but not between these strains.

To search for packaging signals specific to Wa-like strains, pairs of single nucleotide sites ("columns" in multiple alignments) that were complementary in all Wa-like strains (Wa-complementary sites) were identified within and between genomic segments using the multiple alignments made above. Nucleotide sites containing gaps were eliminated from the analysis. Not only the Watson–Crick base pairs of U and A and C and G, but also a wobble base pair of U and G, was regarded as complementary. For each pair of single Wa-complementary sites, complementarity was further examined between Wa-like and DS-1-like strains; if no complementarity was observed, these sites were considered to differentiate Wa-like strains from DS-1-like strains (Wa-differentiating sites). The proportion of Wa-differentiating sites among single Wa-complementary sites was designated as p_{Wa} (= [number of Wa-differentiating sites]/[number of single Wa-complementary sites]). Wa-complementary sites longer than one were also identified within and between genomic segments. For each length of Wa-complementary sites, the probability of containing a particular number or more of Wa-differentiating sites under the null hypothesis of random distribution for Wa-differentiating sites among Wa-complementary sites was computed using the binomial distribution, the probability of occurrence of a Wa-differentiating site being given by p_{Wa}. Wa-complementary sites containing a significantly large number of Wa-differentiating sites were identified by conducting the Bonferroni correction for multiple testing using the observed number of Wa-complementary sites and the family-wise significance level of $P < 0.05$ (26). Similar analysis as described above, but exchanging Wa and DS-1, was also conducted to search for packaging signals specific to DS-1-like strains.

In an analysis of five Wa-like and seven DS-1-like strains (Supplementary Table S1), the length of Wa-complementary sites ranged from one to 17 (Table 1). The p_{Wa} value was found to be 0.00533 (= 309,846/58,134,512). One pair of Wa-complementary sites of length 11 was judged as containing a significantly large number (three) of Wa-differentiating sites (Table 1). These Wa-complementary sites were located at positions 2068–2078 in the VP2 segment and at positions 471–481 in the VP6 segment (nucleotide and amino acid positions throughout the text refer to those for Wa) (Table 2) (27, 28). Consensus sequences at these positions in the VP2 and VP6 segments were, 5′-

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Table 1. Number of Wa-complementary sites of length (*X*) containing a given number of Wa-differentiating sites (*Y*)

	Y‡				
X†	3	2	1	0	Total
17	0§	0	0	2	2
16	0	0	0	10	10
15	0	0	2	26	28
14	0	0	7	56	63
13	0	0	15	154	169
12	0	1	27	434	462
11	1	2	75	1245	1323
10	3	6	206	3576	3791
9	4	17	531	10548	11100
8	6	42	1387	30845	32280
7	12	107	3482	91310	94911
6	17	266	8575	272785	281643
5	22	559	20700	801660	822941
4	28	945	49093	2346452	2396518
3	17	1261	112041	6832121	6945440
2	NA	1249	222389	19861168	20084806
1	NA	NA	309846	57824666	58134512

†, no Wa-complementary sites of length 18 or more were observed; ‡,no Wa-complementary sites containing four or more Wa-differentiating sites were observed; §, significantly large numbers of Wa-differentiating sites to be included in each length of Wa-complementary sites are colored gray. NA, not applicable.

UGAAAGACGRA-3′ and 5′-UUUGUUUUUCA-3′ for Wa-like strains, respectively, and 5′-AGAAAUYAGAA-3′ and 5′-UUYACAUUYCA-3′ for DS-1-like strains, respectively (Table 2). Multiple alignments of VP2 and VP6 segments appeared to be reliable at these positions, because no gap was observed at upstream 1014 sites and downstream 639 sites of positions 2068–2078 in the VP2 segment (Supplementary Fig. S1), and at upstream 470 sites and downstream 875 sites of positions 471–481 in the VP6 segment (Supplementary Fig. S2). In particular, multiple alignment of VP6 segment was gap-free over the entire region of 1356 sites. No pair of DS-1-complementary sites was judged as containing a significantly large number of DS-1-differentiating sites.

Potential for interaction between the Wa-complementary sites identified above was examined by analyzing secondary structures of +RNAs for VP2 and VP6 segments. Complementary sites in different genomic segments may be required to be free from base-pairing within each genomic segment to interact with each other. Secondary structures of +RNAs for VP2 and VP6 segments were predicted for each of five Wa-like and seven DS-1-like strains using RNAFOLD (29). In Wa, 10 sites of positions 2068–2078 in the VP2 segment and seven sites of positions 471–481 in the VP6 segment were observed to be free from base-pairing (Supplementary Fig. S3). To investigate whether these positions contained a significantly large number of non-base-pairing sites, sliding window analysis with window size 11 and step size one was conducted to obtain an empirical probability distribution for the number of non-base-pairing sites included in 11 nucleotide sites of VP2 and VP6 segments for Wa. It was observed that the empirical probability that 10 or more sites were free from base-pairing in the VP2 segment was 0.0635 and that seven or more sites were free from base-pairing in the VP6 segment was 0.168. However, the joint probability that, in a pair of 11 nucleotide sites in the VP2 and VP6 segments, one contained 10 or more and the other contained seven or more non-base-pairing sites was 0.0154. Among five Wa-like and seven DS-1-like strains,

Table 2. Wa-complementary sites containing a significantly large number of Wa-differentiating sites

	VP2		VP6		
Strain name†	Positions	Sequence‡	Positions	Sequence‡	Joint probability§
RVA/Human-tc/USA/Wa/1974/G1P[8]	2068–2078	5′–UGAAAGACGAA–3′	471–481	5′–UUUGUUUUUCA–3′	*0.0154*
RVA/Human-xx/Japan/KU/1995/G1P[8]	2074–2084	5′–UGAAAGACGAA–3′	471–481	5′–UUUGUUUUUCA–3′	0.811
RVA/Human-wt/BGD/Dhaka16/2003/G1P[8]	2080–2090	5′–UGAAAGACGAA–3′	471–481	5′–UUUGUUUUUCA–3′	0.352
RVA/Human-tc/BGD/MMC71/2005/G1P[8]	2083–2093	5′–UGAAAGACGGA–3′	471–481	5′–UUUGUUUUUCA–3′	0.496
RVA/Human-tc/IND/AM06-I/2006/G1P[8]	2080–2090	5′–UGAAAGACGAA–3′	471–481	5′–UUUGUUUUUCA–3′	0.135
RVA/Human-tc/USA/DS-1/1976/G2P[4]	2035–2045	5′–AGAAAUCAGAA–3′	471–481	5′–UUUACAUUUCA–3′	*0.0480*
RVA/Human-xx/KEN/D205/1989/G2P[4]	2038–2048	5′–AGAAAUCAGAA–3′	471–481	5′–UUCACAUUCCA–3′	0.894
RVA/Human-wt/CHN/TB-Chen/1996/G2P[4]	2035–2045	5′–AGAAAUCAGAA–3′	471–481	5′–UUUACAUUUCA–3′	*0.0483*
RVA/Human-xx/BGD/MMC6/2005/G2P[4]	2035–2045	5′–AGAAAUUAGAA–3′	471–481	5′–UUUACAUUUCA–3′	0.0786
RVA/Human-xx/BGD/MMC88/2005/G2P[4]	2035–2045	5′–AGAAAUCAGAA–3′	471–481	5′–UUUACAUUUCA–3′	*0.0402*
RVA/Human-wt/ITA/PA17/2008/G2P[4]	2035–2045	5′–AGAAAUCAGAA$^{-3'}$	471–481	5′–UUUACAUUUCA$^{-3'}$	*0.0259*
RVA/Human-wt/ZAF/3203WC/2009/G2P[4]	2035–2045	5′–AGAAAUCAGAA$^{-3'}$	471-481	5′–UUUACAUUUCA$^{-3'}$	0.204

†, suffixes G1P[8] and G2P[4] indicate Wa-like and DS-1-like strains, respectively; ‡, nucleotides that are free from base-pairing in the secondary structures predicted for +RNAs of VP2 and VP6 segments are underlined; §, joint probabilities smaller than 0.05 are italicized.

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the joint probability was less than 0.05 in five cases ($p = 0.000184$) (Table 2), suggesting that the pair of Wa-complementary sites identified above tends to be free of base-pairing in human RVA.

The Wa-complementary sites identified in the present study (positions 2068–2078 of VP2 segment and positions 471–481 of VP6 segment) do not reside in the terminal ≈200 nucleotide sites of genomic segments, where packaging signals are thought to be located (6, 12). It should be noted, however, that in *Influenza A virus* packaging signals have been identified in both terminal and internal regions of genomic segments (25, 30, 31). In the RVA virion, VP2 and VP6 form the core shell and the inner capsid, respectively, and are known to interact with each other (5). In addition, protein compatibility has been proposed as a major determinant of genomic constellation for RVA (32, 33). The Wa-complementary sites encode amino acid positions 684–688 of VP2 and amino acid positions 150–153 of VP6. However, amino acid positions 228, 236, 252, 849 and 851 of VP2 (34) and amino acid positions 31–39 and 63–76 of VP6 (34–36) are reportedly involved in the interaction between VP2 and VP6, suggesting that the Wa-complementary sites are unlikely to be related to protein compatibility. The results obtained in the present study suggest that the Wa-complementary sites may serve as a packaging signal between +RNAs of VP2 and VP6 segments in Wa-like strains, possibly through forming a kissing loop complex (17). It would be interesting to conduct experiments aimed at examining whether packaging is impaired by disrupting complementarity at these sites with synonymous mutations using site-directed mutagenesis (37).

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DISCLOSURE

The author declares no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Multiple alignment of nucleotide sequences for VP2 segment of five Wa-like and seven DS-1-like strains (Supplementary Table S1) made using MAFFT (23). The Wa-complementary sites containing a significantly large number of Wa-differentiating sites are colored yellow.

Fig. S2. Multiple alignment of nucleotide sequences for VP6 segment of five Wa-like and seven DS-1-like strains (Supplementary Table S1) made using MAFFT (23). The Wa-complementary sites containing a significantly large number of Wa-differentiating sites are colored yellow.

Fig. S3. Local secondary structures of +RNAs for VP2 and VP6 segments of Wa, centered on the Wa-complementary sites containing a significantly large number of Wa-differentiating sites (arrowheads). Secondary structures were predicted using RNAFOLD (29). The 5′ and 3′ ends of the sequences are indicated with "5′" and "3′", respectively. Base-pairing and non-base-pairing probabilities are color-coded for base-pairing and non-base-pairing nucleotide sites, as indicated by the scale bar.

Table S1. Strain names and accession numbers in the International Nucleotide Sequence Database of the genomic segments for five Wa-like and seven DS-1-like strains of human RVA analyzed in the present study.

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