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Putative bundling signals incompatible between influenza C and D viruses

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ABSTRACT

Genomes of influenza C (ICV) and D (IDV) viruses consist of seven segments of single-stranded negative-sense RNAs called viral RNAs (vRNAs). Reportedly, eight segments are contained in the virions of ICV and IDV, in which at least one copy each of seven genomic segments is thought to be selectively packaged. Although the mechanism of selective packaging is not fully understood, it is thought that a set of vRNAs are bundled through intersegmental interactions of bundling signals, which are likely to be pairs of complementary genomic sequences between vRNAs. Here bundling signals incompatible between ICV and IDV were searched for by the analysis of genomic sequences for four ICV and 21 IDV strains. One pair each of genomic loci between the P3 and HEF vRNAs and within the HEF vRNA were found to be complementary within IDV but noncomplementary between ICV and IDV. Many of these genomic loci appeared to be associated with hairpin and internal loops in vRNAs, suggesting that they may be free from nucleoprotein (NP) in viral ribonucleoproteins (vRNPs) and involved in intersegmental interactions as bundling signals. The virion of IDV may contain one copy each of seven genomic segments together with one extra copy of the HEF segment.

1. Introduction

Influenza virus is composed of influenza A (IAV), B (IBV), C (ICV), and D (IDV) viruses, each of which constitutes a single species in the genera *Alphainfluenzavirus*, *Betainfluenzavirus*, *Gammainfluenzavirus*, and *Deltainfluenzavirus*, respectively, in the family *Orthomyxoviridae* (Asha and Kumar, 2019). Genomes of influenza virus are single-stranded negative-sense RNAs called viral RNAs (vRNAs) and are divided into eight segments in IAV and IBV and seven segments in ICV and IDV (Su et al., 2017). Phylogenetic analyses of influenza virus revealed that IAV and IBV as well as ICV and IDV were closely related to each other (Hause et al., 2013, 2014).

The vRNA of influenza virus comprises, from the 5′-end to the 3′-end, the complementary sequences of 3′-untranslated region (3′-UTR), at least one open reading frame (ORF), and 5′-UTR (Hutchinson et al., 2010). Each vRNA wraps around an oligomer of nucleoprotein (NP), where the phosphate-sugar backbone of vRNA binds to NP exposing bases to the surface (Baudin et al., 1994). The 5′- and 3′-terminal ~15 bases of each vRNA are partially complementary and bound by an RNA-dependent RNA polymerase (RdRp) to construct a viral ribonucleoprotein (vRNP) (Pflug et al., 2014; Reich et al., 2014; Hengrung et al., 2015; Peng et al., 2019).

It is thought that vRNPs of influenza virus are selectively packaged in the virion; i.e., a particular set of vRNPs are bundled as a supramolecular complex and incorporated into a budding virion (Noda et al., 2006). Eight vRNPs are arranged in the "1 + 7" configuration in the virions of IAV and IBV, where eight vRNPs appear to contain one copy each of eight vRNAs (Chou et al., 2012; Fournier et al., 2012; Nakatsu et al., 2016). Interestingly, eight vRNPs were also found to be arranged in the "1 + 7" configuration in the virions of ICV and IDV, which possess only seven vRNAs (Nakatsu et al., 2018).

The molecular mechanism for bundling of vRNPs in influenza virus has not been fully understood (McDonald et al., 2016). It has been proposed in IAV that parts of vRNAs are free from NP in vRNPs through forming local secondary structures (Noda et al., 2012) and involved in intersegmental interactions as bundling signals (Goto et al., 2013). However, the nucleotide sequences that have been proposed to constitute bundling signals were not conserved among different strains of IAV (Gavazzi et al., 2013; Dadonaite et al., 2019), which may contradict the fact that reassortment has frequently occurred during evolution of IAV (Holmes et al., 2005; Nelson et al., 2008).

Reportedly, reassortment occurs only within each species of

Abbreviations: HA, hemagglutinin; HEF, hemagglutinin-esterase-fusion; IAV, influenza A virus; IBV, influenza B virus; ICV, influenza C virus; IDV, influenza D virus; INSD, International Nucleotide Sequence Database; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; ORF, open reading frame; P, polymerase; PB, polymerase basic; RdRp, RNA-dependent RNA polymerase; UTR, untranslated region; vRNA, viral RNA; vRNP, viral ribonucleoprotein

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influenza virus (Hause et al., 2013, 2014; Ducatez et al., 2015; White and Lowen, 2018). It has been demonstrated that NP of IBV inhibits the RdRp activity of IAV, suggesting that the lack of reassortment between IAV and IBV may be attributable to the incompatibility of genomic segments at the protein level (Wanitchang et al., 2012). However, viable viruses could be produced by replacing the ORFs for hemagglutinin (HA) and neuraminidase (NA) of IAV with those of IBV, suggesting that the lack of reassortment between IAV and IBV may also be attributable to the incompatibility of genomic segments at the RNA level; i.e., the incompatibility of bundling signals (Baker et al., 2014).

The bundling signals that are incompatible between different species may constitute pairs of genomic loci with characteristic signatures in nucleotide sequences. Under the assumption that genomic locations of bundling signals have been maintained after speciation, bundling signals may constitute pairs of genomic loci where nucleotide sequences are complementary within each species but noncomplementary between different species, namely the pairs of co-evolving complementary genomic loci (Suzuki, 2014, 2016, 2018). In contrast, under the assumption that genomic locations of bundling signals have not necessarily been maintained after speciation, bundling signals may constitute pairs of genomic loci where nucleotide sequences are complementary within one species but noncomplementary between different species, namely the pairs of species-discriminating complementary genomic loci (Suzuki, 2015). The purpose of the present study was to detect pairs of co-evolving and species-discriminating complementary genomic loci from the analysis of genomic sequences for ICV and IDV.

2. Materials and methods

2.1. Sequence data

Genomic segments 1–7 of ICV and IDV encode polymerase basic protein 2 (PB2), PB1, polymerase protein 3 (P3), hemagglutinin-esterase-fusion protein (HEF), NP, matrix proteins (M), and nonstructural proteins (NS), respectively. Nucleotide sequences for the entire regions of all genomic segments were available for four ICV and 21 IDV strains in the International Nucleotide Sequence Database (INSD) (Karsch-Mizrachi et al., 2018) on July 17, 2019. The INSD accession numbers for these sequences are listed in Supplementary Table S1. Multiple alignment for the total of 25 nucleotide sequences was made for each of seven genomic segments using the computer program MAFFT (version 7.305b) (Katoh et al., 2002). Seven multiple alignments obtained were compared to detect pairs of co-evolving and species-discriminating complementary genomic loci in ICV and IDV.

2.2. Data analysis

To detect pairs of co-evolving complementary genomic loci, all pairs of complementary nucleotide positions, in which the complementarity was observed for each of ICV and IDV strains, were enumerated within and between genomic segments. Here only the Watson-Crick base pairs of U-A and C-G were regarded as complementary, even though U-G may also form a wobble base pair, to reduce false positives in detecting functionally relevant pairs of complementary genomic loci (Boyce et al., 2016). Among the pairs of complementary nucleotide positions obtained were included the pairs with different bases in ICV and IDV, which were called the pairs of co-varying nucleotide positions, with the proportion designated as p_{cov}. Then, all pairs of complementary genomic loci with the length of l nucleotide positions, consisting of l pairs of complementary nucleotide positions, were enumerated within and between genomic segments. For each of pairs of complementary genomic loci with the length of l nucleotide positions, a statistical test based on the binomial probability was conducted to examine whether the number of pairs of co-varying nucleotide positions included (n) was significantly larger than expected from p_{cov}. The Bonferroni (Sokal and Rohlf, 1995) and Benjamini-Hochberg (Benjamini and Hochberg, 1995) corrections for multiple testing were performed using the family-wise error rate and the false-positive rate of 0.05, respectively. The pairs of complementary genomic loci including significantly large numbers of pairs of co-varying nucleotide positions were detected as the pairs of co-evolving complementary genomic loci, which were considered to be selected for diversifying ICV and IDV.

To detect pairs of ICV-discriminating complementary genomic loci, all pairs of ICV-complementary nucleotide positions, in which the complementarity was observed for each of ICV strains, were enumerated within and between genomic segments. Among the pairs of ICV-complementary nucleotide positions obtained were included the pairs without complementarity between ICV and IDV, which were called the pairs of ICV-IDV-noncomplementary nucleotide positions, with the proportion designated as p_{ICV}. Then, all pairs of ICV-complementary genomic loci with the length of l nucleotide positions, consisting of l pairs of ICV-complementary nucleotide positions, were enumerated within and between genomic segments. For each of pairs of ICV-complementary genomic loci with the length of l nucleotide positions, a statistical test based on the binomial probability was conducted to examine whether the number of pairs of ICV-IDV-noncomplementary nucleotide positions included (n) was significantly larger than expected from p_{ICV}. The Bonferroni (Sokal and Rohlf, 1995) and Benjamini-Hochberg (Benjamini and Hochberg, 1995) corrections for multiple testing were performed using the family-wise error rate and the false-positive rate of 0.05, respectively. The pairs of ICV-complementary genomic loci including significantly large numbers of pairs of ICV-IDV-noncomplementary nucleotide positions were detected as the pairs of ICV-discriminating complementary genomic loci, which were considered to be selected for diversifying ICV and IDV. The above procedure was repeated, with ICV and IDV exchanged, to detect pairs of IDV-discriminating complementary genomic loci.

For the parts of vRNAs to be involved in intersegmental interactions as bundling signals, they should be free from NP in vRNPs. Although the binding positions of NP in vRNAs have not been clarified (Lee et al., 2017; Williams et al., 2018; Dadonaite et al., 2019), parts of vRNAs may be free from NP through forming local secondary structures (Noda et al., 2012; Gavazzi et al., 2013; Dadonaite et al., 2019). Therefore, secondary structures were inferred for each of the genomic loci detected above together with 5′- and 3′-flanking 25 and 50 bases. Inference of secondary structure was carried out with the aligned nucleotide sequences of ICV or IDV using RNAalifold (version 2.4.13) (Bernhart et al., 2008).

3. Results

3.1. Putative bundling signals incompatible between ICV and IDV

In the comparison of multiple alignments for seven genomic segments of four ICV and 21 IDV strains, the length of pairs of complementary genomic loci ranged from two to eight nucleotide positions and the p_{cov} was 0.119 (Supplementary Table S2). Consequently, no pairs of co-evolving complementary genomic loci were detected. In addition, the length of pairs of ICV-complementary genomic loci ranged from two to twelve nucleotide positions and the p_{ICV} was 0.144 (Supplementary Table S3), producing no pairs of ICV-discriminating complementary genomic loci.

In contrast, the length of pairs of IDV-complementary genomic loci ranged from two to twelve nucleotide positions and the p_{IDV} was 0.148 (Table 1). As a result, two pairs of IDV-discriminating complementary genomic loci with the length of seven and eight nucleotide positions were detected using the Bonferroni correction (Sokal and Rohlf, 1995) and three additional pairs with the length of six and eight nucleotide positions were detected using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). One pair of IDV-discriminating complementary genomic loci consisted of positions 1943–1950 and

Table 1
Numbers of pairs of IDV-complementary genomic loci with the length of *l* nucleotide positions containing particular numbers (*n*) of pairs of IDV-ICV-non-complementary nucleotide positions.

l	*n*								Total
	7	6	5	4	3	2	1	0	
12	0	0	0	0	0	0	1	0	1
11	0	0	0	0	1	0	2	0	3
10	0	0	1	1	3	4	8	4	21
9	0	0	1	4	11	22	30	24	92
8	**1**[a]	*1*	1	10	42	89	121	115	380
7	1	1	9	32	140	327	534	593	1637
6	N.A.[b]	*2*	22	100	430	1233	2480	3135	7402
5	N.A.	N.A.	21	266	1383	4680	11246	17048	34644
4	N.A.	N.A.	N.A.	424	4022	17046	50517	89321	161330
3	N.A.	N.A.	N.A.	N.A.	8086	54568	213187	466156	741997
2	N.A.	N.A.	N.A.	N.A.	N.A.	131610	802102	2543999	3477711
1	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	2405000	13874214	16279214

[a] Numbers of pairs of IDV-discriminating complementary genomic loci detected using the Bonferroni correction (Sokal and Rohlf, 1995) are bold-faced and those additionally detected using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) are italicized.
[b] Not applicable.

1984–1991 in the P3 and HEF vRNAs of IDV, respectively, which were aligned with positions 1935–1942 and 2017–2024 in the P3 and HEF vRNAs of ICV, respectively (Table 2; Supplementary Figs. S1 and S2). The remaining four pairs overlapped with each other and were included in the pair consisting of positions 702–709 and 1984–1991 in the HEF vRNA of IDV, which were aligned with positions 750–757 and 2017–2024 in the HEF vRNA of ICV, respectively (Table 2; Supplementary Fig. S2). Note that positions 1984–1991 in the HEF vRNA of IDV, which were aligned with positions 2017–2024 in the HEF vRNA of ICV, were involved in both of the pairs of IDV-discriminating complementary genomic loci detected above.

3.2. Secondary structures of putative bundling signals

Secondary structure was inferred for each of the genomic loci with the length of eight nucleotide positions detected above together with 5′- and 3′-flanking 25 and 50 bases in the P3 and HEF vRNAs of ICV and IDV. Note that the numbers of 3′-flanking bases available for positions 2017–2024 and 1984–1991 in the HEF vRNAs of ICV and IDV were 34 and 43, respectively, after excluding the 3′-terminal 15 bases, which were known to interact with the 5′-terminal 15 bases (Pflug et al., 2014; Reich et al., 2014; Hengrung et al., 2015; Peng et al., 2019). In the inferred secondary structures, many of the detected genomic loci appeared to be associated with hairpin and internal loops in ICV and IDV, suggesting that they may be free from NP in vRNPs and involved in intersegmental interactions as bundling signals (Supplementary Figs. S3 and S4).

4. Discussion

In the present study, bundling signals incompatible between ICV and IDV were searched for by the analysis of genomic sequences for four ICV and 21 IDV strains. Statistical methods were devised to detect pairs of co-evolving and species-discriminating complementary genomic loci, which were considered to be selected for diversifying ICV and IDV. Although two pairs of IDV-discriminating complementary genomic loci were detected, no pairs of co-evolving and ICV-discriminating complementary genomic loci were detected, which may reflect relatively small numbers of genomic sequences analyzed, especially for ICV. It should also be noted that natural selection for diversifying bundling signals of ICV and IDV may operate only after establishment of incompatibility of genomic segments for other reasons (Wanitchang et al., 2012; Baker et al., 2014). It may therefore be necessary to devise statistical methods for detecting the bundling signals that are compatible between different species as well as those that are incompatible without natural selection for detecting remaining bundling signals in ICV and IDV.

In the analysis of genomic sequences for detecting pairs of co-evolving and species-discriminating complementary genomic loci, the wobble base pair was not regarded as complementary, to reduce false positives in detecting functionally relevant pairs of complementary genomic loci (Boyce et al., 2016), as mentioned above. However, in the inference of secondary structure for each of the genomic loci detected as putative bundling signals, the wobble base pair was allowed to be formed, to examine the potential for the formation of local secondary structures. Although many of the secondary structures appeared to be associated with hairpin and internal loops, as mentioned above, some of the nucleotide positions appeared to be involved in the formation of stems, which may not seem to be favorable for constructing intersegmental interactions. It should be noted, however, that a stem is composed of complementary nucleotide sequences. Therefore, a pair of complementary genomic loci may tightly bundle vRNAs when each of the complementary genomic loci forms a stem in the local secondary structure Gavazzi et al., 2013.

The pair of IDV-discriminating complementary genomic loci detected between the P3 and HEF vRNAs of IDV were located at positions 1943–1950 and 1984–1991, respectively. Since the P3 and HEF vRNAs of IDV comprise 2195 and 2049 bases, respectively, these genomic loci correspond to positions 246–253 and 59–66 from the 3′-ends of the P3 and HEF vRNAs, respectively (Table 2). Reportedly, bundling signals are located within the terminal ~150–300 nucleotide positions in the 5′- and 3′-ends of each vRNA in IAV and IBV (Muramoto et al., 2006; Sherry et al., 2016). These observations suggest that the pair of IDV-discriminating complementary genomic loci detected between the P3 and HEF vRNAs may interact as a bundling signal in IDV.

The pair of IDV-discriminating complementary genomic loci detected at positions 702–709 and 1984–1991 within the HEF vRNA of IDV may interact with each other either intrasegmentally or intersegmentally. It should be noted, however, that the HEF vRNA of IDV comprises 2049 bases, as mentioned above. In addition, the vRNP is generally known to be rod-shaped (Arranz et al., 2012) with the size roughly proportional to the number of bases in the vRNA (Fournier et al., 2012). Therefore, these genomic loci are likely to be located near the opposite ends of the HEF vRNP and thus unlikely to interact with each other intrasegmentally. These genomic loci may interact intersegmentally as a bundling signal in IDV, which may cause incorporation of an extra copy of the HEF segment together with one copy each of seven genomic segments into the virion of IDV. It would be interesting to validate the results obtained in the present study experimentally.

Table 2
Pairs of IDV-discriminating complementary genomic loci with the length of *l* nucleotide positions containing particular numbers (*n*) of pairs of IDV-ICV-noncomplementary nucleotide positions.

l	*n*	Genomic segment	Positions in ICV	Sequence in ICV	Positions in IDV	Sequence in IDV	Genomic segment	Positions in ICV	Sequence in ICV	Positions in IDV	Sequence in IDV
8[a]	***6***	***P3***	***1935–1942***	***5′UUGUAAAG3′***[c]	***1943–1950***	***5′CUGCUUGC3′***	***HEF***	***2017–2024***	***5′UGUGAGGC3′***	***1984–1991***	***5′GCAAGCAG3′***
8	**7**	**HEF**	**750-757**	**5′CACUGGUG3′**	**702-709**	**5′CUGCUUGC3′**	**HEF**	**2017–2024**	**5′UGUGAGGC3′**	**1984–1991**	**5′GCAAGCAG3′**
7[b]	7	HEF	751-757	5′ACUGGUG3′	703–709	5′UGCUUGC3′	HEF	2017–2023	5′UGUGAGG3′	1984–1990	5′GCAAGCA3′
6	*6*	*HEF*	*751–756*	*5′ACUGGU3′*	*703–708*	*5′UGCUUG3′*	*HEF*	*2018–2023*	*5′GUGAGG3′*	*1985–1990*	*5′CAAGCA3′*
6	*6*	*HEF*	*752–757*	*5′CUGGUG3′*	*704–709*	*5′GCUUGC3′*	*HEF*	*2017–2022*	*5′UGUGAG3′*	*1984–1989*	*5′GCAAGC3′*

[a] The pairs retained after eliminating overlaps are bold-faced.
[b] The pairs detected using the Bonferroni correction (Sokal and Rohlf, 1995) are described in the plain text and those additionally detected using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) are italicized.
[c] The pairs of IDV-ICV-noncomplementary nucleotide positions are underlined.

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CRediT authorship contribution statement

Yoshiyuki Suzuki: Conceptualization, Methodology, Software, Writing - original draft.**Tatsuya Inoue:**Investigation, Visualization.**Maho Nishimura:**Investigation, Visualization.**Yuki Kobayashi:**Validation, Writing - review & editing.

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Declaration of competing interest

The authors declare no conflict of interest.

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