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IBDV particles packaged with only segment A dsRNA

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ABSTRACT

Multi-segmented dsRNA viruses have been suggested to utilize *cis*-acting elements in the plus-strand RNA to accomplish genomic RNA assortment during viral packaging. It is not clear if bi-segmented dsRNA birnavirus uses the same strategy. By applying a reverse genetic technique, we generated IBDV particles packaged with only segment A by co-transfection DF-1 cells of cDNA from segment A and VP1 (without 5′ and 3′ noncoding region of segment B) supporting random assortment mechanism and indicating the packaging elements of segment B include sequences in the 5′ and 3′ NCR. However, *gfp*-containing IBDV could not be generated in the presence of *gfp* cDNA constructs flanked by 5′ and 3′ NCR from segment A or segment B. The data suggest additional packaging signals are required for IBDV genomic packaging. The presence of VP1 protein in the IBDV-A particles also suggests the formation of ribonucleoprotein (RNP) complexes might be involved in the assembly of viral particles.

Introduction

Successful packaging of complete viral genome component is necessary for the viral progeny to be infectious. The specific selection can be achieved by utilizing *cis*-acting elements as the packaging signals to ensure genomic incorporation as well as to tell apart from the cellular nucleic acids. Segmented RNA viruses have one more challenge to take: to encapsidate at least one copy of each genomic segment into the assembling particles. Segmented RNA viruses are proposed to use either random or selective strategy for genomic assortment and packaging. For multi-segmented dsRNA viruses such as rotavirus, *cis*-acting elements reside in 5′ and 3′ noncoding regions (NCR) of plus-strand RNAs (+RNAs) are thought to determine segment selection (McDonald and Patton, 2011). The coding sequences within the open reading frames (ORFs) are not necessary for assortment and packaging and can be partially replaced and still form viruses (Roner and Joklik, 2001). On the other hand, less is known for bi-segmented dsRNA viruses in the Birnaviridae family. Birnavirus has been shown to be very different from multi-segmented dsRNA viruses in the Reoviridae family. Although both viruses have nonenveloped, icosahedral capsid, the capsid of birnavirus is composed of a single layer of VP2 ($T=13$) while the capsid of reovirus possess multiple concentric shells (Coulibaly et al., 2005; Li et al., 2009). Inside the capsid, the 5′ ends of the segment A and segment B are covalently linked to VP1, the RNA-dependent RNA polymerase encoded by segment B (Calvert et al., 1991). Furthermore, the dsRNA of birnavirus is also associated with the VP3 nucleocapsid protein, together with VP1 forming ribonucleoprotein (RNP) complexes (Luque et al., 2009b; Tacken et al., 2002). These RNP complexes have the ability to initiate replication cycle without the VP2 capsid protein (Dalton and Rodríguez, 2014). The structure and replication differences described above between birnavirus and reovirus support the idea that birnavirus might use a different strategy for their genome assortment and packaging.

Infectious bursal disease virus (IBDV) one of the prototype birnaviruses has been shown to be a polyploid virus (Luque et al., 2009a). The polyploidy feature of IBDV indicates that birnavirus might utilize random assortment strategy for genome packaging. Because polyploidy allows viral particles to pack up to 4 genomic dsRNA segments and it significantly increases the chance for the progeny viruses to contain both segment A and segment B within the same viral particle (Luque et al., 2009a). With random assortment strategy, *cis*-acting packaging/selection elements are still necessary to discriminate viral RNAs from cellular RNAs. The predicted secondary structure of IBDV +RNAs showed features shared by both segments including a 5′ long stem loop and a 3′ stem loop with free cytosine tails formed by 5′ and 3′ interaction, suggesting these structures within the 5′ and 3′ NCR might be involved in viral genome packaging (Mosley et al., 2013). However, it is not clear if any viral proteins are involved with genomic RNA encapsidation for IBDV.

In the present study, we provided evidence to confirm that IBDV employed random assortment to package its genome by utilizing the reverse genetics system. Our results also showed the

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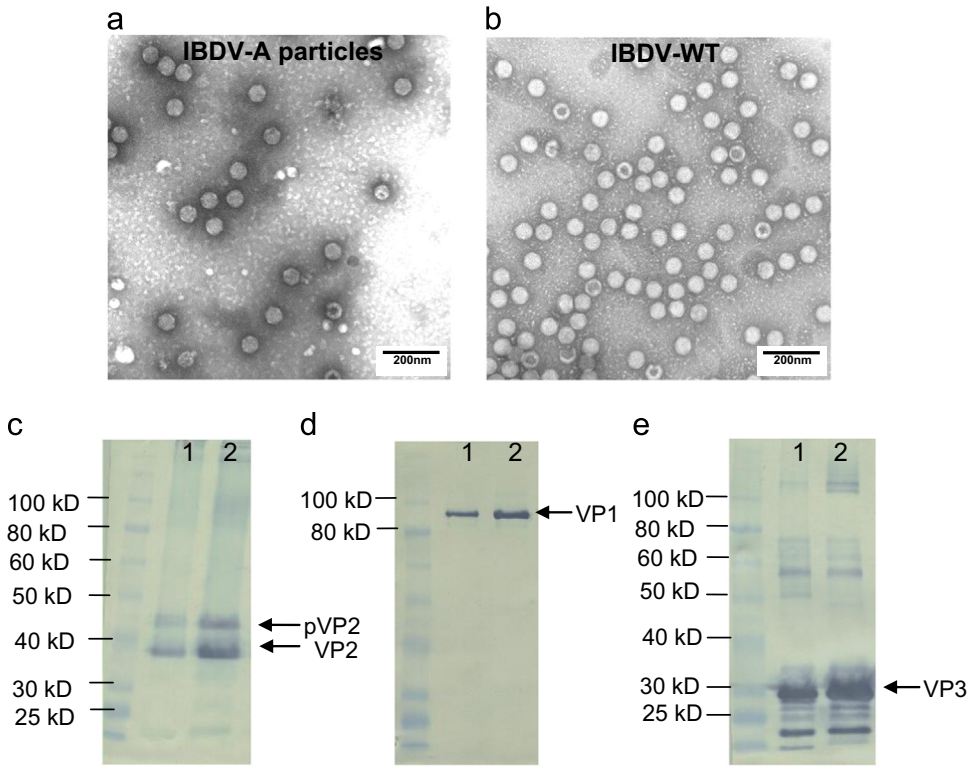

Fig. 1. Characterization of IBDV-A particles by EM and Western blotting. IBDV-A particles were concentrated by sucrose cushion and purified by CsCl gradient centrifugation. Purified IBDV-A particles (a) have similar viral particle morphology and size to those of IBDV WT control (b). Purified IBDV-A particles were also subjected to Western blotting with primary antibody against VP2 (c), VP1 (d) or VP3 (e). Lane 1 represents IBDV-A viral particles and lane 2 was the IBDV WT control.

packaging elements of Segment B resided in the 5′ and 3′ NCR but these signals alone were not enough for genomic packaging. In addition, VP1 and/or RNPs might be required for genomic packaging.

Results

IBDV particles packaged with only SegA can be formed by reverse genetics

By co-transfection of cDNA of segment A (SegA) and segment B (SegB), IBDV-WT could be generated from DF-1 cells (Mosley et al., 2013). To investigate if the packaging elements reside in the 5′ and 3′ NCR of IBDV genomic RNA, we attempted to generate IBDV with SegA and a modified SegB without 5′ and 3′ NCR (only the ORF of segment B in a pcDNA vector), named *SegA+VP1*. The transfection of *SegA+VP1* was able to produce viral-like particles and these particles were further characterized and compared to those of IBDV-WT. We found out these particles have the same appearance and size to IBDV-WT under electron microscope (Fig. 1a and b). In addition, these particles composed the same viral proteins as IBDV-WT such as pVP2/VP2, VP3 and VP1 (Fig. 1c–e). We then examined the genomic composition of these particles by electrophoresis and confirmed these particles were packaged with only SegA (Fig. 2), hence named IBDV-A viral particles. The absence of SegB RNA in the viral particles was also confirmed by RT-PCR (data not shown). Plaque assay of IBDV-A viral particles showed no plaque formation (data not shown) indicating these particles cannot complete its life cycle in infected cells to produce progeny.

Our results showed that IBDV-A particles obtained from transfection of SegA and VP1 were IBDV-like particles packaged only with SegA dsRNA. The fact that these IBDV-like particles could be packaged with only one population of segment indicated

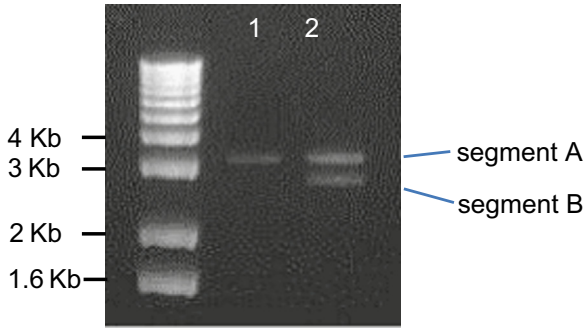

Fig. 2. Genome composition of IBDV-A particles. dsRNAs extracted from IBDV-A viral particles (lane 1) and IBDV-WT (lane 2) by the proteinase K method were examined in a 0.6% native agaorse gel.

that the packaging process of IBDV dsRNA does not require the interaction between the two genomic segments and provided evidence to support random assortment strategy. Our results also suggested that the *cis*-acting packaging elements of IBDV at least for SegB might include sequences in 5′ and 3′ NCR since VP1 dsRNA was not packaged in IBDV-A particles when 5′ and 3′ NCR of SegB were missing.

Signals reside in 5′ and 3′ noncoding regions of IBDV genomic RNA are not enough for genomic packaging

To elucidate if the genomic RNA packaging elements for SegB resided in 5′ and 3′ noncoding regions (NCR), we performed co-transfection of *SegA+SegB* with an additional cDNA construct of green fluorescence protein (GFP) flanked by 5′ and 3′ NCR of SegA or SegB (Fig. 3a). The expression of GFP was confirmed by IFA in transfected cells. However, the viruses collected from transfection contained only IBDV-WT (generated by *SegA+SegB*), no *gfp*-encoding

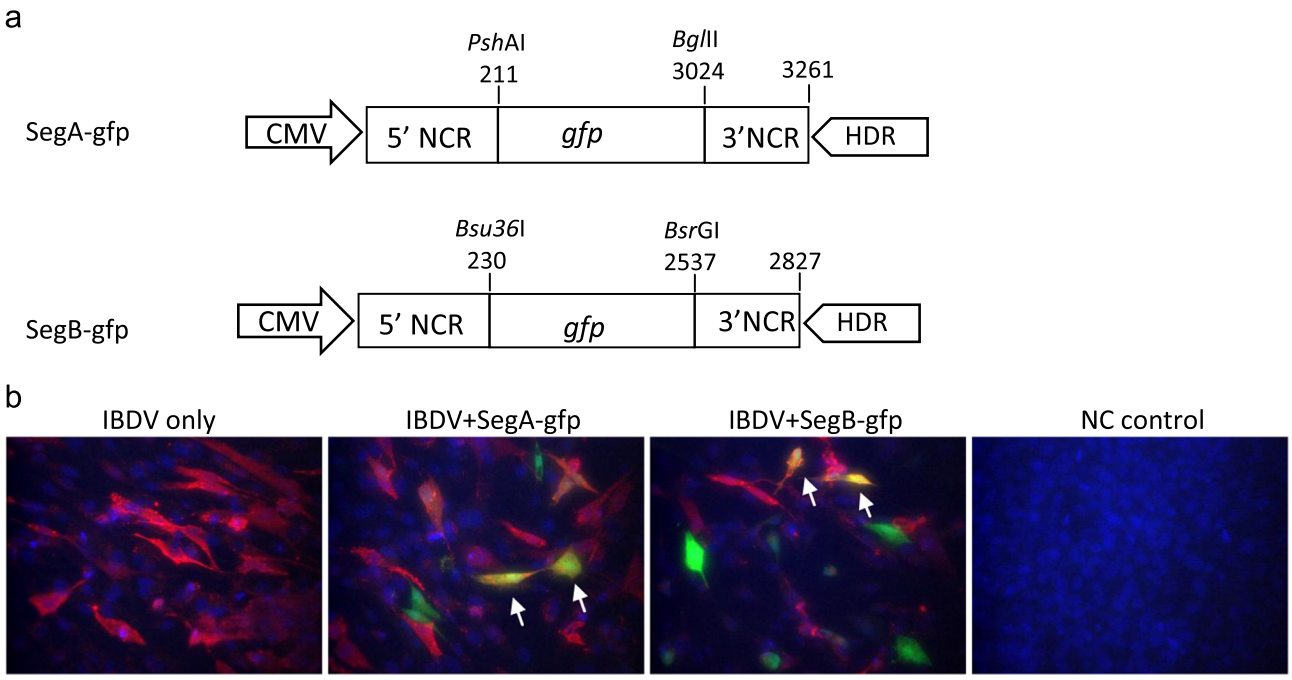

Fig. 3. *The transcript of SegA-gfp and SegB-gfp could not be encapsidated into IBDV*. To elucidate if 5′ and 3′ NCR are sufficient for genome packaging by IBDV, GFP-expressing cDNA containing the 5′ and 3′ NCR of SegA or SegB were constructed (a). DF-1 cells were transfected with either SegA-gfp or SegB-gfp for 24 h, followed by IBDV infection (MOI = 10). After 16 h of IBDV infection, cells were fixed with paraformaldehyde and submitted to IFA for GFP (green) or VP2 (red) protein expression. White arrow points to the cells co-expresses GFP and VP2 (yellow) (40 ×).

genome was packaged in those IBDVs. It was confirmed by RT-PCR for *gfp* sequence from viral particles and there was no GFP expression in those IBDVs-infected cell by IFA (data not shown). To further confirm this finding, we infected DF-1 cells with IBDV-LP1 strain (MOI = 10) followed by transfection of SegA-gfp or SegB-gfp or performed transfection first followed by IBDV infection. Although the expression of GFP and IBDV VP2 was confirmed by IFA in infected and transfected cells (Fig. 3b), the obtained IBDVs did not contain any dsRNA genome with *gfp* sequence nor express GFP in infected cells (data not shown). Our data suggested that *cis*-acting elements in the 5′ and 3′ NCR sequences are not sufficient for IBDV genomic packaging and there is additional packaging signal required for packaging both segments.

VP1 is required for efficient IBDV-A viral particle formation

To examine if VP1 is necessary to form IBDV-A viral particles, we compared the transfection of SegA alone and *SegA + VP1*. The expression of *vp2* in transfected cells was confirmed by RT-PCR (Fig. 4a) and expression of *vp1* was only found in *SegA + VP1* transfected cells (Fig. 4b). However, there was no IBDV-A viral particles generated after transfection from SegA alone. While there was cytopathic effect (CPE) caused by *SegA + VP1*, DF-1 cells remained healthy upon infection from supernatant collected from SegA transfection (Fig. 4c). The absence of viral protein expression was further confirmed by IFA. VP2 and VP1 proteins were not detected in cells infected with the supernatant collected from SegA transfection. On the other hand, both viral proteins were found in *SegA + VP1* infected DF-1 cells (Fig. 4d and e). This results suggested that the existence of VP1 is necessary to generate IBDV-A viral particles. It has been shown that co-exist of VP1, VP3 and dsRNA forms RNP complexes for IBDV. We suspect RNP complexes were formed in IBDV-A particles since VP1 and VP3 were detected by Western blotting (Fig. 1d and e) and dsRNA of SegA were revealed by electrophoresis (Fig. 2). Hence, coordination of replication (RNP complex formation) and encapsidation may be the mechanism IBDV employs for genomic packaging.

Discussion

Our data provided direct evidence for IBDV random assortment as well as showed that the *cis*-acting packaging elements reside in the 5′ and 3′ NCR, but additional signal is required to fulfill genome packaging. It would be interesting to compensate the 5′ NCR and 3′ NCR separately back to the VP1/pcDNA construct to confirm if both NCRs are required for IBDV genome packaging.

It has been reported that the expression of pVP2-VP4-VP3, the second ORF in SegA could result in the formation of viral-like particles (VLPs) in mammalian or insect cells (Fernández-Arias et al., 1998; Kibenge et al., 1999). In our results, the efficiency of VLPs from SegA transfection was quite low since only a few VP2-expressing cells were detected by IFA when the supernatant from transfection was used to infect DF-1 cells. The expression of SegA ORFs (including VP5 and pVP2-VP4-VP3) was enhanced by co-transfection of VP1 (since VP1 is the RNA-dependent RNA polymerase). But we argue this enhancement was not the reason we obtained IBDV-A particles. It has been known that VP3 is required to provide the scaffold to initiate the viral capsid assembly process (Saugar et al., 2010). During capsid assembly, VP3 also interacts with VP1 (Tacken et al., 2002). This interaction not only brings VP1 into viral capsid but also makes IBDV particle formation more efficiently (Lombardo et al., 1999). In addition, VP3, VP1 and genomic dsRNAs can form RNP complexes inside the capsid which have the transcription and replication ability (Luque et al., 2009b). We suspect RNP complexes were generated in IBDV-A particles. However, due to the lack of SegB dsRNA, IBDV-A viral particles could not synthesize VP1 +RNA and thus there was no active VP1 in the infected cells and no IBDV progeny could be generated after the second round of infection. It is possible that encapsidation of the IBDV genome is coupled with RNP complex formation.

The IBDV non-structural protein, VP5 is related to inducing cell apoptosis. Interesting, when we performed immunostaining in *SegA + VP1* transfected cells, we observed cell membrane-associated VP5 expression, but not in SegA-transfected cells (Fig. S1). We suspected the existence of VP1 helped and possibly regulated the

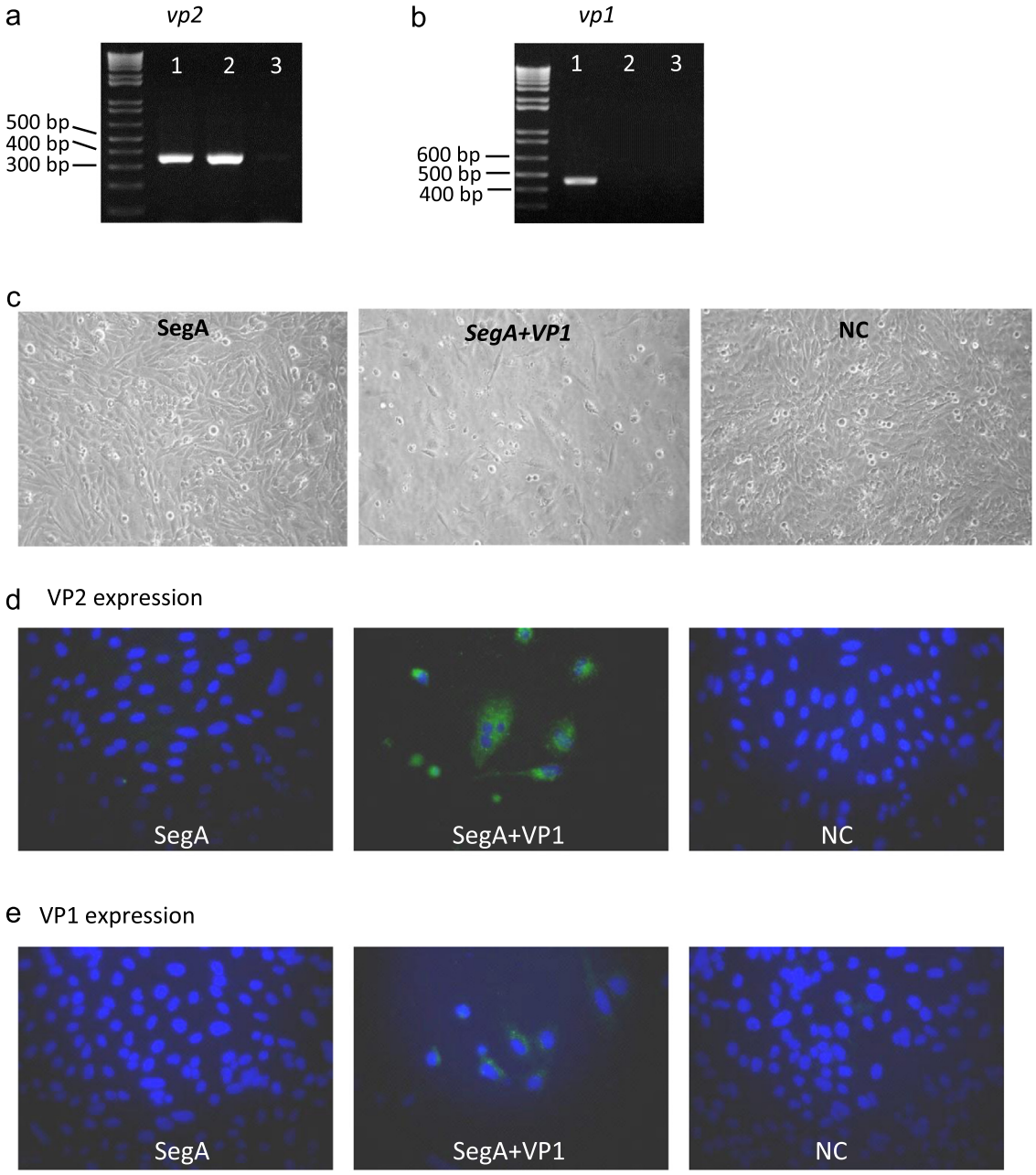

Fig. 4. *VP1 is required for IBDV-A particles formation.* Expression of *vp1* and *vp2* from transfection was confirmed by RT-PCR. RNA was extracted from transfected DF-1 cells and followed by RT-PCR for *vp2* gene (350 bp) (a) and *vp1* gene (454 bp) (b). The RT-PCR template of lane 1 was from *SegA+VP1* transfected cells, lane 2 was from SegA transfected cells, and lane 3 was from mock transfected cells. DF-1 cells were infected with supernatant collected from transfection of SegA, *SegA+VP1* or mock transfection for 48 h (c). Immunofluorescence antibody assay for VP2 (d) and VP1 (e) in infected cells (40 ×).

first ORF expression in SegA, which encode for VP5. And this VP5 expression might be the cause of CPE we observed in the infectivity test for IBDV-A viral particles (Fig. 4c).

In summary, we have shown that IBDV particles containing only segment A could be assembled by the presence of VP1 protein. The *cis*-acting packaging elements for IBDV segment B RNA encapsidation include sequences in the 5′ and 3′ NCR sequence and the packaging process does not require the interaction between the two segments. However, additional packaging signal is required for IBDV genome packaging. Based on our previous publication, the +RNA of segment A and segment B share similar predicted secondary structure at 5′ and 3′ NCR (Mosley et al., 2013). Whether these structures are the components of *cis*-acting signals for IBDV packaging or there are *cis*-acting signals in the ORF warrant further investigation.

Materials and methods

Obtaining IBDV-A viral particles

DF-1 cells (ATCC, Manassas, VA, USA) were co-transfected with SegA and VP1/pcDNA with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and collected as described previously (Mosley et al., 2014) with collecting time at 48 h.

Electron microscopy for IBDV-A viral particles

IBDV-A viral particles were produced by co-transfection in DF-1 cells by TransIT®-LT1 (Mirus Bio, Madison, WI, USA) in 14 6-wells. After 48 h of transfection, supernatant and cells were collected by scraping and subjected to freeze-thawed cycles for three times. IBDV-A viral particles were collected by centrifugation at 2 krpm for 20 min at 20 °C and concentrated by sucrose cushion (25% sucrose) in SW28 tubes (Optima XL-100K, Beckman Coulter, Brea, CA, USA) at 25 krpm for 3 h at 4 °C. Then, IBDV-A viral particles were purified by CsCl gradient (1.327 g/ml) in SW41 Ti rotor (Optima XL-100K, Beckman Coulter) at 25.8 krpm for 18 h at 4 °C and washed with TNE buffer (10 mM Tris–HCl, pH 7.2; 100 mM NaCl; 1 mM EDTA) by Amicon Ultra-4 MWCO 100K filter (Millipore, Billerica, MA, USA) at 2.5 krcf for 5 min. Negative staining was performed with 2% uranyl acetate acid on grids and viewed by a transmission electron microscope (CM100 Biotwin, Philips/FEI Corporation, Eindhoven, Holland).

Western blotting for IBDV-A viral particles

Purified IBDV-A viral particles (described as above) were subjected to SDS-PAGE in 12.5% acrylamide gel, followed by transferring separated proteins to a Nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), Western blotting was performed using anti-VP2 monoclonal antibody R63 (ATCC), anti-VP3 monoclonal antibody (Meridian, Saco, ME, USA) or anti-VP1 rabbit polyclonal antibody (a gift from Dr. Mundt, the University of Georgia) as the primary antibody and horseradish peroxidase (HRPO)-labeled goat anti-mouse or goat anti-rabbit secondary antibody (KPL). The stained protein bands were revealed by 3,3′,5,5′-tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL, Gaithersburg, MD, USA).

Genome composition of IBDV-A viral particles by agarose gel

DF-1 cells were transfected in 10 cm dishes with *SegA+VP1* by TransIT®-LT1 (Mirus Bio) by manufacture's instruction. Briefly, 45 μl of TransIT®-LT1 was diluted in 1.5 ml OPTI-MEM® (Invitrogen). Then, 15 μg of each cDNA construct was added to the mix and incubated for 15 min. Before dropwising the transfection mixture to each dish, cell medium was replaced with fresh medium. After 75 h of incubation at 37 °C incubator, cells and supernatant were collected by scraping, and followed by freeze-thawed for three times. Viral particles were concentrated by SW28 rotor in a high speed centrifuge (Optima XL-100K, Beckman Coulter) at 20 krpm for 2 h at 4 °C. The pellet was resuspended in 200 μl of sterile phosphate buffered saline (SPBS) and subjected to dsRNA extraction by a proteinase K method as described (Mosley et al., 2013). Final dsRNA pellet was dissolved in 20 μl of diethyl pyrocarbonate (DEPC)-treated water and examined in 0.6% agaorse gel (The buffer was prepared in DEPC-treated water).

Expression of VP2 and VP1 by RT-PCR

RNA was extracted by the proteinase K method from transected DF-1 cells as described. RT-PCR was followed for IBDV VP2 and VP1 gene by QIAGEN One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA) as instructed by manufacture's manual. Primers used for VP2 gene are #6 (5′-ACAATCACACTGTTCTCAGCC-3′) and #7 (5′-ATAGTTGCCACCGTGGATCG-3′). Primers used for VP1 gene are with primer 2304F (5′-GAGTGGTCTCGTCCTTCTAGC-3′) and VP1R (5′-ATCATGGCTATTGGCGGC-3′).

Infectivity test and immunofluorescent staining

DF-1 cells in 24-wells were infected with 300 μl of collected supernatant from SegA transfection or *SegA+VP1* (IBDV-A viral particles). After absorption for 1.5 h, medium was added to each well and incubated for 48 h. Viral proteins in infected cells were detected by immunofluorescent staining as described previously (Mosley et al., 2013) with anti-VP2 monoclonal antibody R63 (ATCC) or anti-VP5 rabbit polyclonal antibody (a gift from Dr. Mundt) as the primary antibody and followed by FITC-labeled secondary antibody (KPL).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.001.

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