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BRIEF REPORT

Efficient rescue of infectious bursal disease virus using a simplified RNA polymerase II-based reverse genetics strategy

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Abstract We describe a simplified RNA polymerase II-based reverse genetics approach that allows for the efficient rescue of high-titer infectious bursal disease virus (IBDV) from cloned cDNAs of genomic segments A and B. Unlike the previously reported RNA polymerase II-based methods, the developed strategy does not necessitate the introduction of a ribozyme sequence at both ends of the genomic cDNA sequences. This was achieved by fusing the 5′ terminal sequence of the cDNA of each segment to the transcription start site of the immediate early cytomegalovirus promoter, while a ribozyme sequence was only introduced at the 3′ end. Using this strategy, and without complementing with IBDV structural proteins, titers as high as 10^{11} tissue culture infectious dose 50 were reproducibly obtained in chicken embryo fibroblast cells immediately upon co-transfection with cDNAs of both segments. We anticipate that this modification could improve reverse genetics for any other RNA virus and may be beneficial for vaccine development and dissection of the viral life cycle.

Infectious bursal disease virus (IBDV), a member of the family *Birnaviridae*, genus *Avibirnavirus*, is the causative agent of an avian-restricted disease, known as Gumboro. This disease has a major economic impact on the poultry industry. IBDV displays a bi-segmented double-stranded RNA (dsRNA) genome. The genomic segment A (3.3 kbp) contains two overlapping open reading frames (ORFs) [1, 10]. The smaller ORF encodes VP5, a non structural, membrane-bound accessory protein, and the second ORF specifies a polyprotein which ultimately yields the two capsid proteins, VP2 and VP3, as well as VP4, the viral protease. The smaller segment B (2.9 kbp) encodes VP1, a protein associated with an RNA-dependent RNA-polymerase and capping enzyme activities [9, 26, 29]. In virions, VP1 protein is bound to both genome segments [27].

Reverse genetics has significantly increased our knowledge on aspects relating to the antigenicity and virulence of IBDV [16]. It also offers the advantage of conceiving modified viruses and improved vaccine strains [3–5, 12, 15, 18, 24, 25, 28, 31]. Like many other RNA viruses [6, 20, 23], generation of IBDV particles through reverse genetics was initially achieved by co-transfecting eukaryotic cells with in vitro-synthesised RNA transcripts of both genomic segments [17]. This method involves partial exposure of the RNA, which decreases the efficiency of the experiment, as witnessed by the recovered low virus titers. Lim et al. [12] succeeded in generating IBDV progeny upon transfection of cells with cDNAs cloned downstream of the cytomegalovirus (CMV) promoter of a eukaryotic expression vector. According to the constructs made in this report, the synthesized transcripts must have contained both 5′ and 3′ plasmid-born sequences, thus, limiting the recovery efficiency. Another recovery method based on intracellularly synthesised transcripts with authentic termini was devised [2, 3]. For this purpose, the 5′ end of each genomic segment cDNA was fused to the T7 promoter and cloned into plasmid vectors. To ensure the generation of correct 3′ ends, the hepatitis delta virus (HDV) ribozyme sequence was cloned downstream of each 3′ end. Co-transfection of both

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plasmids into fowlpox-T7 virus-infected cells, which express T7 polymerase, resulted in the formation of infectious IBDV particles. This cDNA-based approach proved to be more efficient than the widely used cRNA method [5].

Recently, a strategy based on RNA polymerase II was reported and proved to be very efficient in producing IBDV [19]. In this method, the synthesis of authentic genomic cRNAs was ensured by flanking the genomic cDNA with hammerhead and HDV ribozyme sequences. Thus, rescue of IBDV occurs through direct transfection of various cell lines. Here, we provide a simpler alternative that does not necessitate a ribozyme sequence at the 5′ end of genomic segments. Our approach consists in fusing the 5′ end of each genomic cDNA to the transcription start site of the immediate early CMV promoter while preserving the HDV ribozyme sequence at its 3′ end.

To construct IBDV infectious clones, plasmids FKP2A and FKP2B, containing the full-length cDNAs of genomic segments A and B, respectively, of the IBDV tissue-culture-adapted attenuated P2 strain (Germany), were used. These plasmids were kindly provided to us by Dr. Egbert Mundt (Friedrich–Loeffler-Institut, Federal Research Institute for Animal Health, Germany). The plasmids were used as templates for PCR amplification of both genomic segments with the primer pairs SASAC/Aext3 and SBSAC/Bext3 (Table 1). PCR reactions were performed with the Expand High Fidelity *Taq* polymerase mix (Roche Applied Science, Germany) and were carried out for 30 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. The amplicons were used for the fusion of the genomic cDNAs (segments A and B) to the CMV promoter of the pVAX vector (Invitrogen) at the *Sac*I site (Fig. 1). A two-step cloning procedure was required to clone the segment A amplicon in pVAX as its sequence contains an internal natural *Sac*I site at position 867. The amplified segment A cDNA was first simultaneously digested with *Sac*I and *Eco*RI, and the largest fragment was directionally inserted into *Sac*I/*Eco*RI-cleaved pVAX. Then the full-length cDNA of segment A was reconstituted by inserting the smaller *Sac*I–*Sac*I fragment. The plasmid containing the latter insert in the right orientation was selected and termed pVAXSA. The amplicon corresponding to segment B was digested with *Sac*I and *Pst*I and was directly subcloned into similarly restricted pVAX vector, yielding the pVAXSB plasmid construct.

The sequence of the HDV ribozyme [7] was assembled from two overlapping PCR fragments, products of the two primer pairs, Rib1int/Rib2 and Rib1ext/Rib2ext. Briefly, the overlapping primers Rib1int and Rib2 were mixed, denatured, and allowed to anneal for 5-min periods at decreasing temperatures (5°C increment) from 80 to 40°C. *Pfu* DNA polymerase was added for a 1-h extension at 72°C. Next, a PCR was carried out for 30 cycles with *Taq* DNA polymerase (Amersham Biosciences) under cyclic conditions: 30 sec at 94°C; 30 s at 55°C; 30 s at 72°C, using the primer pair Rib1ext/Rib2ext. The resulting PCR product, corresponding to the full-length HDV ribozyme

Table 1 Nucleotide sequence, orientation, and positions on genomic segments of the primers used in this work

Primer designation	Sequence (5′–3′)	Orientation	Nucleotide no	Restriction sites
SASAC	CACGAGCTCGTTTAGTGAACCGGGATACGATCGGTCTGACCCCG	+	1–22	*Sac*I
Aext3	AGAGAATTCTGTACAGGGGACCCGCGAACGGATCCAATT	–	3,237–3,261	*Eco*RI
SBSAC	CAGAGCTCGTTTAGTGAACCGGGATACGATGGGTCTGAC	+	1–18	*Sac*I
Bext3	CGATCTGCTGCAGGGGGCCCCCGCAGGCGAAGG	–	2,806–2,827	*Pst*I
Rib1int	CTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCACGTCC	+		
Rib2	AGTGGCTCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTC	–		
Rib1ext	GGGTCGGCATGGCATCTCCACCTCCTCGCGGTC	–		
Rib1Fus	ATCCGTTCGCGGGTCCCCTGGGTCGGCATGGCATCTCC	+		
Rib2ext	AGTGGCTCTCCCTTAGCCATC	+		
SABG	ATCAGATCTGCTCTTGACTGCGATGG	+	3,025–3,041	*Bgl*II
SAFus	AGGGGACCCGCGAACGGAT	–	3,242–3,261	
Rib5apa	TGCGGGGGCCCCCGGGTCGGCATGGCATCTCC	+		*Apa*I
Rib3apa	TACGGGCCCGAATTCAGTGGCTCTCCCTTAGCCATC	–		*Apa*I, *Eco*RI[a]
U2	ATGTGAGGCTTGGTGAC	+	660–676	
L2	CCTGTTGCCACTCTTTC	–	1,192–1,209	

The restriction sites, wherever present, are underlined in the sequence

[a] *Eco*RI in this primer was used to verify the orientation of the cloned ribozyme sequence

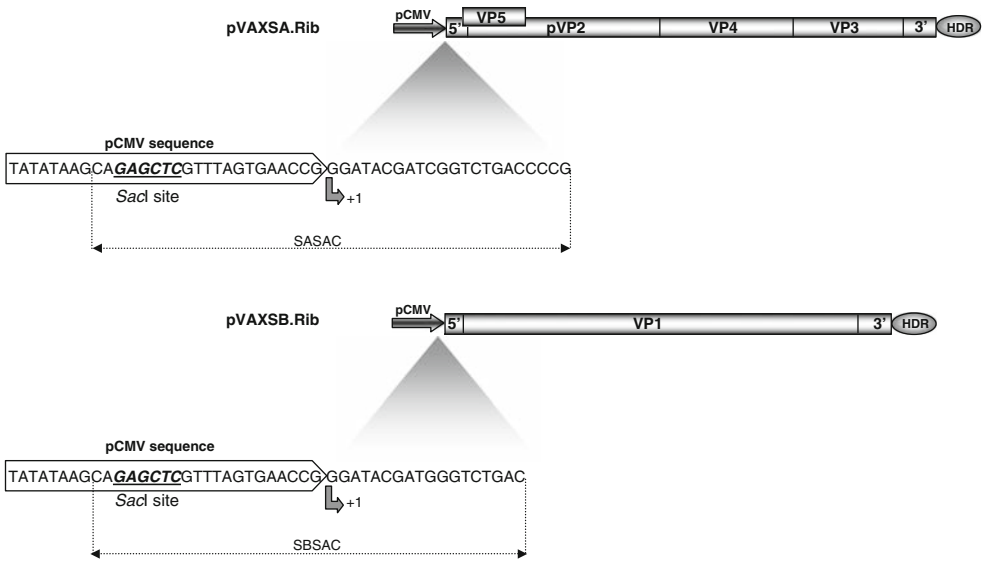

Fig. 1 Schematic representation of plasmids containing the full-length cDNA sequences of the P2 strain segments A (pVAXSA.Rib) and B (pVAXSB.Rib). In both constructs, the cDNAs of both segments were placed so that transcription from the CMV promoter starts at the first 5′ nucleotide, whereas the HDR sequence was introduced downstream of both segments to ensure the generation of authentic 3′ ends. The segment A-encoded viral proteins VP5, pVP2, VP3, and VP4, as well as the segment B VP1 protein are shown. 5′ and 3′ refers to the untranslated regions

sequence, was cloned in pCR2.1 vector (Invitrogen), yielding the pCR.Rib plasmid construct.

Insertion of the HDV ribozyme sequence at the 3′ end of the segment A cDNA in the pVAXSA expression plasmid was achieved by first fusing the 3′ end of segment A cDNA to the ribozyme sequence by assembly PCR. For this purpose, two independent PCR reactions using the primer pairs SABG/SAFus and Rib1Fus/Rib2ext were performed on the templates pVAXSA and pCR.Rib, respectively. The amplicons of the two PCR reactions were then combined to produce a PCR product using the external primers SABG and Rib2ext. The latter PCR product, which contains the 3′ end of the segment A cDNA fused to the HDV ribozyme sequence, was digested with *Bgl*II and *Eco*RI and cloned into the similarly digested pVAXSA to yield pVAXSA.Rib.

Introduction of the HDV ribozyme sequence at the 3′ end of the segment B cDNA was readily achieved through the natural 3′ end *Apa*I site (nucleotide position 2,820). The HDV ribozyme sequence was amplified with Rib5apa/Rib3apa oligonucleotide primers, restricted with *Apa*I (the site of this enzyme was added to the 5′ end of the primers) and inserted into pVAXSB. A clone containing a properly oriented ribozyme sequence was selected, using the *Eco*RI restriction site of the reverse primer Rib3apa, and named pVAXSB.Rib. The 5′ and 3′ manipulated regions were checked by sequencing for both pVAXSA.Rib and pVAXSB.Rib constructs.

Primary chicken embryo fibroblast (CEF) cells [22], growing in 100-mm dishes at 80% confluence, were co-transfected with 5 μg each of plasmid constructs pVAXSA.Rib and pVAXSB.Rib using FuGENE 6 according to the manufacturer's protocol (Roche Applied Science, Germany). Three days later, consistent cytopathic effect (CPE) reminiscent of IBDV replication was obvious in several foci and was maximal by the sixth day post-transfection (data not shown). No such CPE was observed in CEF cells transfected with 10 μg of the original pVAX vector. The experiment was reproduced twice using two new CEF cell preparations, and the same results were obtained.

At 6 days post-transfection, the supernatant was collected (Passage 0; P_0), clarified by centrifugation at $200\times g$ for 15 min, and used to re-infect freshly prepared CEF cells at a 1/10 dilution (P_1). A consistent CPE was observed as soon as 2 days post-infection (Fig. 2). Three additional serial passages (P_2–P_4) were performed, and all produced the same CPE. To confirm that the observed CPE was accompanied by IBDV release, we subjected the supernatant of the first passage to RT-PCR amplification targeting both the minus- and the plus-sense strand of the genomic double-stranded RNA. The latter was prepared as described previously [8], including a DNase I treatment to eliminate any residual plasmid DNA. As shown in Fig. 3a, a PCR product corresponding to the expected size (a 549-bp fragment lying in the hypervariable region of VP2 sequence) was obtained from both the plus- and the minus-sense strand. Corresponding products could not be amplified directly from the RNA preparation, eliminating the possibility that amplification could have occurred

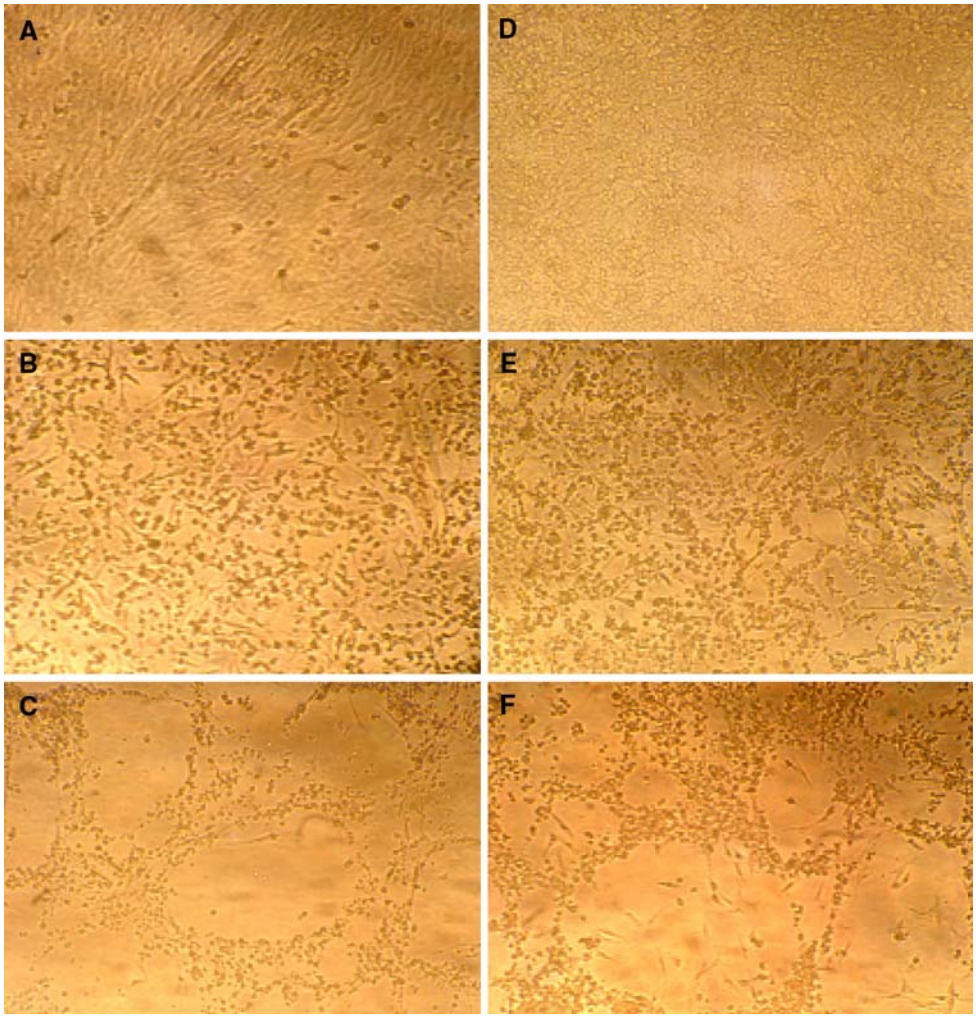

Fig. 2 Detection of cytopathic effect in CEF and Vero cells infected with the supernatant of CEF cells co-transfected with plasmids pVAXSA.Rib and pVAXSB.Rib. The appearance of CPE is visualized in CEF cells (**b**) and in Vero cells (**e**). **a**, **d** Show mock-infected CEF and Vero cells, respectively; **c**, **f** represent CEF and Vero cells infected by the Bursine-2 vaccine strain, respectively

from a residual plasmid DNA. Thus, transcripts generated from pVAXSA.Rib and pVAXSB.Rib yielded double-stranded RNA that was released into the supernatant and passaged as infectious (passage-competent) IBDV particles. Next, we confirmed by nucleotide sequencing that the obtained amplicons corresponded to the P2 strain (data not shown).

To unambiguously demonstrate that the observed CPE was accompanied by the release of IBDV particles, the supernatant of passage 1 (P_1) was pelleted and subjected to immunoprecipitation using an anti-VP2 monoclonal antibody, kindly provided to us by Dr. Bernard Delmas (Unité de virologie et immunologie moléculaires, INRA, Jouy-en-Josas, France). As shown in Fig. 3b, the anti-VP2 monoclonal antibody recognized a protein band of approximately 40 kDa, the expected molecular mass of the IBDV VP2 protein, which was also revealed in a Bursine-2 concentrated virus stock. Electron microscopy analysis performed on the same pellet further confirmed the presence of viral particles typical of IBDV virus (data not shown). Taken together, these data indicate that co-transfection of CEF cells with constructs pVAXSA.Rib and pVAXSB.Rib yielded infectious IBDV virions.

The supernatant of co-transfected CEF cells (P_0) as well as of passages P_1–P_4 were subjected to virus titration in both CEF and Vero cells according to the Reed and Muench method [21]. The titration was performed in triplicate, and the results are shown in Fig. 4. Strikingly, titers in CEF cells reached unprecedented values varying from $10^{10.5}$ (P_0) to $10^{9.5}$ (P_4) tissue culture infectious dose 50 ($TCID_{50}$/ml), peaking at $10^{11.8}$ in P_3. Similar results were obtained when titration was performed on Vero cells (Fig. 4) and when viral stocks obtained from independent co-transfection experiments were titered. The difference in titers between passages may stem from the fact that different CEF preparations were used.

Reverse genetics based on the RNA polymerase II system has been recently applied with high efficiency to RNA viruses such as rabies virus [11], borna disease virus [14, 30], and measles virus [14]. The approach consists in flanking the genomic cDNA with ribozyme sequences so that transcripts with authentic 5′ and 3′ termini can be

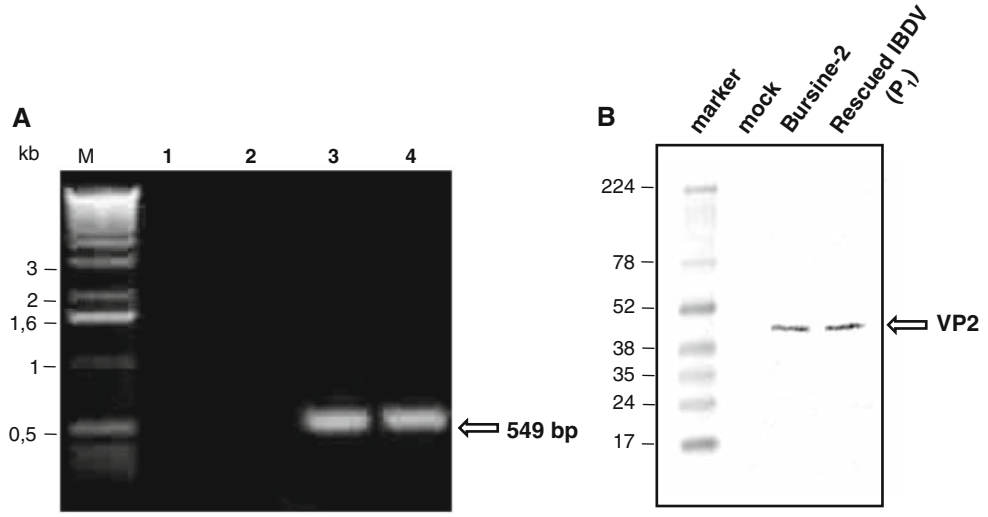

Fig. 3 **a** Amplification of the VP2 hypervariable region using RNA extracted from the clarified supernatant of passage 1 (P_1) of rescued IBDV. RT-PCR using the primer pair U2/L2 targeted either the minus strand (*lane 3*) or the plus strand (*lane 4*) of the genomic segment A by initiating reverse transcription with U2 and L2, respectively. As negative control, RT-PCR was performed using RNA extracted from the P_1 supernatant of pVAX-transfected CEFs cells (*lane 1*). In addition, a PCR was performed directly on DNAse I-treated RNA extracts (without RT reaction) to exclude the possibility that amplification might have occurred through residual plasmid DNA (*lane 2*). M: 1-kb DNA ladder (Invitrogen). **b** Western blot analysis of the pellet fraction of the P_1 supernatant (in CEF cells) of rescued virus using anti-VP2. As positive control, we used the pellet fraction of the supernatant of CEF cells infected with Bursine-2 vaccine strain. Mock-infected CEF cells were analysed in parallel. Molecular sizes (in kDa) of the high-range rainbow molecular weight marker (Amersham Biosciences) are given on the *left*

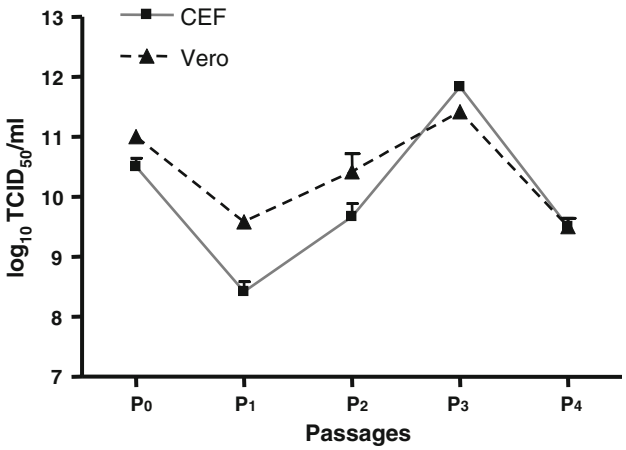

Fig. 4 Evolution of the titer (in $TCID_{50}$/ml) of CEF-passaged rescued virus. Titering was performed in both CEF and Vero cells. The supernatant of cells co-transfected with plasmid constructs pVAXSA.Rib and pVAXSB.Rib (passage 0; P_0) was serially passaged (P_1–P_4) at a dilution of 1/10. The titer of each passage represents the mean value of three independent experiments. As mentioned in the text, variations in titers could be attributed to the use, at each passage, of a different CEF preparation

obtained. In comparison to the T7 RNA polymerase-based reverse genetics approaches, methods based on RNA polymerase II benefit from the increased genome expression induced by the viral structural proteins, thus resulting in higher rescue efficiency [11, 14]. In this respect, it is worthy of mentioning that the T7-based reverse genetics yielded very high IBDV titers, reaching $10^{10.3}$ $TCID_{50}$/ml with prior transfection with a plasmid construct encoding the polyprotein [18].

Following a similar strategy, and using the CMV enhancer and beta chicken actin promoter, Qi et al. [19] have recently increased the rescue efficiency of IBDV. In the present report, we have further simplified the RNA polymerase II approach for IBDV rescue by designing a strategy that does not necessitate the introduction of a ribozyme sequence at the 5′ end of the genomic cDNA sequences. For this purpose, we simply fused the 5′ terminal sequence of the genomic segments to the CMV promoter transcription start site. Our strategy resulted in a high yield of infectious particles and proved to be reproducible. Although the RNA ends of both segments produced with our method have not been verified for authenticity by nucleotide sequencing, the regular high titers of the recovered IBDV suggest that both genomic RNAs were produced in an optimal infectious state, thus validating the developed approach. It is, of course, very difficult, or even impossible, to make any comparison with the results obtained by Qi et al. [19], as the two approaches must be compared under identical conditions. Indeed, the difference in titres may be inherent to the nature of the strains used. In fact, using this refined reverse genetics strategy, we have recently been able to generate a tissue-culture-adapted mosaic strain with a mutated segment A of a very virulent Tunisian IBDV isolate [13], whose titer

reached 10^{13} $TCID_{50}$/ml in CEF and Vero cells prior to passaging (manuscript in preparation). The transfection protocol may also account for the higher titers obtained in this work. In our case, we used 2.5-fold higher amounts of DNA and transfected CEF cells with FuGENE 6, which in our hands allowed higher transfection rates in CEF cells than does lipofectamine™ 2000. In addition, the CMV-based plasmid used in this work may direct increased transcription levels, thus resulting in higher rescue rates.

In summary, we have developed a simplified RNA polymerase II-based reverse genetics strategy that proved to be reproducible and allowed the rescue of very-high-titer IBDV stocks. We have recently successfully applied this method to generate a minigenome of segment A which, in complementation assays, proved to be competent for both replication and packaging (manuscript submitted). Furthermore, the availability of such a simplified and reproducible reverse genetics strategy could be applied to other viruses and will help to foster the generation of genetically modified, high titer viruses, for vaccine development.

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