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Synthesis of Reassortant Infectious Bursal Disease Virus in Chickens Injected Directly with Infectious Clones from Different Virus Strains

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Abstract The infectious bursal disease virus (IBDV), a member of the *Birnaviridae* family, containing a bisegmented double-stranded RNA genome, encodes four structural viral proteins, VP1, VP2, VP3, and VP4, as well as a non-structural protein, VP5. In the present paper, the segment A from two IBDV strains, field isolate ZJ2000 and attenuated strain HZ2, were inserted into one *Nae*I site by site-directed silent mutagenesis and subcloned into the eukaryotic expression plasmid pCI under the control of the human cytomegalovirus (hCMV) immediate early enhancer and promoter to construct the recombinant plasmids pCI-AKZJ2000 and pCI-AKHZ2, respectively. Each of the two recombinants was combined with another recombinant pCI plasmid containing the marked segment B of strain HZ2 (pCI-mB), and injected intramuscularly into non-immunized chickens. Two chimeric IBDV strains were recovered from the chickens. Two out of eight chickens in each of two groups showed the bursal histopathological change. The reassortant virus derived from pCI-AKZJ2000/pCI-mB can infect chicken embryos and shows relatively low virulence. We have developed a novel virus reverse genetic approach for the study of IBDV. The results also form the basis for investigating the role of VP1 in viral replication and pathogenecity.

Key words infectious bursal disease; infectious bursal disease virus; reverse genetics; reassortant virus; animal model

The infectious bursal disease virus (IBDV) is the causative agent of infectious bursal disease (IBD) in chickens. It causes high mortality in young chickens and establishes an immunosuppression state by destroying the precursors of B lymphocytes in the bursa of Fabricius and the post-bursal B lymphocytes, leading to vaccination failure against other pathogens [1].

IBDV is a member of the *Birnaviridae* family and has two double-stranded RNA segments (A and B) in its genome. The segment A (about 3.3 kb) contains two partial overlapping open reading frames (ORFs). The larger ORF of the segment A encodes a 110 kDa precursor polyprotein (VP2/VP4/VP3) that can be post-translationally cleaved into three mature proteins, VP2 (40 kDa), VP4 (28 kDa) and VP3 (32 kDa) [2–4]. It has been reported that VP2 is the major protective immunogen of the virus and contains the antigenic epitopes responsible for the induction of neutralizing antibodies. VP2 is also responsible for the virulence determinants, cell tropism and pathogenic phenotype of IBDV [5]. The smaller ORF encodes a non-structural protein VP5 (17 kDa). The segment B (about 2.9 kb) is present within the viral virions, and encodes the viral RNA-dependent RNA polymerase VP1.

Virus reverse genetics is the synthesis of live viruses or virus-like structures in cells or hosts that are transfected with infectious clones. Infectious clones, which are bacterial plasmids containing the full genomic cDNA of the virus that are under the control of suitable promoters, cause the infectious cDNA or cRNA to be transcribed extracellularly. The RNA virus rescue system makes it

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possible to understand both the relationship between the function of the virus and its viral gene structure, and the interaction between viruses and hosts. In recent years, the full-length genomic RNA has been cloned from several IBDV strains [6,7], which makes the recovery of IBDV possible. The first reverse genetic technique for IBDV was developed in 1996 [8] based on the cell lines transfected with the viral genomic cRNA that was transcribed extracellularly. As a benefit of the reverse genetic system, some new viral function-related proteins have been identified [9,10].

We have established an IBDV recovery method based on the RNA polymerase II (pol II) system in Vero cells [11], which makes the IBDV recovery easier and quicker. In this method, co-transfection of eukaryotic expression vectors (pCI) with the modified segment A and segment B from IBDV into the Vero cells causes the transcription of the viral RNA and the expression of the proteins to be initiated by RNA pol II. One chimeric virus strain Xihu2002 was recovered in this system from the Vero cells after the cells were transfected with the plasmids pCI-mA and pCI-mB derived from the IBDV attenuated strain HZ2.

It has been well demonstrated that the IBDV virulent field isolates can not adapt to cell lines until after several passages, but the virus may be attenuated after adaptation because of the nucleotide changes in segment A [2,12]. So it is not easy to study the characteristics of the virulent IBDV strain using the reverse genetic system on cell lines. Animal models provide another way for virus recovery. It has been reported that several viruses have been recovered in animal models; for instance, the hepatitis C virus HCV, hepatitis E virus and porcine circovirus [13,14], etc..

In the present study, we report for the first time that the chimeric cDNA clones of segment A from the IBDV virulent strain and segment B from the IBDV attenuated strain are infectious when co-injected directly into the muscles of non-immunized chickens. The immunogenicity and pathogenicity of the chimeric infectious DNA clones are primarily characterized in chickens.

Materials and Methods

Restriction enzymes and reagents

One-shot long and accurate-PCR (LA-PCR) mix and all restriction enzymes were purchased from TaKaRa Biotech (Dalian Company Limited, Dalian, China). The Superscript II preamplification system, Concert™ high-purity plasmid purification system kit, Lipofectamine reagent 2000 and Trizol were purchased from Invitrogen Company Limited (Carlsbad, California, USA). The rabbit anti-IBDV serum was previously prepared in our laboratory. The serum titer was higher than 1:6400.

Site-directed mutagenesis of the genomic segment A of IBDV

The recombinant plasmids pGEM-T-A/ZJ2000 and pGEM-T-A/HZ2 containing the genomic segment A from the IBDV strains ZJ2000 and HZ2 [6] were used as two templates for site-directed mutagenesis. First, a 157 bp DNA segment (named PCR1) was amplified with the primer pair Afl5/Nae3, and a 915 bp segment (named PCR2) was amplified with the primer pair Nae5/Kpn3. PCR1 and PCR2 were then fused with the primer pair Afl5/Kpn3 (**Table 1**) and the fused segment was named PCR3 (1050 bp). PCR3 and pGEM-T-A were digested with *Afl*II and *Spe*I (**Table 1**), and the resulting fragments were then ligated by fragment change to obtain the mutated plasmids pTAK/ZJ2000 and pTAK/HZ2.

Construction of eukaryotic expression plasmids containing mutated segment A

First, the pTAK/ZJ2000 or the pTAK/HZ2 was digested

Table 1 Sequences of primers used in the experiment

Primer	Nucleotide sequence	Location	Restriction site
Afl5	CTTGGC*CTTAAG*TTGGCTGGT	2225–2245	*Afl*II
Nae3	GGTACTGGCG*gCCgGC*ATTGGGTGGA	2356–2381	*Nae*I
Nae5	CCCAAT*GCcGGc*CGCCAGTACCACC	2360–2384	*Nae*I
Kpn3	*GGACTAGTGGTACC*GGGGACCCGCGAACGGATCCCAATTTGGA	3232–3260	*Spe*I *and Kpn*I
A5	GGATACGATCGGTCTGACCCCGG	1–23	–
A3	GGGGACCCGCGAACGGAT	3243–3260	–
B5	ATGAATTCGGATACGATGGGTCTGACCCT	1–21	*Eco*RI
B3	ATTTCTAGAGGGGGCCCCCGCAGGCGAA	2809–2827	*Xba*I

with both *Eco*RI and *Kpn*I. The products were further digested with *Dra*I to cleave the vectors. Then the largest segments were inserted into pCI plasmids that had been digested with *Eco*RI and *Kpn*I, respectively. The resulting recombinant plasmids were named pCI-AKZJ2000 and pCI-AKHZ2.

Plasmid preparation and animal inoculation

High-purity plasmids pCI-AKZJ2000, pCI-AKHZ2 and pCI-mB [11] were prepared using the Concert™ high-purity plasmid purification system kit (Invitrogen). Plasmid DNA was incorporated into Lipofectamine half an hour before injection.

Twenty-two 14-day-old non-immunized chickens were divided randomly into three groups. Group A (eight chickens) was co-inoculated intramuscularly with pCI-AKZJ2000 and pCI-mB, and group B (eight chickens) was co-inoculated intramuscularly with pCI-AKHZ2 and pCI-mB. The dose of each plasmid that was administered to each chicken was 10 μg. Group C (six chickens) received 0.2 ml of phosphate buffered saline (PBS) (0.02 M, pH 7.4) per chicken, and served as the blank control. The three groups of chickens were raised in isolation.

All groups were observed three times every day after inoculation. Fourteen days after inoculation, all chickens were killed for serum and bursa collection. A portion of every bursa was preserved in 10% formaldehyde for further histopathological analysis, and another portion was frozen at –40 °C for IBDV antigen detection and observation by electron microscope.

Detection of the serum antibody of IBDV

Enzyme-linked immunosorbent assay (ELISA) was used to determine the anti-IBDV antibodies. Two weeks after the injection of plasmids, three serum samples from each test group were randomly selected for antibody titer measurement. The coating antigen (National Institute for Supervision of Veterinary Pharmaceuticals, Beijing, China) and test sera were diluted 200 times. The horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Dingguo Biotech Development Center, Beijing, China) was used as the secondary antibody and 3,3′,5,5′-tetramethyl benzidine (TMB) was used as the substrate. Absorbance was measured at 492 nm with an automated plate reader (Labsystem Multiscan Ascent, Helsinki, Finland). None of the chickens' sera before the plasmid injection reacted with the positive IBDV antigen.

Histopathological examination of bursal samples

All the bursal samples were preserved in 10% formaldehyde and embedded in wax. Thin sections were prepared for hematoxylin-eosin (HE) staining and histopathological examination of bursal lesions. The lesions on the bursa of Fabricius were scored acording to Shaw and Davison [15], with the following modifications: score 0 (no bursal damage or lesion in any follicle, clear demarcation of medulla and cortex) and score 1 (mild to severe necrosis and lymphocyte depletion of follicles).

Detection of IBDV antigens and IBDV-like virions in bursas

Three bursal samples in every group were randomly selected for detection. The samples were then frozen and thawed three times, and the bursas were skived and centrifugated for viral particle separation. The supernatant served as the source of the IBDV antigen after incubation at 60 °C for 30 min. The agar immuno-diffusion test (AIDT) was carried out to detect the IBDV antigen in the bursas. The supernatant was also examined for IBDV-like virions using the electron microscope. At the same time, thin sections of the Fabricius bursas were also examined by electron microscope after they were double stained with uranyl acetate and lead citrate.

Chicken embryo infection with reassortant IBDV

After filtering to within 0.22 μm, every supernatant sample obtained from above was inoculated onto the chorioallantoic membranes of five 11-day-old chicken embryos. Five embryos inoculated with the IBDV strain ZJ2000 served as the positive control, while five embryos inoculated with 0.2 ml of PBS served as the negative control. Seven days after inoculation, the allantoic fluids were collected for further antigen detection using ELISA. First, the allantoic fluids of embryos infected with the same supernatant sample were mixed. The mixtures were centrifuged at 12,000 *g* for 15 min and served as the coating antigens after incubation at 60 °C for 30 min. Rabbit anti-IBDV serum (2000 times dilution) and HRP-conjugated goat anti-rabbit IgG (Dingguo Biotech Development Center, Beijing, China) were used as the primary and secondary antibodies, respectively.

Molecular mark detection of chimeric IBDV genomic segments

The cDNA amplification of the virus genomic segments A and B was carried out using long and accurate reverse transcription polymerase chain reaction (LA RT-PCR) using the primer pairs A3/A5 and B3/B5 respectively [11,15]. The PCR product of segment A was digested with *Nae*I to identify the mutated GCcGGc (2366–2371 bp) sites. The

PCR product of segment B was digested with *Eco*RV to identify the mutated GaTAtc (2282–2287 bp) sites.

Results

Site-directed mutagenesis and eukaryotic plasmid construction of genomic segment A

After fusion PCR, one unique *Nae*I site for segment A was inserted into PCR3 and the *Nae*I site was located in the 2366–2371 bp position, with silent mutation of A (2368) to C, and A (2371) to C. One *Kpn*I site was inserted just behind the segment A. Then the mutated segment A was cleaved from the pGEM-T easy vector that was digested with *Eco*RI/*Kpn*I and inserted into the plasmid pCI to produce the plasmids pCI-AKZJ2000 and pCI-AKHZ2 (**Fig. 1**).

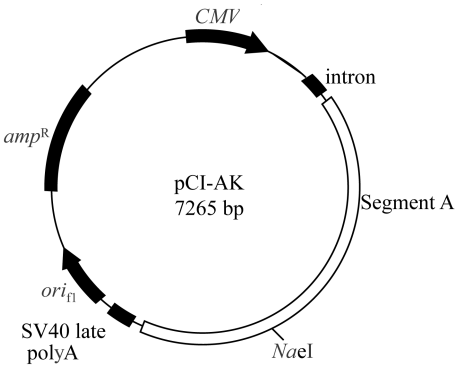

Fig. 1 Construction of eukaryotic plasmids encoding the site-mutated full-length cDNA of IBDV genomic segment A

The mutated segment A of two IBDV strains, ZJ2000 and HZ2, were inserted into pCI vectors to produce the plasmids pCI-AKZJ2000 and pCI-AKHZ2.

Serum antibody assays

It can be seen from **Fig. 2** that all selected serum samples reacted with the positive antigen, and the results were significantly different for negative serum ($P<0.05$). This suggests that antibodies with high titer are induced in chickens.

Clinical symptoms and the bursal histopathological examination

None of the chickens showed typical clinical symptoms from injection until euthanasia. Two of eight bursas showed histopathological changes in both test groups (25%). The

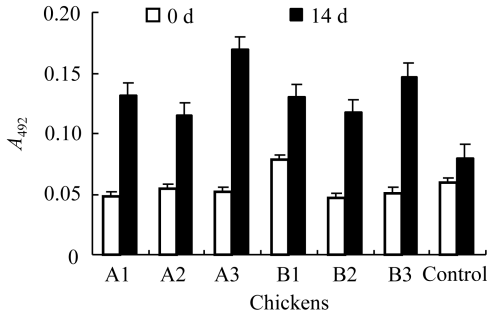

Fig. 2 ELISA detection of the serum antibody of IBDV [a]

A1, A2 and A3, serum samples selected randomly from group A; B1, B2 and B3, serum samples selected randomly from group B; Control, negative chicken serum. The IBDV positive antigen and negative chicken serum were purchased from the National Institute for Supervision of Veterinary Pharmaceuticals, Beijing, China. The coating IBDV antigen and serum samples were diluted 200 times. [a]the linear range of the ELISA curves was located at 80 to 640 times dilution.

pathological change in the bursa represents mild necrosis and mild lymphocyte depletion of a few follicles, with the overall bursal architecture being maintained (**Fig. 3**). These pathological changes were similar to those of chickens infected with the parent IBDV. None of the bursas in group C displayed any pathological changes.

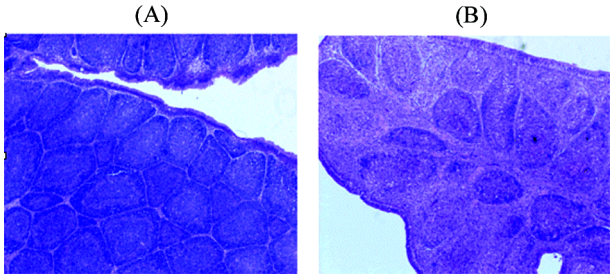

Fig. 3 Histopathological change in bursas infected with the recovered virus

(A) No bursal damage or lesion in any follicle, with clear demarcation of the medulla and cortex. (B) Mild necrosis and lymphocyte depletion of follicles. Magnification, 200×.

Detection of IBDV antigens and IBDV-like virions in bursas

All the bursal samples reacted with positive antibodies; however, no reaction was observed between normal bursas and positive antibodies (**Table 2**). This suggests that the IBDV antigen was expressed in bursas and induced anti-IBDV antibodies, as shown in **Fig. 2**. As **Fig. 4** shows, IBDV-like viral particles (55–60 nm, non-enveloped and

Table 2 AIDT detection of the IBDV antigen in bursas

Bursal sample	A1	A2	A3	B1	B2	B3	C1	C2	Positive antigen
Positive serum	+	+	+	+	+	+	–	–	+
Negative serum	–	–	–	–	–	–	–	–	–

Bursal samples A1–A3, B1–B3, C1 and C2 were selected randomly from Groups A, B and C, respectively. All antigen samples reacted with the positive serum except C1 and C2. However, no bursal sample reacted with the negative serum.

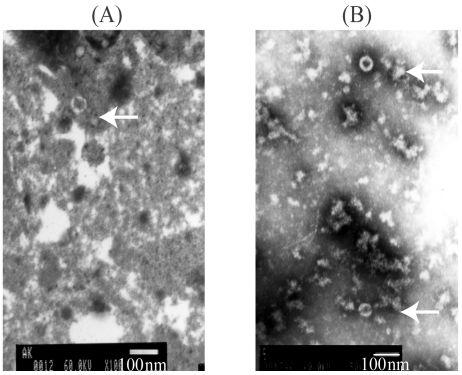

Fig. 4 Detection of IBDV-like virion structures through the electron microscope

Some IBDV-like virions (55–60 nm, non-enveloped and icosahedral-shaped) were observed in the thin sections (A) and extractions of bursas (B) through the electron microscope, as indicated by the arrows.

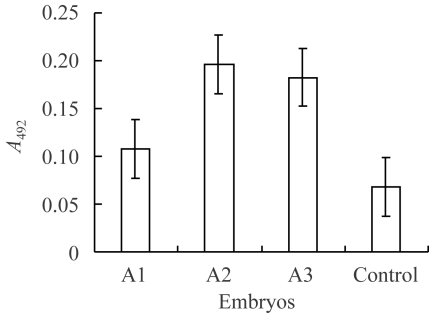

Fig. 5 ELISA detection of the IBDV antigen in allantoic fluids after infection [a]

A1, A2 and A3, allantoic fluid mixtures of the embryos infected with the supernatants of bursal samples A1, A2 and A3; Control, allantoic fluid mixture of the embryos inoculated with the mixture of bursal samples C1 and C2. All allantoic fluid mixtures were incubated at 60 °C for 30 min and diluted 5120 times for coating. [a] the linear range of the ELISA curves was located at 1280 to 20,480 times dilution.

icosahedral-shaped) were also observed under the electron microscope. These results indicate that the reassortant IBDV derived from pCI-AKZJ2000/pCI-mB was recovered in chickens after the plasmids were injected. The virus was named IBDV strain rAKZJ/HZ.

Embryo infection test of reassortant IBDV

None of the chicken embryos infected with the IBDV strain rAKZJ/HZ died. Seven days after inoculation, the allantoic fluids were collected for further analysis. All embryos died within 96 h after infection with the ZJ2000 strain, while none of the embryos in the negative control group died. As shown in **Fig. 5**, the allantoic fluids reacted with rabbit anti-IBDV serum and the linear range was located at 5120 times dilution. Similar results were obtained when the virus propagated in chicken embryos to the second and third generations. These results indicate that the IBDV strain rAKZJ/HZ can reproduce in embryos and shows low virulence.

Molecular mark detection in reassortant IBDV genomic segments

The genomic segment A (about 3300 bp) from the rAKZJ/HZ strain can be amplified both from bursal samples and allantoic fluids regulated with primers A3 and A5. However, the cDNA of segment B (about 2800 bp) can only be amplified from the allantoic fluid sample (**Fig. 6**). The segment A can be cleaved with *Nae*I into two fragments (about 900 bp and 2500 bp, respectively). The segment B was digested with *Eco*RV into two suitable segments as shown in **Fig. 6**. No specific bands were shown

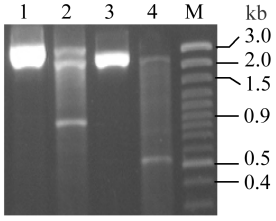

Fig. 6 Identification of the molecular marks in genomic segments of the recovered IBDV

1, RT-PCR products of segment A of the recovered IBDV (about 3300 bp); 2, PCR products of segment A of the recovered IBDV that were digested with *Nae*I (about 2500 bp and 900 bp, respectively); 3, PCR products of segment B of the recovered IBDV (about 2800 bp); 4, PCR products of segment B of the recovered IBDV that were digested with *Eco*RV (about 2250 bp and 550 bp, respectively); M, GeneRuler DNA ladder mix marker.

in the negative control. These results suggest that the cDNA of viral genomic RNA was transcribed correctly and that the genomic RNA of the recovered virus was site-mutated as expected.

Discussion

The creation of the virus recovery technique has paved the way for the basic research of viral proteins and genomes, especially RNA viruses. There are four main kinds of recovery methods for RNA viruses based on full-length infectious clones: (1) the recombinant vaccinia virus system that expresses the bacteriophage T7 RNA polymerase (VV/T7); (2) the extracellular transcription system; (3) the RNA polymerase I (pol I) system; and (4) the RNA polymerase II (pol II) system. Extra promoters included in the above systems initiate the viral RNA transcription and protein synthesis in cells or hosts. Then, the artificial virions are packaged with these RNAs and proteins under suitable conditions. In the pol II system, the transcriptions of infectious cDNA clones are initiated by the RNA pol II. The relatively high fidelity of the pol II and the stability of DNA transfection make this system more effective and feasible. The system also makes it possible to recover, in animal models, viruses benefiting from the transfection of cDNA.

Obtaining and marking the full-length genome of the target virus is the first step for virus recovery. We have developed a system to clone the IBDV segments A and B by LA RT-PCR [16], and several genomic cDNAs of IBDV strains have been cloned. The method based on cRNA transfection is the first established method for IBDV recovery. According to this method, the cDNA of the virus genome is transcribed into cRNA extracellularly and then transfected into cells. However, this method involves partial exposure of the RNA in question. The cDNA transfection system makes it possible to recover the RNA virus in eukaryotic cells or animal models transfected with cDNA clones. An infectious clone transfection recovery system based on the cell line for IBDV has also been established in our laboratory [11].

It has been reported that virulent strains of IBDV can not adapt to the cell culture and that the cell tropism determinants of IBDV are located in the VP2 protein. The IBDV field isolate strain ZJ2000 also can not replicate well in chicken embryonic fibrillation (CEF) cells. This makes it difficult to study the relationship between the viral genes and viral proteins of virulent strains of IBDV, such as the ZJ2000 strain and the reassortant virus that contains segment A from the ZJ2000 strain, by recovering the virus on cell lines. However, reverse genetics is one of the most important bases for studying viral characteristics. Animal models have provided another way to conduct reverse genetic research on virulent strains benefiting from the infectious clone transfection system. In the present paper, we report a novel recovery method for IBDV that uses chickens as the animal model.

Acting as a molecular marker, an *Nae*I site (GCcGGc) was inserted into segment A of IBDV strains by site-directed mutagenesis, resulting in the construction of pCI-AKZJ2000 and pCI-AKHZ2. The pCI-mB derived from the HZ2 strain was inserted into an *Eco*RV site. Fourteen-day-old non-immunized chickens received these plasmids in different combinations (Groups A and B). Anti-IBDV antibodies were induced in both Groups A and B after an injection of a low dose of plasmids (10 μg per chicken), and there was no significant difference between the groups (**Fig. 2**). As we have reported previously, pCI-VP2/4/3, acting as a DNA vaccine, can not induce antibodies effectively at doses lower than 50 μg per chicken [17]. This suggests that the high antibody titers in this study were induced not only because of the plasmid expression in chickens. There was another more efficient agent that induced the high antibody titers. Several bursas in both Groups A and B displayed a histopathological response similar to that caused by IBDV. IBDV-like virion structures were observed using the electron microscope. The IBDV antigen was also detected in bursas. As **Fig. 5** shows, the viral genomic RNA segments were amplified from bursal and allantoic fluid samples. The two segments were digested into two suitable fragments with *Nae*I and *Eco*RV, respectively, and the results showed that the recombinant viruses were recovered from bursas. The recovered virus showed good immunogenicity in inducing high humoral immune responses as shown in **Fig. 2**. The reassortant virus derived from pCI-AKZJ2000/pCI-mB was named the IBDV rAKZJ/HZ strain.

All the embryos died within 96 h after infection with the ZJ2000 strain. However, none of the embryos died 6 days after infection with the rAKZJ/HZ strain. This indicates that the reassortant virus strain rAKZJ/HZ might be relatively attenuated as compared with the ZJ2000 strain. Other data in this study also support this hypothesis. Two of eight chickens showed bursal histopathological changes in both Groups A and B. This implies that the viral protein 1 (VP1) encoded by segment B might play an important role in virus replication and pathogenicity. It has been reported that VP1 is associated with viral replication [18]; however, more experiments are necessary to investigate

how VP1 affects the replication and pathogenicity of IBDV.

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