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A single amino acid in the C-terminus of VP3 protein influences the replication of attenuated infectious bursal disease virus *in vitro* and *in vivo*

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ABSTRACT

The very virulent infectious bursal disease virus (vvIBDV) Gx strain causes over 60% mortality in chickens but cannot replicate in CEF cultures. The attenuated Gt strain, however, is not virulent in chickens and replicates well in CEF cultures. The two strains display differences in 6 amino acids in VP4 and 4 amino acids in VP3. To determine whether VP4 and VP3 are involved in the virulence and replication of IBDV, three chimeric viruses, in which the VP4/VP3/3′UTR, VP3/3′UTR or VP4 region of Gt were replaced by the corresponding region of Gx, were constructed and characterized *in vitro* and *in vivo*. The substituted regions in VP4 or VP3 did not affect virulence of Gt. While the substituted region in VP4 had no effect on viral replication of Gt in CEF cultures, substitution of the VP3/3′UTR region did reduce the replicative capacity of the virus. Through site-directed mutagenesis, three rescued recombinant viruses with a single amino acid substitution in the C-terminus of VP3 of the Gt strain (L981P, A990V and T1005A) were characterized in a similar manner. Amino acid substitution at position 990 reduced viral replication of Gt and reduced its efficacy of protection against vvIBDV Gx challenge *in vivo*. This study provides important information for the design and development of more effective IBDV vaccines using reverse genetics.

1. Introduction

Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens that causes considerable economic loss to the poultry industry worldwide (Cosgrove, 1962). The causative agent is infectious bursal disease virus (IBDV), which belongs to the genus *avibirnavirus* of the family *birnaviridae*. The genome consists of two segments, A and B, of double-stranded RNA. Segment B encodes the VP1 protein (90 kDa), the viral RNA-dependent RNA polymerase (von Einem et al., 2004). Segment A contains two partially overlapping open reading frames (ORF) (Hudson et al., 1986). The smaller ORF encodes the non-structural protein VP5 (17 kDa) (Mundt et al., 1995), while the larger one encodes a polyprotein of approximately 110 kDa (NH_2-pVP$_2$-VP$_4$-VP$_3$-COOH) that is proteolytically cleaved by the viral protease VP4 (Birghan et al., 2000) to form the proteins pVP2 (48 kDa), VP3 (32 kDa) and VP4 (24 kDa) (Hudson et al., 1986). VP2 and four peptides are derived from the maturation of pVP2 (Da Costa et al., 2002).

VP2 (amino acids [aa] 1–512) and VP3 (aa 756–1012) form the outer and inner capsids of the virus, respectively (Böttcher et al., 1997). VP2 is the main factor for the virulence, cell tropism and pathogenic phenotype of IBDV (Brandt et al., 2001). However, it is not the sole determinant of virulence (Boot et al., 2000). VP3, accounting for 40% of the total protein, is a multifunctional protein that interacts with pVP2, VP1, dsRNA and itself (Chevalier et al., 2002, 2004; González et al., 2005; Lombardo et al., 1999; Maraver et al., 2003a,b; Oña et al., 2004; Tacken et al., 2000, 2002, 2003). VP3 accomplishes numerous roles during the viral cycle and acts as a scaffolding protein required for assembly control (Luque et al., 2007). Previously, we attenuated the vvIBDV Gx strain to produce the Gt strain through continuous passage in specific-pathogen-free (SPF) chicken embryos (5 generations) and in CEF cultures (20 generations) (Wang et al., 2004). These two strains provide a good model system for studying the virulence, cell tropism and viral replication of IBDV.

Using our previously developed RNA polymerase II-based reverse genetic system (Qi et al., 2007), we generated three chimeric viruses based on the Gt strain as a backbone to carry various domains from the Gx strain which contain amino acid differences between the two strains. From characterizations of these chimeric viruses, we also constructed three Gt based viruses with a single amino acid substitution in the VP3 protein to further define

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the influence of this protein on virus replication. These viruses were also characterized *in vivo* to determine whether the sequence differences were responsible for IBDV replication and virulence in chickens.

2. Materials and methods

2.1. Plasmids, viruses, cells and animals

The recombinant plasmids, pUC18GtA, pCDNA4B-2GtA and pCDNA4B-2GxA, were constructed in our laboratory; pUC18GtA and pCDNA4B-2GtA contain the segment A cDNA sequence of the attenuated Gt strain, and pCDNA4B-2GxA contains the segment A cDNA sequence of the vvIBDV Gx strain. The eukaryotic expression vector pCAGGS (Niwa et al., 1991) was a kind gift from Dr. J. Miyazaki, University of Tokyo, Japan. The plasmid pCAGGmGtBHRT containing the segment B cDNA of Gt strain with genetic tags (PstI) and flanked by hammerhead ribozyme (HamRz) and hepatitis delta ribozyme (HdvRz) sequences was constructed by our laboratory previously. The virus rmGt was rescued from the genome of Gt in which there were EcoRV and PstI sites (as genetic tags) in segments A and B, respectively (Qi et al., 2007). DF-1 cells used for transfection were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator. Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old SPF chicken embryos. Further propagation and evaluation of replication kinetics of the rescued virus and indirect immunofluorescence were carried out in secondary CEF cultures. SPF chickens were purchased from and housed in the Experimental Animal Center of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China. All experiments were approved by the Animal Ethics Committee, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Deduced amino acid sequence analysis

The deduced amino acid sequences of polyprotein between Gx and Gt strains were analyzed with the sequence analysis software MegAlign (DNAStar Inc., Madison, WI, USA). Sequence alignment was performed using the Clustal W multiple sequence alignment program.

2.3. Construction of the segment A chimeras

Three chimeric IBDV plasmids were constructed. In these plasmids, the VP4/VP3/3′UTR (1727–3260 bp of segment A), VP3/3′UTR (2859–3260 bp of segment A) and VP4 (1727–2858 bp of segment A) regions of Gt were each replaced by the corresponding regions of Gx (Fig. 2).

To replace the VP4/VP3/3′UTR region of Gt with the corresponding region of Gx, the recombinant plasmid pCDNA4B-2GxA was digested with HincII/EcoRI to yield a fragment of 1534 bp containing VP4, VP3 and 3′UTR regions of Gx. The fragment was then ligated into pCDNA4B-2GtA that was digested with the same restriction enzymes to obtain the recombinant plasmid pCDNA4B-2GtA-GxVP4/VP3/3′UTR. Using the same strategy, the recombinant plasmid pCDNA4B-2GxA was digested with SphI and EcoRI to produce a short fragment of 402 bp containing the C-terminus of VP3 and 3′UTR of Gx. After ligation into SphI and EcoRI digested pCDNA4B-2GtA, the recombinant plasmid pCDNA4B-2GtA-GxVP3/3′UTR was established. To obtain the recombinant plasmid pCDNA4B-2GtA-GxVP4, a fragment of 1132 bp containing the C-terminus of VP4 and the N-terminus of VP3 of Gx was obtained by digesting the recombinant plasmid pCDNA4B-2GxA using HincII and SphI, followed by ligation of the fragment into HincII and SphI cleaved pCDNA4B-2GtA.

Using the three plasmids (pCDNA4B-2GtA-GxVP4/VP3/3′UTR, pCDNA4B-2GtA-GxVP3/3′UTR and pCDNA4B-2GtA-GxVP4) as the backbone templates, cDNA sequences of the HamRz (5′TGTTAAGCGTCTGATGAGTCCGTGAGGACGAAACTATAG GAAAGGAATTCCTATAGTC3′) and the HdvRz (5′GGGTCGGCATGGCATCTCCACCTC CTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGCACGTCCACTCG-GATG GCTAAGGGAGGGCG3′) were used to flank the segment A of the virus cDNAs with three pairs of primers P5/P6, P7/P8 and P7/P9 (Table 1) by three steps of PCR as described previously (Qi et al., 2007). The resulting PCR products were then digested with ClaI and KpnI and cloned downstream of the beta chicken actin promoter in the vector pCAGGS, which was digested with the same restriction enzymes. In this manner, recombinant eukaryotic expression vectors pCAGGGtA-GxVP4/VP3/3′UTRHRT, pCAGGGtA-GxVP3/3′UTRHRT and pCAGGGtA-GxVP4HRT (Fig. 2) were obtained. To confirm the correct sequence of these vectors, restriction mapping, electrophoresis of PCR products and sequencing analysis were performed.

2.4. Site-directed mutagenesis

The plasmid pUC18GtA was used as the backbone for the following site-directed mutagenesis. Mutations were introduced following protocols described previously (Qi et al., 2007). The mutagenic primers were 20–30 bases in length, and the desired mutation was in the center of the primer (Gt3072U/Gt3072L, Gt3099U/Gt3099L and Gt3143U/Gt3143L, Table 1). Sequence analysis showed that the mutations were successfully introduced. HamRz and HdvRz were then used to flank the segment A of the virus cDNAs and recombinant eukaryotic expression vectors were constructed as described above. After the size and restriction patterns were confirmed by PCR, the three recombinant eukaryotic vectors (pCAGGGtA-L981PHRT, pCAGGGtA-A990VHRT and pCAGGGtA-T1005AHRT) were confirmed by sequencing analysis (Fig. 2).

2.5. Virus rescue from plasmids

To rescue the three chimeric and three mutated IBDV strains, the plasmids pCAGGGtA-GxVP4/VP3/3′UTRHRT, pCAGGGtA-GxVP3/3′UTRHRT, pCAGGGtA-GxVP4HRT, pCAGGGtA-L981PHRT, pCAGGGtA-A990VHRT and pCAGGGtA-T1005AHRT were purified using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the Manufacturer's protocol, then each plasmid was co-transfected with pCAGGmGtBHRT into DF-1 cells, following the previously published procedure (Qi et al., 2007). The rescued virus stocks (rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4, rGtA-L981P, rGtA-A990V and rGtA-T1005A) were serially passaged seven times in the secondary CEF cultures.

2.6. Identification of rescued virus

To determine the specificity of the rescued viruses, indirect immunofluorescence assay (IFA) was performed on secondary CEF monolayers using monoclonal antibodies (mAbs, 1:100 dilution) against the VP2 protein of the IBDV Gt strain (generated by our laboratory). To verify whether the rescued viruses were chimeric, viral RNAs were extracted and analyzed by RT-PCR, as described previously (Qi et al., 2007). For each virus, two fragments were amplified using the primer pairs F9VP2Q1512U/A38 (1421–3260 bp of segment A) and B3P/B37 (2009–2827 bp of segment B) (Table 1) and then sequenced. The full-length genomes of the rescued viruses were also amplified and sequenced as described previously (Qi et al., 2007).

Table 1
PCR primers used in the study.[a].

Name	Sequence	Orientation	Position (nt)
P5	tgaggacgaaactataggaaaggaattcctatagtcGGATACGATCGGTCTGAC	Sense	A: -36~18
P6	cggaccgcgaggaggtggagatgccatgccgacccGGGGACCCGCGAACGGATC	Antisense	A: 3242~+35
P7	ATTAATCGATtgttaagcgtctgatgagtccgtgaggacgaaactataggaaag	Sense	A: -58~-15
P8	gagtggacgtgcgtcctccttcggatgcccaggtcggaccgcgaggaggtggag	Antisense	A: +16~+69
P9	ATTAGGTACCcgccctcccttagccatccgagtggacgtgcgtcctccttc	Antisense	A: +48~+88
A38	TAGGTACCGGGGACCCGCGAACGGATCCAATTTGGGAT	Antisense	A: 3231~3260
B37	GCTCTAGAGGGGGCCCCCGCAGGCGAAGGCCGGGGAT	Antisense	B: 2799~2827
F9VP2Q1512U	GACCTCAACTCTCCCCTGAAG	Sense	A: 1421~1441
B3P	ACTACCCACTCCTGAACAAA	Sense	B: 2009~2028
Gt3072U	CAGGCGGGCTCcACCAAAGCCCAAG	Sense	A: 3061~3085
Gt3072L	CTTGGGCTTTGGTgGAGCCCGCCTG	Antisense	A: 176~200
Gt3099U	CCAAAACCCAATGtTCCAACACAGAG	Sense	A: 3086~3111
Gt3099L	CTCTGTGTTGGAaCATTGGGTTTTGG	Antisense	A: 150~175
Gt3143U	CGCTGGATCAGGgCCGTCTCTGATGAG	Sense	A: 3131~3157
Gt3143L	CTCATCAGAGACGGcCCTGATCCAGCG	Antisense	A: 103~130

[a] The binding positions of primers B37 and B3P are in accordance with the published sequence of IBDV Gx strain (GenBank accession numbers: AY444873 and AY705393). The binding positions of other primers are in accordance with the published sequence of the Gt strain (GenBank accession numbers: DQ403248 and DQ403249). Virus specific sequences are underlined. Ribozyme sequences are in lower-case characters, and the introduced restriction sites are highlighted by boxes. Symbols "+" and "−" in front of the positions of nucleotides indicate the nucleotides are localized upstream and downstream of the genome, respectively. Primer pairs of Gt3072U/Gt3072L, Gt3099U/Gt3099L and Gt3143U/Gt3143L were used for site-directed mutagenesis at positions of 3072, 3099 and 3143, respectively. The introduced mutated nucleotides are in lower-case letters in the center of the primers.

2.7. Evaluation of replication kinetics of rescued viruses in CEF cells

To evaluate the replication ability of rescued viruses (rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4, rGtA-L981P, rGtA-A990V and rGtA-T1005A) compared with rmGt, growth curve assays were performed. Cell culture flasks (25 cm^2) each containing a confluent monolayer of CEF cells were inoculated with 0.1 ml of viral suspension containing 10^4 50% cell culture infectious doses ($CCID_{50}$) of rescued viruses based on the titer determined in CEF cells. After a 2 h absorption period at 37 °C, the inoculum was removed. Cells were then washed with DMEM and 5 ml of maintenance media was added to each flask before the flasks were put back in the incubator. The supernatant was removed every 12 h to determine the titer of the infectious progeny in terms of $CCID_{50}$ per milliliter using the Reed–Muench formula. All experiments were repeated three times.

2.8. Characterization of rescued virus in SPF chickens

The virulence, replicative properties in bursa of fabricius (BF) and ability to protect against virulent Gx virus challenge of the rescued viruses were evaluated *in vivo*. One hundred and fifty, 2-week-old, SPF broiler chickens were divided randomly into six groups. Each group was maintained in isolators with negative pressure and filtered air. Chickens were infected intranasally and intraocularly with $10^{4.4}$ $CCID_{50}$/0.1 ml (200 μl/chicken) rescued virus or DMEM medium (group 1: rmGt, 45 chickens; group 2: rGtA-A990V, 45 chickens; group 3: rGt-GxVP4/VP3/3′UTR, 15 chickens; group 4: rGt-GxVP3/3′UTR, 15 chickens; group 5: rGt-GxVP4, 15 chickens; group 6: DMEM, 15 chickens).

Chickens were monitored for clinical signs daily. At 1, 3, 5, 7 and 14 days post-inoculation (d p.i.), 3 chickens were randomly selected and euthanized for necropsy from groups 1 to 6. The bursa and body weights of each chicken were determined, and the bursa:body-weight index (BBIX) was calculated according to the following formula: BBIX = (bursa:body-weight ratios)/(bursa:body-weight ratios in negative group). Bursae with a BBIX lower than 0.70 were considered atrophied (Lucio and Hitcher, 1979). Each bursa was divided into two sections: one for histopathological analysis as described (Boot et al., 2002) from which the severity of bursal follicular necrosis was recorded using average histopathologic bursal lesion scores (HBLS) (Schröder et al., 2000); the other was used for confirmation of virus identity during infection. RNAs of rescued viruses were amplified by RT-PCR using the primer pairs F9VP2Q1512U/A38 (1421–3260 bp of segment A) and B3P/B37 (2009–2827 bp of segment B) and sequenced.

A real-time RT-PCR assay was performed to quantify the level of viral RNA in the bursal tissue of SPF chickens infected with rGtA-A990V or rmGt. At 1, 3, 5, 7 and 14 d p.i., three chickens from group 6 and six chickens randomly selected from groups 1 and 2 were then euthanized. The BF of each chicken was removed for quantitation of viral RNA loads using real-time RT-PCR. The bursal tissue was collected and either stored at −70 °C or processed immediately. Bursal tissue was homogenized in chilled sterile phosphate-buffered saline. The viral RNA was extracted using PureLink™ Viral RNA/DNA kit (Invitrogen) according to the Manufacturer's instructions. Then, reverse transcription and real-time RT-PCR were both performed as described previously (Wang et al., 2009).

Sixty-four, 11-day-old SPF chickens were used for evaluating protective efficacy between rmGt and rGtA-A990V. The chick-

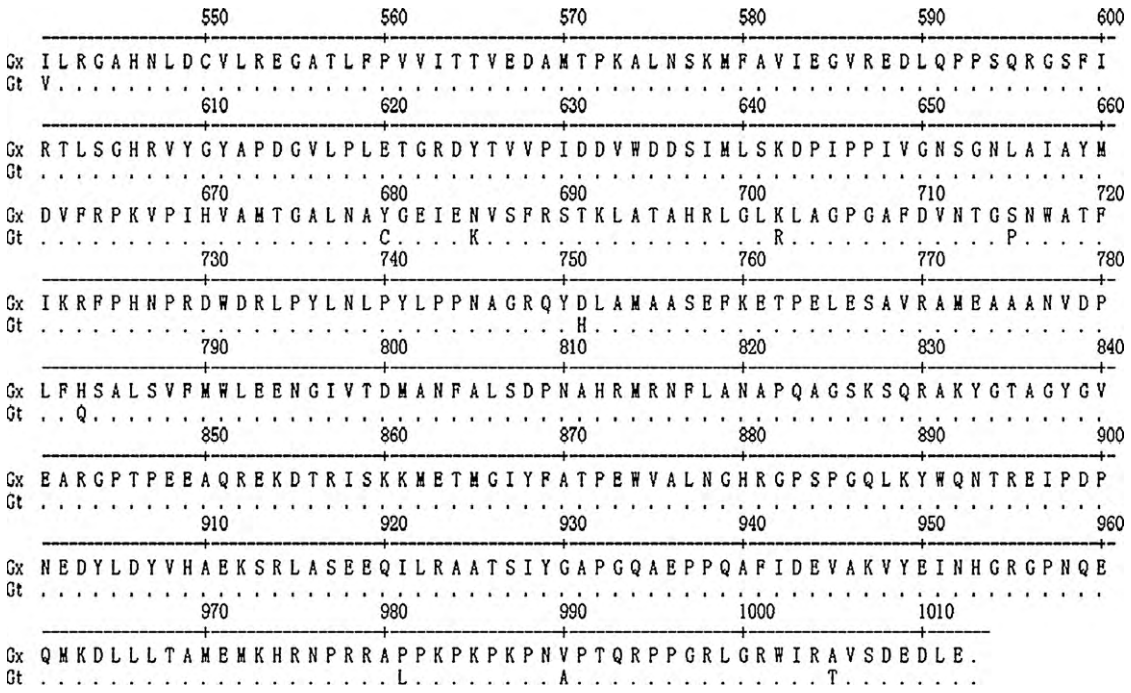

Fig. 1. Alignment of deduced amino acid sequences of VP4 (513–755 amino acids of polyprotein) and VP3 (756–1012 amino acids of polyprotein) between Gx (AY444873) and Gt (DQ403248).

ens were immunized intranasally and intraocularly with $10^{4.4}$ $CCID_{50}$/0.1 ml (200 μl/chicken) rmGt or rGtA-A990V. In addition, 20 chickens which received an equal volume of DMEM were used as the challenge control. Serum was collected from blood of six chickens randomly selected from each group at 14 days post-immunization and antibody titers against IBDV were measured by a commercial antigen coated ELISA kit (IDEXX, Westbrook, Maine). All vaccinated and unvaccinated control chickens were challenged with $10^{2.72}$ ELD_{50} (50% embryo lethal dose) of vvIBDV Gx strain through the intranasal and intraocular routes at 14 days post-immunization. The mortality was calculated at 10 days post-infection.

2.9. Statistical analysis

The viral titers both *in vitro* and *in vivo* were presented as average ± standard deviation. One-way ANOVA with Tukey's post hoc test was employed to evaluate the difference of viral titers among different groups using SPSS 17.0 (SPSS Inc., Chicago, IL). Statistical significance was set at $P < 0.05$ for all tests.

3. Results

3.1. Deduced amino acid sequence analysis

The deduced amino acid sequences were compared between Gx and Gt strains. Alignment results indicated that 6 aa were different in VP4, and 4 aa were different in VP3 between the two strains (Fig. 1).

3.2. Rescue of virus

The rescued IBDV strains named rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4, rGtA-L981P, rGtA-A990V and rGtA-T1005A were serially passaged for seven generations in the secondary CEF cultures. Positive signals were detected by IFA using mAbs against VP2 of IBDV in rescued virus-inoculated CEF cultures (data not shown), but not in the control monolayer. RT-PCR results showed fragment size patterns as expected from the CEF samples infected with rescued viruses. In addition, sequencing analysis indicated that the nucleotide sequence of these rescued viruses were successfully replaced or mutated (data not shown).

3.3. Amino acid substitution at position 990 of VP3 C-terminus of rmGt reduces viral replication in vitro

To compare the replication ability of different rescued viruses, the replication kinetics of rescued viruses in CEF cultures were obtained in triplicate. As shown in Fig. 3, strain rGt-GxVP4/VP3/3′UTR exhibited a slight delay at 36 h post-inoculation (h p.i.) compared to other viruses, and strain rGt-GxVP3/3′UTR replicated very slowly in the CEF cultures after 36 h p.i. The replication kinetics of rGt-GxVP4 were nearly the same as that of the parental virus rmGt. However, the titers of rGt-GxVP4/VP3/3′UTR and rGt-GxVP3/3′UTR were about 1.5 log units lower than that of rmGt after 48 h p.i. suggesting that the VP3/3′UTR region influenced viral replication in CEF cultures. Moreover, the replication kinetics of rGtA-L981P and rGtA-T1005A were almost the same as the parental virus rmGt, while rGtA-A990V was similar to rGt-GxVP3/3′UTR, yielding about 1.5 log units lower than that of rmGt. The viral titers of rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR and rGtA-A990V were significantly lower ($P < 0.05$) than that of rmGt, rGt-GxVP4, rGtA-L981P and rGtA-T1005A at 60 h p.i. These results indicate that the amino acid at position 990 plays a critical role in viral replication of rmGt *in vitro*.

3.4. Amino acid substitution at position 990 in VP3 C-terminus of rmGt reduces viral replication in vivo

The viral replication properties of rmGt and rGtA-A990V in bursa were determined by real-time RT-PCR. After 1 day post-inoculation (d p.i.), the viral load began to increase in bursal tissue inoculated with rmGt. The peak load of rmGt was detected at 5 d p.i. and maintained to 7 d p.i. Compared with rmGt, rGtA-A990V replicated slowly in bursa and had a load approximately 2 log units lower ($P < 0.05$) than that of rmGt at 3, 5 and 7 d p.i. (Fig. 4). These results

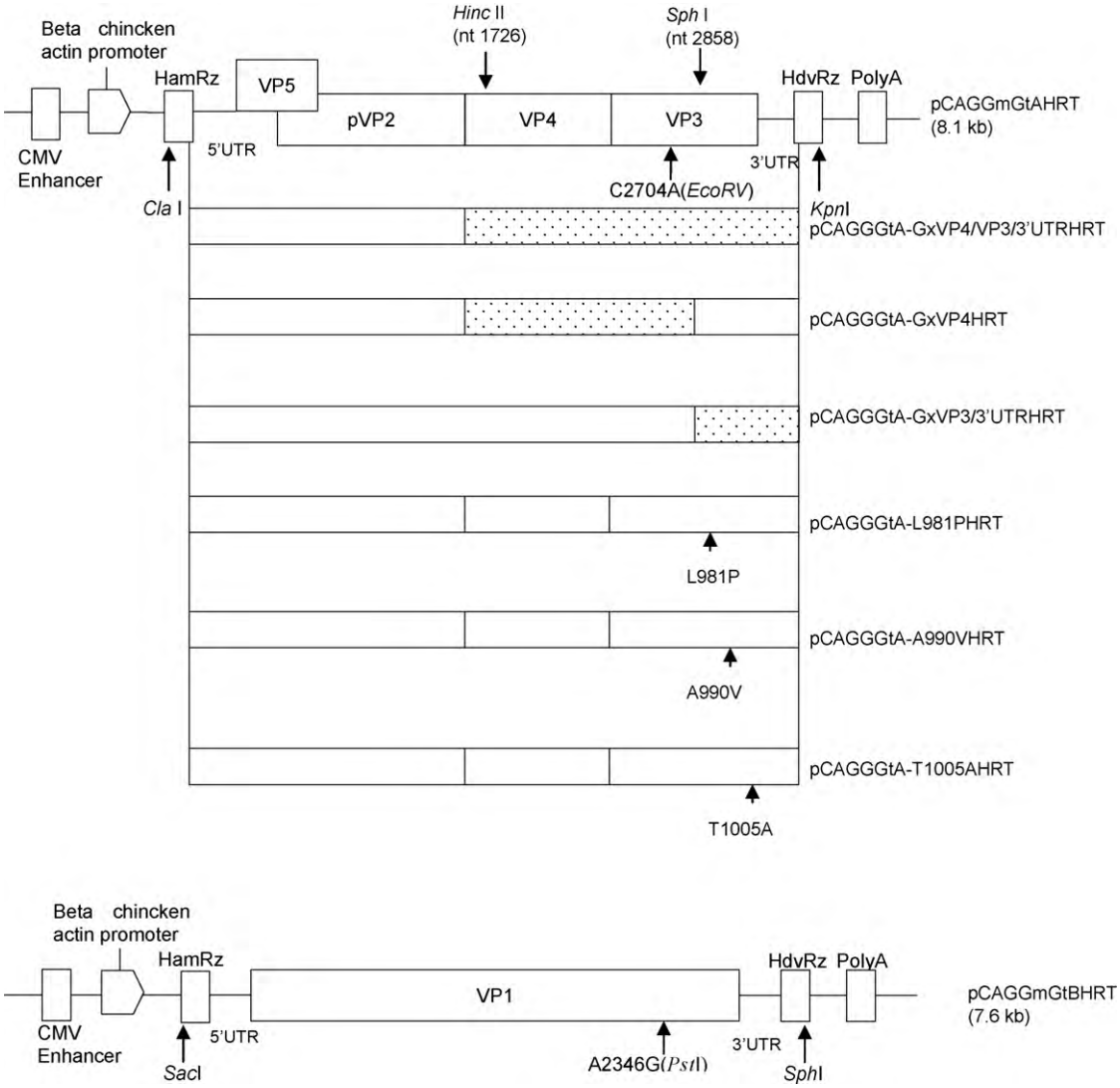

Fig. 2. Schematic diagrams of the recombinant eukaryotic expression vectors containing the chimeric cDNAs of segment A and segment B of infectious bursal disease virus (IBDV) (not drawn to scale). Sequences of strain Gt are depicted by an open box and sequences of strain Gx are marked by a shaded box. The cDNA sequences are preceded by a cytomegalovirus enhancer and a beta chicken actin promoter. cDNAs of HamRz and HdvRz flank each side of the gene of interest. The restriction enzyme sites (ClaI, EcoRI, HincII, KpnI, and SphI) used for the construction of recombinant vectors are shown. The genetic tags (C2704A and A2346G) that introduce unique restriction enzyme site (EcoRV or PstI) into segment A or B of Gt are also indicated.

further suggest that amino acid 990 plays a critical role in viral replication of rmGt *in vivo*.

3.5. Amino acid substitution at position 990 in VP3 C-terminus reduces protective efficacy of rmGt against vvIBDV challenge

The results of antibody response and challenge are summarized in Table 2. Thirty-two chickens immunized with rmGt had 100% protection against challenge by vvIBDV Gx, and five out of six chickens (83.3%) that were randomly tested had positive anti-IBDV antibodies in this group. However, the rescued virus rGtA-A990V could not offer complete protection against vvIBDV Gx with only 29 out of 32 chickens (90.6%) surviving, and only 2 out of 6 chickens (33.3%) had positive anti-IBDV antibodies in that group. By contrast, the mortality rate of the non-immunized control group was 60%. These results indicate that the amino acid substitution at 990 reduced the efficacy of the rmGt strain to protect against vvIBDV challenge.

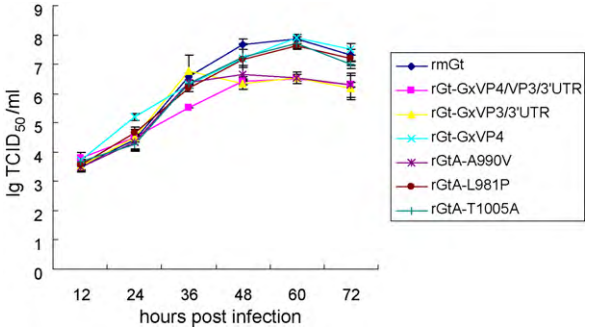

Fig. 3. Replication kinetics of rescued viruses *in vitro*. Cell culture flasks (25 cm^2) containing confluent monolayers of chicken embryo fibroblasts (CEF) cells were inoculated with 0.1 ml of virus suspension containing 10^4 $CCID_{50}$ of rescued viruses (rmGt, rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4, rGtA-L981P, rGtA-A990V and rGtA-T1005A). Virus media were harvested at indicated time points and the titers were determined and expressed as the mean titer (log of $CCID_{50}$). Error bars represent the standard deviation of triplicate assays for each time point.

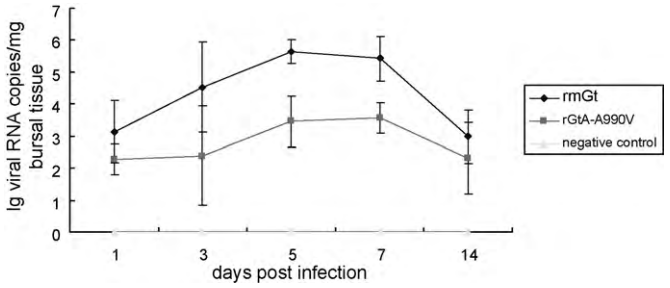

Fig. 4. Replication kinetics of rescued viruses in BF of chickens evaluated by real-time RT-PCR. Two-week-old SPF chickens were infected intranasally and intraocularly with $10^{4.4}$ $CCID_{50}$/0.1 ml (200 μl/chicken) rescued virus (rmGt or rGtA-A990V) or DMEM (negative control). The BF was then collected and homogenized at indicated time points. Subsequently, viral RNA loads were quantified using real-time RT-PCR. Data are the mean titer (log of viral RNA copies) per mg of tissue, and the error bars represent the standard deviations for each time point from six assays.

Table 2
Efficacy of rescued viruses against vvIBDV challenge.

Groups	Number of chickens	Anti-IBDV antibody[a]	Mortality[b]	Protection[c] (%)
rmGt	32	5/6	0/32	100
rGtA-A990V	32	2/6	3/32	90.625
Control	20	0/6	12/20	40

[a] Number of positive samples/total number of sera samples. Sera were obtained at 14 days post-immunization and antibody titers against IBDV were measured by a commercial antigen coated ELISA kit. The results were determined according to the instructions of the Manufacturer.

[b] Number of dead chickens/total number of challenged chickens. The test groups were challenged with $10^{2.72}$ ELD_{50} vvIBDV Gx at 14 days post-immunization. The chickens were monitored daily, and mortality was calculated at 10 days post-challenge.

[c] Protection defined as number of surviving chickens/total number of challenged chickens in that group.

3.6. Pathogenicity of the rescued viruses in SPF chickens

To evaluate the virulence and pathogenic phenotype of the rescued viruses, pathological tests were conducted. Throughout the 14-day experimental period, neither gross pathology nor bursa atrophy was observed in different groups inoculated with rmGt, rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4, rGtA-A990V or DMEM control. In addition, histopathological sections of bursae derived from all groups showed normal follicles and follicular connective tissues, and no microscopic lesions were observed (data not shown).

3.7. Analysis of rescued virus re-isolated after infection in SPF chickens

Based on the RT-PCR results, a 1848 bp fragment (part of segment A) and an 827 bp fragment (part of segment B) were amplified from the bursae of groups inoculated with rmGt, rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR, rGt-GxVP4 or rGtA-A990V. Sequence analysis showed that all rescued viral sequences were 100% identical to their respective parental plasmids (data not shown).

4. Discussion

Since the first successful rescue of IBDV in 1996 (Mundt and Vakharia, 1996), reverse genetics has been an excellent tool for confirming molecular determinants of virulence, cell tropism, and viral replication of IBDV. In our laboratory, vvIBDV Gx strain causes over 60% mortality in chickens but cannot replicate in CEF cultures. The attenuated Gt strain, however, has no virulence in chickens and replicates well in CEF cultures. Mutations occurred in the genome during the attenuation, while antigenicity and immunogenicity were preserved as described in other studies (Lazarus et al., 2008; Yamaguchi et al., 1996). Sequence analysis indicated that there were differences in 4 aa in VP3, 6 aa in VP4, 8 aa in VP5, 13 aa in VP2 and 17 aa in VP1 between Gx and Gt. Additionally, the VP1 of Gx strain has one more aa in C-terminus than that of Gt (data not shown). We previously demonstrated that aa 253 with 284 together in VP2 are responsible for the cell tropism and virulence (Qi et al., 2009). It is well known that VP3 is a multifunctional protein and plays numerous roles during the viral cycle (Luque et al., 2007). Therefore, in this study we focused on whether the aa differences in VP3 and VP4 between Gx and Gt are involved in virulence or viral replication. Three chimeric viruses (rGt-GxVP4/VP3/3′UTR, rGt-GxVP3/3′UTR and rGt-GxVP4) were rescued and subsequently characterized *in vitro* and *in vivo* in comparison with rmGt. The results indicated that the replacement of either VP4/VP3/3′UTR, VP3/3′UTR or VP4 of Gt with the corresponding regions of Gx could not enhance the virulence of IBDV in SPF chickens. Previously, Boot et al. (2000) observed that replacement of neither VP4 nor VP3 of strain CEF94 with the corresponding regions of D6948 could enhance virulence and affect viral replication. Data in this study showed that replacing VP4 of Gt with the corresponding region of Gx did not have an impact on viral replication, which was consistent with earlier studies (Boot et al., 2000; Brandt et al., 2001). However, the VP3/3′UTR region was determined to be involved in viral replication. The C-terminus of VP3 has been defined to be a multifunctional domain that interacts with VP1 (Maraver et al., 2003a) and dsRNA (Tacken et al., 2002). Sequence analysis showed that 3 out of 4 aa differences in VP3 between Gx and Gt were localized at the C-terminus, suggesting that these amino acids might participate in the viral replication through regulation of protein–protein or protein–dsRNA interactions. To confirm whether a single amino acid could impact viral replication, three rescued viruses with a single amino acid substitution in the C-terminus of VP3 were characterized in this study. The results indicated that a substitution of aa 990 reduced the viral replication both *in vitro* and *in vivo*. The level of anti-IBDV antibody was affected since the viral load was about 100-fold lower than that of rmGt in BF, thus reducing the protective efficacy against vvIBDV Gx challenge.

In summary, our findings indicate that the C-terminus of VP3 influences viral replication both *in vitro* and *in vivo*, and more specifically the amino acid at position 235 of VP3 (or position 990 of the polyprotein) plays a critical role. Additionally, other amino acid changes in VP4 or VP3 were determined not to affect viral replication. Our study provides important information for the design and development of more effective IBDV vaccines using reverse genetics.

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