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Naturally occurring mutations at residues 253 and 284 in VP2 contribute to the cell tropism and virulence of very virulent infectious bursal disease virus

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ABSTRACT

Infectious bursal disease virus (IBDV) is responsible for the highly contagious infectious bursal disease in chickens. Previously, by blind passage, a vvIBDV Gx strain was attenuated to the Gt strain, and a strain CEF-9 with intermediate characters was obtained during attenuation. Since CEF-9 exhibited only two interesting amino acid mutations (Q253H and A284T) on loops P_{DE} and P_{FG} at the tip of VP2 spikes, we hypothesized that, either function separately or in combination, they define the cell tropism and virulence of vvIBDV. To test this hypothesis, Q253H and A284T were introduced individually or in combination into VP2 of the Gx or Gt strain to obtain six modified clones. Using reverse genetics, combined mutations of Q253H and A284T could adapt vvIBDV to non-permissive CEF cells (rGx-F9VP2) but any single mutation could not. In vivo, rGx-F9VP2 did not cause mortality while the Gx strain induced 66.7% mortality. Dual evidence from natural and rescued strains identified that the cell tropism of vvIBDV to CEF cells was determined by the combined VP2 mutations Q253H and A284T, but not by single mutation. The two residues were mainly responsible for the virulence of vvIBDV. These findings may be helpful in the design of new tailored IBDV vaccines.

1. Introduction

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease in chickens and has been posing a great threat to the commercial poultry industry worldwide (Cosgrove, 1962; Müller et al., 2003). Since the discovery of the classic strain during the first outbreak of IBD in 1957 (Cosgrove, 1962), antigenic variant (Jackwood and Saif, 1987) and very virulent IBDV (vvIBDV) (Chettle et al., 1989) have emerged and represent new challenges for effective prevention and control of IBD.

In genus *Avibirnavirus* of the *Birnaviridae* family, IBDV has a non-enveloped capsid structure containing a double-stranded RNA genome of two segments, A and B. Segment A contains two partially overlapping open reading frames (ORFs). The smaller ORF encodes the nonstructural viral protein 5 (VP5) and the larger one encodes a polyprotein precursor NH_2–pVP2–VP4–VP3–COOH that is self-cleaved to form viral proteins VP2, VP3, and VP4 (Hudson et al., 1986). VP2 is the major structural protein and the only component of the icosahedral capsid (Coulibaly et al., 2005; Saugar et al., 2005); it is responsible for virulence, cell tropism, as well as antigenic variation and contains conformation-dependent, neutralizing epitopes of IBDV (Boot et al., 2000; Brandt et al., 2001; Jackwood et al., 2008; Letzel et al., 2007; Lim et al., 1999; Van Loon et al., 2002). VP3 is another structural protein of multiple functions involved in the viral cycle and acting as a scaffold protein for virus assembly (Luque et al., 2007). VP4 is a viral protease responsible for self-processing of the IBDV polyprotein (Birghan et al., 2000; Lejal et al., 2000). Segment B encodes the VP1 protein, the viral RNA-dependent RNA polymerase (Von Einem et al., 2004).

It is well known that wild IBDV strain cannot grow in cell culture but can be adapted to culture conditions and attenuated by blind passage. This method of attenuation has been used to develop live vaccine strains of IBDV from wild strain, however, it is a time consuming and laborious process with uncertain results. In the past decade, the role of VP2 in cell tropism or virulence has attracted great attention (Boot et al., 2000; Brandt et al., 2001; Lim et al., 1999; Mundt, 1999; Van Loon et al., 2002). However, the precise molecular determinants are still somehow unclear (Brandt et al., 2001; Lim et al., 1999; Mundt, 1999; Van Loon et al., 2002). It is greatly important to identify potentially critical mutations in the structure protein VP2 that determine the cell tropism and virulence.

In our previous study, by blind passage, a vvIBDV Gx strain isolated in China was adapted to chicken embryo fibroblast (CEF) cell culture and attenuated to the Gt strain (Wang et al., 2003). During attenuation, a strain CEF-9 with intermediate virulence and

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Table 1
Primers for genome cloning and mutagenesis of IBDV[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
P10	tgaggacgaaactataggaaaggaattcctatagtcGGATACGATGGGTCTGAC	sense	B: -36~18
P11	cggaccgcgaggaggtggagatgccatgccgacccGGGGGCCCCCGCAGGCGAA	antisense	B: 2809~+35
P15	ATTACTCGACcgccctcccttagccatccgagtggacgtgcgtcccctte	antisense	B: +48~+88
P16	ATTAGAGCTCtgttaagcgtctgatgagtccgtgaggacgaaactataggaaag	sense	A: -58~-15
GxAU	GGAATTCGGATACGATCGGTCTGAC	sense	A: 1~18
GxA1477L	AGGTAGCCCATGTCTGGT	antisense	A: 1460~1477
GxAA889TU	CAAACAAGCGTCCAtGGCCTTATACTG	sense	A: 875~901
GxAA889TL	CAGTATAAGGCCaTGGACGCTTGTTTG	antisense	A: 875~901
GxAG980AU	CAATGGGCTAACGaCCGGCACTGACAAC	sense	A: 967~994
GxAG980AL	GTTGTCAGTGCCGGtCGTTAGCCCATTG	antisense	A: 967~994
GtAC889AU	CGAACAAGCGTCCAaGGTCTTGTACTG	sense	A: 875~901
GtAC889AL	CAGTACAAGACCtTGGACGCTTGTTCG	antisense	A: 875~901
GtAA980GU	CAATGGGCTGACGgCCGGCACCGACAAC	sense	A: 967~994
GtAA980GL	GTTGTCGGTGCCGGcCGTCAGCCCATTG	antisense	A: 967~994
B3P	ACTACCCACTCCTGAACAAA	sense	B:2009~2028
B37	GCTCTAGAGGGGGCCCCCGCAGGCGAAGGCCGGGGAT	antisense	B: 2799~2827

[a] The positions where the primers GxAU, GxA1477L, GxaA889TU, GxaA889TL, GxAG980AU, GxAG980AL, B3P and B37 bind are in accordance with the published sequence of IBDV strain Gx (GenBank accession nos. AY444873 and AY705393). The positions where other primers bind are in accordance with the published sequence of strain Gt (GenBank accession nos. DQ403248 and DQ403249). The virus-specific sequences are underlined, the ribozyme sequences are lower-case characters, and the introduced restriction sites are highlighted by boxes. Orientation and position of the virus-specific sequences of the primers are shown. The "+" or "−" symbols in front of the positions of nucleotides indicate the upstream or downstream of the genome, respectively. The nucleotides without any symbols are the nucleotides for protecting the digestion sites of the restriction enzymes.

adaptation to cell culture was isolated (Wang et al., 2004, 2007). In this follow-up study, we started from the observation that CEF-9 exhibited an interesting gene pattern in VP2. We investigated the amino acid differences in VP2 by multiple sequence alignment in order to identify the putative amino acids responsible for the cell tropism and virulence of vvIBDV. We then used our previous RNA polymerase II-directed reverse genetic system (Qi et al., 2007) to introduce selected mutations into the backbone of Gx and Gt in order to elucidate the role of the individual amino acids.

2. Materials and methods

2.1. Viruses, cells, and plasmids

The typical vvIBDV strain Gx was previously isolated in China and identified by the INCO-China project (contract ERB IC18-CT98-0330). It had been attenuated to strain Gt by blind passage in vivo (Wang et al., 2003). The virus of CEF-9 is an intermediate strain during the attenuation (Wang et al., 2004, 2007). The virus rmGt was previously rescued from Gt cDNA (Qi et al., 2007). DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. Primary CEF cells were prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos. Further propagation of the recovered virus, the replication kinetics and the indirected immunofluorescence assay (IFA) were carried out in secondary CEF cells. The eukaryotic expression vector pCAGGS (Niwa et al., 1991) was kindly supplied by Dr. J. Miyazaki, University of Tokyo, Tokyo, Japan. The plasmids pBluGxA and pBluGxB, each containing segments A and B of Gx, were constructed previously (Gao et al., 2007). The plasmid pCAGGmGtBHRT (pCmGtBHRT) containing segment B of Gt flanked by a ribozyme sequence was also constructed previously (Qi et al., 2007).

2.2. Animals

Fourteen-day-old SPF chickens were purchased from the Experimental Animal Center of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS), China and housed in the negative-pressure-filtered air isolators. Animal experiments were approved by the Animal Ethics Committee of the Institute.

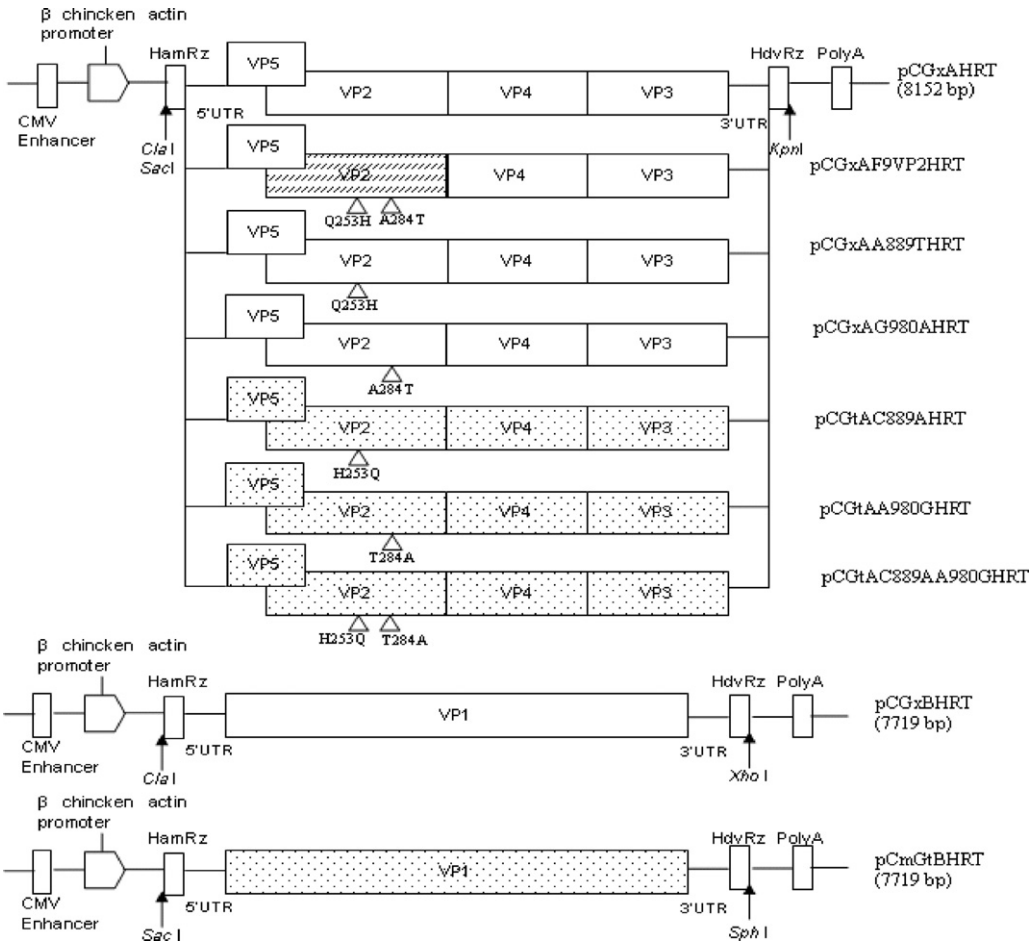

Fig. 1. Schematic diagrams of the recombinant eukaryotic expression plasmids containing the modified cDNAs of segment A and segment B of IBDV (not drawn to scale). Plasmid pCGxAHRT contains the wild type cDNA of vvIBDV Gx without modification (depicted by an open box). In plasmid pCGxAF9VP2HRT, replacement of VP2 gene with that of intermediate strain CEF-9 (a box with oblique lines) resulted in amino acid substitutions Q253H and A284T. In plasmids pCGxAA889THRT and pCGxAG980AHRT, a nucleotide substitutions A889T and G980A resulted in amino acid substitutions Q253H and A284T, respectively. In plasmids pCGtAC889AA980GHRT, pCGtAC889AHRT and pCGtAA980GHRT, nucleotide substitutions C889A and A980G resulted in amino acid substitutions H253Q and T284A in VP2 of attenuated strain Gt (a box with dots), simultaneously and respectively. The genome cDNA sequences are preceded by a cytomegalovirus enhancer and a beta chicken actin promoter and are flanked by the cDNAs of HamRz and HdvRz. The restriction enzyme sites (*Cla*I, *Kpn*I, *Sac*I, *Cla* I, *Xho* I, *Sph* I) used for the construction of recombinant vectors are shown. Only pCGxAF9VP2HRT was successful in generation of rescued recombinant IBDV while all others were unsuccessful.

2.3. Amplification of VP2 gene of CEF-9

Total RNA was extracted from CEF cells infected with the intermediate strain CEF-9 of IBDV using RNAiso Reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Reverse transcription was carried out by M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). The cDNA was used to amplify VP2 gene of CEF-9 using one pair of primers GxAU/GxA1477L (Table 1) directed by PrimeSTAR^{TM} HS DNA Polymerase (Takara Biotechnology Co., Ltd., Dalian, China). The PCR products were purified with Biospin Gel Extraction kit (BioFlux, Hangzhou, China) and then ligated into the pMD18T vector (Takara Biotechnology Co., Ltd., Dalian, China). The recombinant plasmid was confirmed by sequencing and named pTF9VP2.

2.4. Construction of the full-length cDNA clones of Gx

As described previously (Qi et al., 2007), the hammerhead ribozyme (HamRz) cDNA sequence (5′-TGTTAAGCGTCTGATGAGTCCGTGAGGACGAAACTATAGGAAAGGAATTCCTATAGTC-3′) and hepatitis delta ribozyme (HdvRz) cDNA sequence (5′-GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGC ATCCGAAGGAGGACGCACGTCCACTCGGATGGCTAAGGGAGGGCG-3′) were fused to segment A of Gx strain using pBluGxA as template and three primer pairs P5/P6, P16/P8, and P16/P9 were used one by one in the three consecutive PCR reactions. Similarly, the HamRz and HdvRz were fused to segment B of Gx using pBluGxB as template and three primer pairs P10/P11, P7/P8, and P7/P15 were used, respectively, in another three consecutive PCRs. The final PCR products GxAHRT and GxBHRT were digested with *Sac*I/*Kpn*I or *Cla*I/*Sal*I, and ligated into pCAGGS (digested with *Sac*I/*Kpn*I) or pCAGGS (digested with *Cla*I/*Xho*I) to obtain the recombinant eukaryotic expression plasmids pCGxAHRT and pCGxBHRT (Fig. 1). All the modified plasmids were confirmed by sequencing.

2.5. Replacement of VP2 of Gx

To replace the VP2 gene of Gx with the corresponding gene of CEF-9, the fusion PCR was developed. First, with pCGxAHRT as template, two DNA PCR fragments (Fra I, 203 bp; Fra III, 1938 bp) were amplified with primer pairs P16/F9VP2Q172L and F9VP2Q1512U/P9 (Table 1), separately in two reactions. Secondly, using pTF9VP2 as template, another PCR fragment (Fra II, 1327 bp) containing the VP2 gene of CEF-9 was amplified with primer pairs F9VP2Q172U/F9VP2Q1512L (Table 1). Thirdly, the fragments Fra I and Fra II were fused using primer pair P16/F9VP2Q1512L and the

new fragment was named Fra IV (1509 bp). Finally, using primer pair P16/P9, the Fra IV and Fra III fragments were fused to one fragment GxAF9VP2 in which the VP2 of Gx was replaced with the VP2 of CEF-9. After purification, the PCR product GxAF9VP2 was digested with *Sac*I/*Kpn*I and ligated into pCAGGS to obtain the recombinant eukaryotic expression plasmid pCGxAF9VP2HRT (Fig. 1). All the modified plasmids were confirmed by sequencing.

2.6. Site-directed mutagenesis in VP2

To introduce direct mutations into the genome of Gx strain, based on the parental plasmid of pBluGxA, PCR for site-directed mutagenesis with specific primer pairs was developed as described previously (Qi et al., 2007). Based on sequence of GxA in the plasmid pBluGxA, primer pair GxAA889TU/GxAA889TL or GXAG980AU/GXAG980AU (Table 1) was synthesized to introduce direct mutation A889T or G980A (resulted in amino acid mutation of Q253H or A284T in VP2) into segment A of Gx, respectively. The final modified plasmids were named pBluGxAA889T and pBluGxAG980A, respectively. Similarly, based on the sequences of GtA in the parental plasmid pUC18GtA, with the primer pairs GtAC889AU/GtAC889AL or GtAA980GU/GtAA980GL (Table 1), the nucleotide mutation C889A/A980G, C889A, A980G (resulted in amino acid mutations of H253Q/T284A, H253Q, T284A in VP2) were introduced into segment A of Gt and three modified plasmids pUC18GtAC889A/A980G, pUC18GtAC889A, pUC18GtA980G, respectively, were obtained.

Similar to the construction of the full-length cDNA clones of Gx as described above, using the similar sets of primers (P5/P6, P16/P8 and P16/P9 for pBluGxAA889T and pBluGxAG980A; P5/P6, P7/P8 and P7/P9 for other three recombinant plasmids), and HamRz and HdvRz fusions, all the fragment A mutations were subcloned into eukaryotic expression plasmid pCAGGS. Therefore, a new set of recombinant eukaryotic expression plasmids were obtained. They were pCGxAA889THRT, pCGxAG980AHRT, pCGtAC889AA980GHRT, pCGtAC889AHRT, and pCGtAA980GHRT, respectively (Fig. 1). All the modified plasmids were sequenced 3 times.

2.7. Rescue and identification of modified IBDV

Using the reverse genetics system directed by RNA polymerase II (Qi et al., 2007), the modified plasmids were transfected into DF-1 cells to rescue virus. In brief, purified plasmids with the Gx backbone (pCGxAHRT, pCGxAA889THRT, pCGxAG980AHRT) and another group with the Gt backbone (pCGtAC889AA980GHRT, pCGtAC889AHRT, pCGtAA980GHRT) were each co-transfected with pCGxBHRT and pCmGtBHRT (2 μl each plasmid at 1 μg/μl) into DF-1 cells, respectively. The transfection was directed by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). After 72 h, the cell cultures were harvested. After freezing and thawing for 3 times, the modified viruses were harvested from the supernatants by centrifugation at 3000 × *g* for 10 min at 4 °C and used to infect secondary CEF cells. The modified viruses were blind passaged for a few times.

To characterize the modified viruses, IFA with anti-VP2 mAb and electron microscopy assay were performed as described previously (Qi et al., 2007). To verify whether the modified viruses were the origin of mutation, the genomic RNAs were isolated from the rescued viruses and analyzed by RT-PCR and sequencing. For each virus, 2 fragments were amplified by RT-PCR using the primer pairs GxAU/GxA1477L (1–1477 bp of segment A) and B3P/B37 (2009–2827 bp of segment B) (Table 1) and then sequenced. The full-length genomes of the modified viruses were also amplified and sequenced as described previously (Qi et al., 2007).

2.8. Replication kinetics of modified IBDV in CEF cells

Replication kinetic curves were obtained to assess the replication abilities of the modified IBDV and the control rmGt. Confluent secondary CEF cells in 60 mm culture plates (approximately 10^6 cells/plate) were infected with each virus strain at 10^4 50% cell culture infective dose ($CCID_{50}$), and then harvested at 12 h intervals. The titer of the infectious viral progeny was determined in terms of $CCID_{50}$ per milliliter using the Reed–Muench formula. The mean values and standard deviations of the data obtained from three independent experiments were calculated.

2.9. Characterization of the modified IBDV in chicken

To evaluate the virulence of the modified viruses, SPF chickens housed separately in negative-pressure isolators were infected with viruses intranasally and by eye drops. At 14-day-old, chickens were randomly assigned to six groups with 15 chickens in each group. The first and the second groups were infected with $10^{5.8}$ $CCID_{50}$ of rGx-F9VP2, the third and the fourth groups were infected with 200 EID_{50} of Gx, and the fifth group was infected with $10^{5.8}$ $CCID_{50}$ of rmGt. The sixth group received DMEM without any viruses as the negative control.

Chickens were observed daily for clinical symptoms and mortality. At 2, 3, 5, 7, and 14 days post-inoculation (d p.i.), 3 chickens were randomly selected from groups 2, 3, 5, and 6, and euthanized for necropsy. Each chicken was examined for signs of pathological changes. The bursa and body weights of all chickens were determined, and the bursa:body-weight index (BBIX) at 2, 3, 5, 7, and 14 d p.i. was calculated with standard deviation. 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). Then each bursa was split into 2 parts, one part for histopathology assay and the other for amplification of viral RNA.

2.10. Histopathology

The bursae of each group isolated at different days were fixed immediately after necropsy in 10% neutrally buffered formalin at a ratio higher than 10:1. After 7 days, cross sections were cut from paraffin-embedded bursae and stained with hematoxylin and eosin, and examined with a light microscope. The severity of bursal follicular necrosis was recorded using average histopathologic bursa lesion score (HBLS) as described earlier (Schröder et al., 2000).

2.11. Identification of the modified virus from the bursa

To confirm the identity of the modified virus and investigate whether changes in the nucleotide acid sequence occurred during challenge, the viral RNAs obtained from the chicken bursae were amplified by RT-PCR using the primer pairs GxAU/GxA1477L (1–1477 bp of segment A) and B3P/B37 (2009–2827 bp of segment B) (Table 1) and then sequenced.

3. Results

3.1. Amplification and analysis of VP2 gene of CEF-9

Compared with vvIBDV Gx, CEF-9 had only two nucleotide mutations A889T and G980A in VP2 gene, which resulted in the corresponding amino acid mutations Q253H and A284T (Fig. 2). In addition, CEF-9 had the same amino acid residues as the attenuated strain Gt in 253H and 284T of VP2 (Fig. 2).

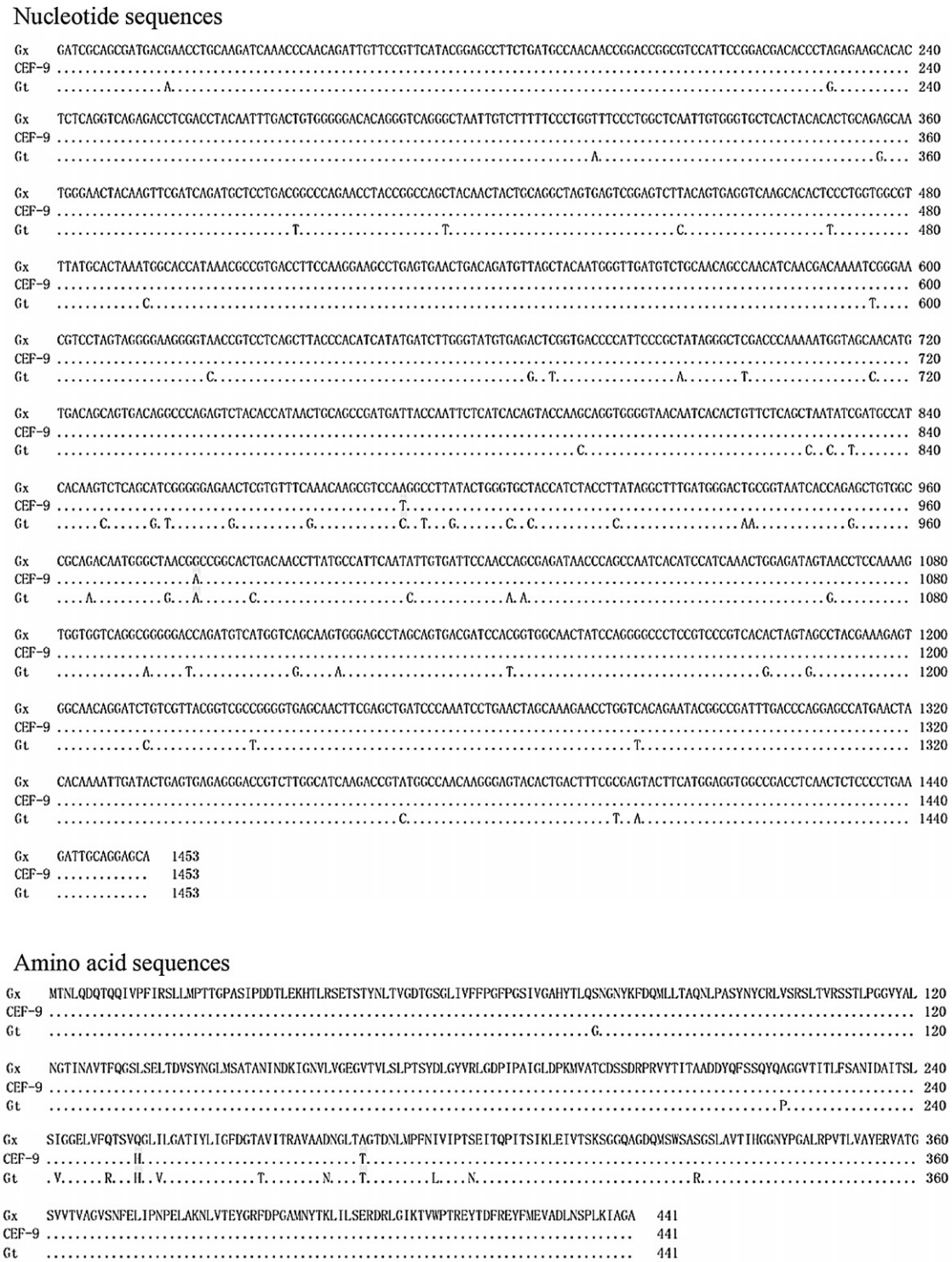

Fig. 2. Alignment of nucleotide sequences and the deduced amino acid sequences among VP2 of the vvIBDV Gx (GenBank accession no. AY444873), the intermediate strain CEF-9 and the attenuated strain Gt (GenBank accession no. DQ403248). Compared with Gx, nucleotide mutations (A889T and G980A) that resulted in amino acid mutations (Q253H and A284T) in VP2 of CEF-9 were highlighted.

3.2. Combined mutations of Q253H/A284T adapted vvIBDV to CEF culture

To test the functionality of the two important amino acids in VP2, a set of recombinant plasmids were constructed as described in detail in Section 2. They were pCGxAHRT, pCGxAF9VP2HRT, pCGxAA889THRT, pCGxAG980AHRT, pCGtAC889AA980GHRT, pCGtAC889AHRT, pCGtAA980GHRT containing single or combined mutations at 253 and 284 of VP2 in either the Gx or Gt backbone. The final constructs were confirmed by sequencing; upon transfection into DF-1 cells, the correct protein expression was further confirmed by immunofluorescence with anti-VP2 mAb (data not shown).

To adapt vvIBDV Gx to CEF culture, the plasmids containing the wild type or the modified segment A cDNAs were co-transfected with the plasmids containing the wild type of segment

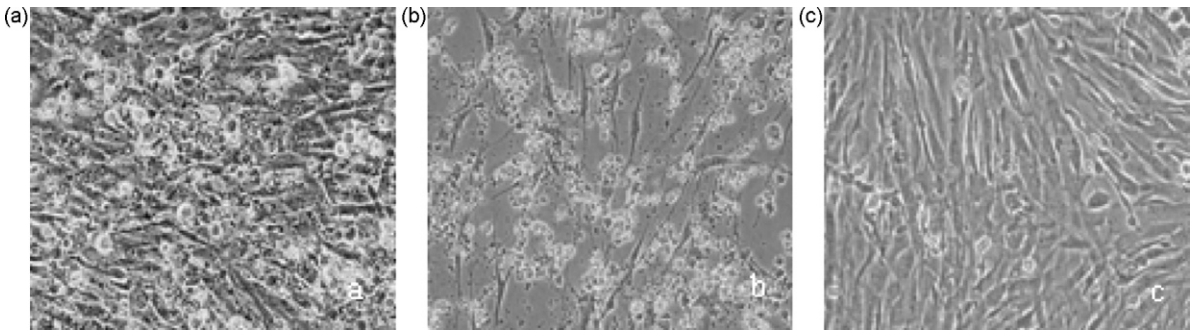

Fig. 3. CPE in the secondary chicken embryo fibroblast (CEF) cell culture by the infection with the modified IBDV (200×). rGx-F9VP2 (a) caused a milder CPE than the control rmGt (b). The normal CEF cell (c) without IBDV infection is also shown.

B cDNAs in cells. Infectious viruses were rescued in cells transfected with pCGxAF9VP2HRT/pCGxBHRT, and the rescued virus was named rGx-F9VP2. No virus was rescued in cells transfected with pCGxA/pCGxBHRT after three repeated experiments. The results showed that VP2 was the determinant of cell tropism of IBDV and the combined mutations Q253H and A284T adapted vvIBDV Gx to CEF culture. To further confirm the results, plasmids pCGtAC889AA980GHRT/pCmGtBHRT carrying the reverse mutations (H253Q and T284A) were also used for transfection but no virus adapted to CEF cells was rescued after three trials.

To further examine whether only single mutation is sufficient to alter the cell tropism of vvIBDV, the plasmid pairs pCGxAA889THRT/pCGxBHRT, pCGxAG980AHRT/pCGxBHRT, pCGtAC889AHRT/pCGtBHRT, and pCGtAA980GHRT/pCGtBHRT were also transfected into cells, but no virus adapted to CEF cells was rescued after three trials. This result indicated that single amino acid mutation at either 253 or 284 of VP2 alone could not adapt vvIBDV Gx to CEF culture.

3.3. Characterization of the modified IBDV in CEF cells

The modified virus rGx-F9VP2 induced a typical cytopathogenic effect (CPE) in CEF cells, which became more significant in CEF cells after blind passage. However, compared with rmGt, rGx-F9VP2 caused pathological changes at a greatly reduced extent. At 72 h post-infection (h p.i.), although many infected cells underwent apoptosis or necrosis and were lysed into small particles, the culture monolayer still existed (Fig. 3a); whereas almost all the rmGt-infected CEF cells were completely dead with cell debris detached from the dish, and the monolayer had disappeared (Fig. 3b).

The replication kinetic curves obtained in triplicates also showed that rGx-F9VP2 replicated more slowly than the control virus rmGt in CEF cells (Fig. 4). Release of rGx-F9VP2 could be detected at 12 h p.i. but slowed down after 24 h p.i., and then eventually reached a plateau at 60 h p.i. with the highest titer of $10^{4.7}$ $CCID_{50}$/ml, which was 1000 times lower than that of rmGt ($10^{7.7}$ $CCID_{50}$/ml).

3.4. Combined mutations Q253H/A284T attenuated vvIBDV in chickens

The virulence of the modified IBDV in vivo was investigated in 14-day-old SPF chickens. All chickens infected with the wild type vvIBDV Gx showed severe clinical symptoms of IBD after 3 d p.i. and the mortality was as high as 66.7% (10/15). In contrast, the mutated rGx-F9VP2 caused only temporal symptoms and the infected chickens were only mildly dispirited at 6 d p.i. No death of chickens was observed. Also, no clinical symptoms of IBD were observed for chickens receiving the rmGt and the virus-free DMEM.

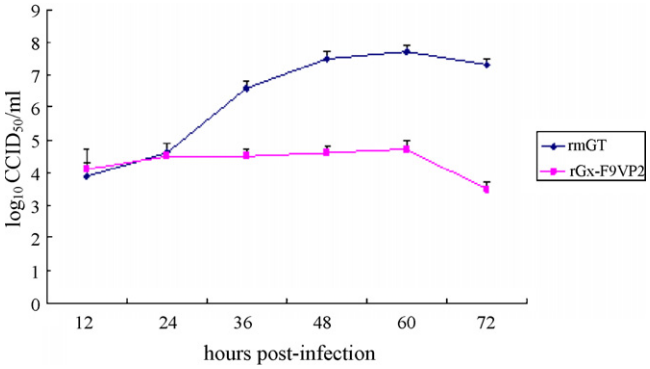

Fig. 4. Replication kinetic curves of modified IBDV strains. Secondary CEF cells (approximately 10^6 cells) were infected with the modified virus rGx-F9VP2 or the control rmGt at 10^4 $CCID_{50}$. The titers of viruses harvested at different time intervals were calculated and expressed as $CCID_{50}$ per milliliter. Average titers and standard deviations (error bars) from 3 independent experiments are shown.

Chickens infected with Gx and those with rGx-F9VP2 both showed gross lesions in bursa but with obvious differences. In the Gx-infected group, the hemorrhage of bursa was observed as early as 2 d p.i.; the atrophy and hemorrhage of bursa were accompanied by the hemorrhage of muscle in the chest and leg, and persisted from 3 d p.i. to 14 d p.i. In contrast, in the group of rGx-F9VP2-infected chickens, no hemorrhage was observed in the bursa, chest and leg. In addition, although the atrophy of bursa was also persistent from 3 d p.i. to 14 d p.i., the average BBIX in this group gradually increased during the same period of time (Fig. 5). The BBIX in the rmGt and DMEM control groups were not changed much and kept above the critical value of 0.7 (Fig. 5).

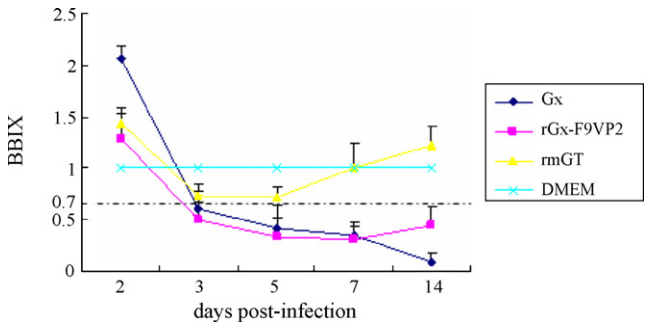

Fig. 5. The kinetic curves of bursa:body-weight index (BBIX) of SPF chickens infected with the modified virus rGx-F9VP2, rmGt, and vvIBDV Gx, or the chickens injected with DMEM alone without viruses (negative control). Bursae with a BBIX lower than 0.70 were considered atrophied.

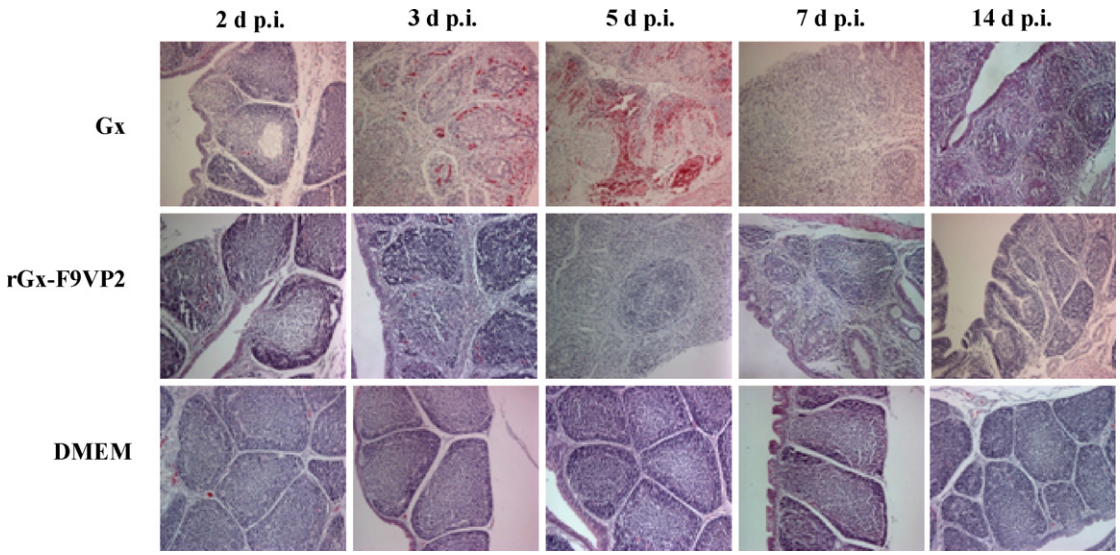

Fig. 6. Histopathological appearance of sections (hematoxylin and eosin) of bursae derived from groups of chickens infected with the modified virus rGx-F9VP2, vvIBDV Gx and the negative control DMEM at different day post-infection (200×). Compared with Gx, the histopathological lesions of bursae caused by rGx-9VP2 were mild obviously.

3.5. Histopathogical examination of the bursa

The vvIBDV Gx strain induced severe and persistent histopathologic bursal lesions. Pathological examination of the infected tissues revealed severe lymphocytic deletion and necrosis, severe hemorrhage and gore, and severe atrophy and fibrosis of the follicles. The average HBLS was 5 (Fig. 6). In contrast, rGx-F9VP2 induced relative smaller bursal lesions with milder pathological changes. At 2 d p.i., only mild damages to medullary lymphocytes (average HBLS of 2) were observed; at 3 d p.i., the depletion of several follicles (average HBLS of 3) was observed. Severe lesions without bleeding or gore (average HBLS of 5) were appeared only at 5 d p.i., showing dropsy of the interstitial tissue, lymphocytic necrosis, and follicular depletions, which resulted in the loss of distinction between the cortex and medulla. The bursal damages began to recover from 7 d p.i. and the tissue returned to normal with distinct outline and structure of follicles at 14 d p.i. (Fig. 6). Also, there were no microscopic lesions of bursa in the control chickens infected with rmGt (data not shown) and DMEM (Fig. 6).

3.6. Confirmation of genetic stability of the modified virus

RT-PCR was performed to confirm the presence of the modified virus in the bursae. First, a 1477 bp sequence in segment A and a 818 bp sequence in segment B were amplified from the bursal tissue samples in the chickens infected with rGx-F9VP2, and then the PCR products were purified and sequenced. The sequence analysis showed that rGx-F9VP2 replicated with high fidelity in the bursae of chickens and its gene did not revert to that of the parental Gx strain. In addition, rGx-F9VP2 was also shown to be genetically stable after 12 passages in the secondary CEF cells.

4. Discussion

Since the development of the reverse genetics system 12 years ago (Mundt and Vakharia, 1996), the molecular determinants of variation, attenuation and cell tropism of IBDV (Boot et al., 1999, 2000, 2001a,b, 2002; Boot and Pritz-Verschuren, 2004; Brandt et al., 2001; Mundt, 1999; Mundt et al., 1997, 2003; Van Loon et al., 2002; Yao et al., 1998) have gradually been elucidated. VP2 has been shown to be the single most important determinant of cell tropism of IBDV (Boot et al., 2000; Brandt et al., 2001), and its amino acid residues 253, 279, 284 and 330 have been studied. While residue 330 has little influence on the ability of IBDV to infect cell culture, the roles of the residues 253, 279, 284 in cell tropism are conflicting (Brandt et al., 2001; Lim et al., 1999; Mundt, 1999; Van Loon et al., 2002).

Previously, we successfully attenuated the vvIBDV Gx strain to the Gt strain through blind passage in SPF chicken embryos for 5 generations and in CEF cell cultures after 20 generations (Wang et al., 2003, 2004, 2007). We also obtained an intermediate strain CEF-9 that was partially attenuated and adapted to the CEF culture. Further analysis of VP2 gene of CEF-9 identified two nucleotide mutations (A889T, G980A) that encode two mutated amino acids Q253H and A284T. To our knowledge, this was the first direct evidence that Q253H and A284T might change the cell tropism of vvIBDV and enable it to adapt to CEF culture.

In this study, this observation was further confirmed by the RNA polymerase II-directed reverse genetics system (Qi et al., 2007), in which the VP2 of Gx was replaced with the corresponding gene of CEF-9, and the modified recombinant virus rGx-F9VP2 was successfully rescued. In addition, after the two mutations C889A and A980G (corresponding to amino acids mutations H253Q and T284A) were introduced to VP2 gene of the Gt strain, the modified Gt lost the ability to infect CEF cells. The results observed from different backbones showed that VP2 was the sole determinant of the cell tropism of IBDV and the amino acid residues 253 and 284 were the key elements. To some extent, the results were consistent with the previous reports (Mundt, 1999; Van Loon et al., 2002). Mundt (1999) reported that mutations at residues 253 and 284 could adapt the chimeric virus with two background of variant strain E/Del and attenuated strain D78 to chicken embryo cells (CEC) and quail-drived cells (QM-7). Certainly, a complex backbone is not ideal for gene function research. Van Loon et al. (2002) reported that mutations of 253 and 284 residues could adapt vvIBDV UK661 to CEC culture. However, these two reports did not study the replication properties of the rescued viruses in much detail.

We tried to answer whether the single mutation Q253H or A284T could adapt vvIBDV to CEF. Different researchers have reported different results. Van Loon et al. (2002) reported that single mutation Q253H could render UK661 to replicate in CEC cells at some extent and additional mutation of A284T increased the

replication efficiency. On the other hand, single mutation A284T did not change the cell tropism of vvIBDV. Also, Mundt (1999) found single mutation Q253H or A284T could not change the cell tropism of IBDV. However, in the same paper, Mundt (1999) reported that the single mutation A284T was sufficient for the alteration of the cell tropism of GLS-BU to GLS-TC. To solve the discrepancy, we introduced a single mutation Q253H or A284T of VP2 in the backbone of Gx, and the single reverse mutation H253Q or T284A in the backbone of Gt, no viruses were rescued in CEF cells after three experiments. These results showed that single mutation Q253H or A284T in VP2 was not sufficient to adapt vvIBDV to CEF cells.

Wild type IBDV cannot grow in cell culture but can replicate inside CEF cells after being transfected into the cells, indicating that virus entry is the definitive requirement for cell tropism of IBDV (Liu and Vakharia, 2004). As the capsid protein, VP2 contains the domains for binding to the corresponding receptors at the cell surface. Each subunit of VP2 is folded mainly into three distinct domains, designated as the base (B), the shell (S), and the projection (P) domains (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). The three dimensional structure of VP2 build by the SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace) shows that the amino acid residues 253 and 284 are located on loops P_{DE} and P_{FG} at the tip of the VP2 spikes, respectively (data not shown). These two loops are involved in virus–cell receptor binding (Lee et al., 2006). The change of amino acid residue from linear Q to annulate H at the 253 site favors the formation of the β turn. In addition, the change of amino acid residue from nonpolar A to polar T at the 284 site favors the formation of the β turn and creates a potential new site for casein kinase II phosphorylation. Also, the H and T residues at 253 and 284 have their side chains pointing outwards; they do not participate in contacts important for the VP2 folding nor the interactions between subunits that stabilize the virion (Coulibaly et al., 2005). Therefore, it is highly possible that the combined mutations at 253 and 284 of VP2 alter the affinity of viral domain for binding to the corresponding receptors, so that the cell tropism is changed.

In this study, we also found that, compared with rmGt, rGx-F9VP2 caused less CPE in CEF cells. Also, rGx-F9VP2 replicated very slowly and its highest titer was 1000 times lower than that of rmGt. This indicates that, besides 253 and 284 of VP2, other amino acid residues or genes may also contribute to the replication efficiency of IBDV.

To study the relationship between the amino acid mutations (Q253H/A284T) and the virulence, the modified virus rGx-F9VP2 was used to infect SPF chickens. Similar to the results shown in the previous report (Van Loon et al., 2002), vvIBDV was markedly attenuated by Q253H/A284T since the modified virus rGx-F9VP2 caused no mortality. However, such attenuation was not complete since rGx-F9VP2 still induced severe gross changes and histopathologic lesions in bursa. Thus, amino acid residues at 253 and 284 of VP2 mainly contribute to the virulence of vvIBDV, but they are not the sole determinant. Recently, VP1 and VP5 are also shown to serve as the virulent gene of IBDV (Islam et al., 2001; Wei et al., 2006).

In summary, for the first time, this study provides two lines of evidence with natural and genetically engineered strains to show that the cell tropism of vvIBDV to CEF can be achieved by the combined mutations of Q253H and A284T in VP2 but not by any of the single mutations. Moreover, these amino acid residues at 253 and 284 are important to maintain the virulence of vvIBDV. These findings are not only useful to our understanding of the mechanism of viral virulence but also to the design of new IBDV vaccines using the reverse genetics technology. Certainly, in addition to the two residues, it would be interesting to study other residues in VP2 or other genes that may also influence the replication efficiency and virulence of IBDV.

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