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Towards construction of viral vectors based on avian coronavirus infectious bronchitis virus for gene delivery and vaccine development

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Manipulation of the coronavirus genome to accommodate and express foreign genes is an attractive approach for gene delivery and vaccine development. By using an infectious cloning system developed recently for the avian coronavirus infectious bronchitis virus (IBV), the enhanced green fluorescent protein (EGFP) gene, the firefly luciferase gene and several host and viral genes (eIF3f, SARS ORF6, Dengue virus 1 core protein gene) were inserted into various positions of the IBV genome, and the effects on gene expression, virus recovery, and stability in cell culture were studied. Selected viruses were also inoculated into chicken embryos for studies of foreign gene expression at different tissue level. The results demonstrated the stability of recombinant viruses depends on the intrinsic properties of the foreign gene itself as well as the position at which the foreign genes were inserted. For unstable viruses, the loss of expression of the inserted genes was found to result from a large deletion of the inserted gene and even IBV backbone sequences. This represents a promising system for development of coronavirus-based gene delivery vectors and vaccines against coronavirus and other viral infections in chicken.

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1. Introduction

Interest in the use of viruses as gene delivery vectors and vaccine development has increased considerably because they can be subjected to genetic manipulation. While this seems to apply particularly to DNA viruses and retroviruses, it has become clear that there might also be a role for RNA viruses. These viruses, however, are inherently more prone to genetic instability, and this might particularly be the case for coronaviruses in view of the documented high frequency of recombination (Lai, 1996). Nevertheless, coronaviruses have been shown to accept and express foreign genes, making them attractive candidates for gene therapy vectors and vaccine development (Alonso et al., 2002; Curtis et al., 2002; de Haan et al., 2003, 2005; Enjuanes et al., 2001; Hackney et al., 2003; Ortego et al., 2002; Sola et al., 2003; Thiel et al., 2003).

IBV causes an acute and contagious disease in chickens with significant impact on the poultry industry worldwide. It contains a 27.6 kb single-stranded, positive-sense RNA genome. In the virus-infected cells, six mRNA species, including the genome-length RNA (gRNA) 1 and five subgenomic RNA (sgRNA) species (mRNA 2–6),

are produced by a discontinuous RNA transcription mechanism (Pasternak et al., 2006; Sawicki and Sawicki, 2005). Each mRNA species possesses a 64 nucleotides leader sequence derived from the 5' end of the genome (Bournell et al., 1987). Based on the current discontinuous transcription model, the leader sequence is added to the sgRNAs during transcription of the negative strand sgRNAs by base-pairing with the transcription regulatory sequences (TRS), a highly conserved core sequence CUU(G)AACAA, in the genome (Pasternak et al., 2006; Sawicki and Sawicki, 2005; Stirrups et al., 2000). SgRNAs 2, 3, 4, and 6 encode the four structural proteins, i.e., spike glycoprotein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N), respectively. The 5' two-third region of mRNA1 comprises two large ORFs, 1a and 1b, and encodes two polyproteins. The two polyproteins are cleaved proteolytically by two virus-encoded proteinases, the papain-like and 3C-like proteinases, into 15 functional proteins (Nsp2–Nsp16) (Liu et al., 1995, 1997; Ng and Liu, 1998, 2000, 2002; Xu et al., 2001). Compared to other coronaviruses, Nsp1 is absent in IBV but Nsp2 is considerably larger (Lim and Liu, 1998a,b; Lim et al., 2000; Liu et al., 1998). In addition, four accessory proteins, or group-specific proteins, are encoded by ORF3a, 3b, 5a and 5b, respectively (Liu et al., 1991; Liu and Inglis, 1992).

The unique transcription mechanism of coronavirus and the development of coronavirus reverse genetics systems (Almazán et al., 2000; Casais et al., 2001; Yount et al., 2000, 2002, 2003), or targeted recombination (Hajjema et al., 2003; Kuo et al., 2000)

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make it possible to use coronaviruses to express foreign genes. Two types of coronavirus-derived expression systems were attempted (Enjuanes et al., 2001). The first system, the so-called helper-dependent expression systems, was employed to express several heterogenous genes, such as chloramphenicol acetyltransferase (CAT) gene, β -glucuronidase (GUS) gene, green fluorescent protein (GFP) gene, firefly luciferase (LUC) gene and interferon- γ (IFN- γ) gene by using coronavirus-derived minigenomes from mouse hepatitis virus (MHV), transmissible gastroenteritis virus (TGEV), human coronavirus 229E (HCoV-229E) and IBV (Alonso et al., 2002; Hackney et al., 2003; Izeta et al., 1997; Lin and Lai, 1993; Thiel et al., 2003; Zhang et al., 1997). The second type of expression systems based on reverse genetics of coronaviruses permits the expression of heterogenous genes by construction of recombinant viruses carrying foreign genes in the viral genome (de Haan et al., 2003, 2005; Le et al., 2007; Youn et al., 2005).

In this study, the possibility of developing IBV vectors was assessed systematically by using an infectious cloning system based on avian coronavirus infectious bronchitis virus (IBV) (Tan et al., 2006). Two reporter genes, the enhanced green fluorescent protein (EGFP) and the firefly luciferase gene, were placed under the control of a TRS sequence derived from ORF5a and inserted into different positions of the IBV genome. EGFP was also fused to four structural proteins, S, M, E and N. Recombinant viruses with variable degrees of stability and expression efficiency of the inserted foreign genes were recovered and characterized. The results showed that stable recombinant viruses were recovered when the luciferase gene was used to replace the ORF3a3b gene cluster. Recombinant IBV expressing other heterogeneous genes derived from host cells and other viruses were also recovered and efficient expression of the target genes was observed in both cultured cells and chicken embryos. This study presents a detailed assessment of using IBV for the development of gene delivery vectors and potential vaccines.

2. Materials and methods

2.1. Viruses and cells

All recombinant IBV viruses were propagated in Vero cell line. Cells were maintained in Dulbecco's modified eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 37 °C in a humid environment with 5% CO₂. Viruses were propagated in Vero cells in FBS-free DMEM.

2.2. Construction of plasmids and generation of recombinant viruses

All IBV-related nucleotide numbers refer to the positions in the Vero cell-adapted strain P65 from Beaudette strain of IBV, GenBank accession no. DQ001339. To produce the construct containing EGFP gene by replacing IBV ORF 3a and 3b, two fragments, corresponding to nucleotides 20884–23856 and 24202–27611, respectively, were amplified by PCR with the primer pair IBV20884F (5'-²⁰⁸⁸⁴GT-TTACACCTCTAATGAGACCATAG) and IBV23856R (5'-cgggtctc²³⁸⁵⁶CATCAAACAGACTTTTTTGG, IBV-TRSE-F (5'-cgggtctctgaacaaaaacttaacaaatcggagc²⁴²⁰²ATGATGAATTTATTGAATAAG) and IBV27611R (5'-cgggtctcG(T)₃₀-²⁷⁶¹¹TGCTCTAACTCTATACTAGC) (nucleotides in bold represent the introduced Bsal site, the sequence flanking ORF5a TRS are underlined and the gene-specific sequences are capitalized). To ensure the transcription and translation of E gene, a preceding sequence of 25 nucleotides flanking ORF5a TRS was introduced. EGFP ORF sequence was also amplified using primers 5'-cgggtctctgATGGTGAGCAAGGGCGAG and 5'-cgggtctctgttCTATTGTACAGCTCGTCCATGC. *In vitro* ligation

of these three Bsal-digested fragments resulted in the deletion of the nucleotides 23853 to 24201 including ORF 3a and 3b and the insertion of EGFP gene at the corresponding position. To insert the EGFP gene cassette between IBV M gene and 5a5b gene, EGFP gene preceded by an IBV ORF5a TRS and flanking sequences was amplified using the primers 5'-cgggtctcacacataacaaaaacttaacaaatcggagcATGGTGAGCAAGGGCGAG and 5'-cgggtctcacattaCTGTACAGCTCGTCCATGC. Primer pair IBV20884F and IBV25178R (5'-cgggtctc²⁵¹⁷⁸ATGTGTAAGACTACTTC) was used to produce a fragment containing IBV nucleotides 20884 to 25178, while primer pair IBV25179F (5'-cgggtctc²⁵¹⁷⁹AAATGTGTGTAGAGAG) and IBV27611R was used for the amplification of a fragment containing IBV nucleotides 25179 to the 3'-end. Similarly, for the insertion of EGFP gene between the N gene and the 3'-UTR, two fragments containing IBV nucleotides from 20884 to 27107 (IBV20884F and IBV27107R 5'-cgggtctc²⁷¹⁰⁷ACTCAAAGTTTCTCT) and 27106 to 27611 (IBV27106F 5'-cgggtctc²⁷¹⁰⁶GTAACATAATGGACCTGT and IBV27611R), and the EGFP gene expression cassette plus the ORF5a TRS and flanking sequences was amplified with primers (5'-cgggtctcTTGAGaacaaaaacttaacaaatcggagcATGGTGAGCAAGGGCGAG and 5'-cgggtctctgtaCTGTACAGCTCGTCCAT). A Bsal site was introduced at both ends of all fragments. All amplified PCR fragments were cloned into pCR-TOPO-XL (Invitrogen) or pGEM-T easy vector (Promega) and verified by sequencing. Plasmids were then digested with Bsal, and fragments of interest were purified. *In vitro* ligation of Bsal-digested fragments would result in the insertion of EGFP gene expression cassette between nucleotides 25180 and 25181, 27105 and 27106. The same strategies were employed for constructing recombinant viruses expressing the firefly luciferase gene and other foreign genes using various gene-specific primers. Briefly, three fragments containing luciferase gene were amplified with primer pairs: 5'-cgggtctctgATGGAAGACGCCAAAACAT and 5'-cgggtctcTGTTACACGGCGATCTTCCGCCCT; 5'-cgggtctcacacataacaaaaacttaacaaatcggagcATGGAAGACGCCAAAACAT and 5'-cgggtctcACATTACCGGCGATCTTCCGCCCT; and 5'-cgggtctcTTGAGaacaaaaacttaacaaatcggagcATGGAAGACGCCAAAACAT and 5'-cgggtctctgtaCACGGCGATCTTCC, respectively. The luciferase gene was introduced separately into IBV genome at different positions: replacing 3a3b gene, insertion between nucleotides 25180 and 25181 and between nucleotides 27105 and 27106, resulting in the production of three recombinant viruses IBV-Luc Δ 3a3b, IBV-Luc25180 and IBV-Luc27105, respectively. The amplified ORF6 of SARS-CoV Sin2774 (GenBank accession no. AY283798) (5'-cgggtctcacacataacaaaaacttaacaaatcggagcATGTTTCATCTTGTGAC and 5'-cgggtctcacattaTGGATAATCTAACT) and core gene of Dengue virus 1 (5'-cgggtctcTTGAGaacaaaaacttaacaaatcggagcATGAACAACCAACGAAAAAGAC and 5'-cgggtctctgtaCGCCAAGGCTGTGGCAGCAG) were inserted separately between nucleotides 25180 and 25181 and between nucleotides 27105 and 27106, while the eukaryotic translation initiation factor 3f (eIF3f) amplified with primers (5'-cgggtctctg ATGGCCACACCGGGGTACC and 5'-cgggtctctgtTCACAGTTTACAAGTTTTTC) was introduced into IBV genome by replacing 3a3b. The recovered recombinant viruses were designated IBV-SARS6, IBV-DenC and IBV-eIF3f, respectively. In addition, constructs IBV-S-EGFP, IBV-EGFP-E, IBV-M-EGFP, IBV-EGFP-M and IBV-N-EGFP were made by insertion of EGFP gene between nucleotides 23850 and 23851, 24505 and 24506, 25177 and 25178, 24505 and 24506, 27102 and 27103, respectively, resulting in the expression of EGFP fused with these structural proteins.

In vitro assembly of full-length cDNA was performed as described previously (Fang et al., 2007; Tan et al., 2006). The *in vitro* transcribed full-length transcripts were generated using the mMessage mMachine T7 kit (Ambion, Austin, TX) and electro-

porated together with the *in vitro* transcribed IBV N transcripts into Vero cells with one pulse at 450 V, 50 μ F with a Bio-Rad Gene Pulser II electroporator. The electroporated Vero cells were cultured overnight in 1% FBS-containing MEM and further cultured in MEM without FBS. The transfected cells were monitored daily for formation of cytopathic effect (CPE), and the recovered recombinant viruses were plaque-purified and propagated in Vero cells.

2.3. Viral growth assays

Confluent monolayers of Vero cells on six-well plates were infected in triplicates with recovered recombinant viruses and harvested at different times post-infection. Viral stocks were prepared by freezing/thawing of the cells three times. The 50% tissue culture infection dose (TCID₅₀) of each sample was determined by infecting five wells of Vero cells on 96-well plates with 10-fold serial dilution of each viral stock. Luciferase assays were performed according to manufacturer's instructions (Promega, Madison, USA).

2.4. Northern hybridization

Vero cells were infected with wild type and recombinant viruses at 2 PFU per cell. Ten micrograms of total RNA extracted from the infected cells were denatured by heating at 65 °C for 20 min in a MOPS-based buffer containing formamide and formaldehyde prior to denaturing-agarose gel electrophoresis. The separated RNA bands were transferred onto a Hybond N⁺ membrane (Amersham Biosciences, Sunnyvale, USA) via capillary action overnight and fixed by ultraviolet crosslinking (Stratalinker, Stratagene, Austin, USA). Hybridization of Dig-labeled DNA probes corresponding to the IBV 3'-UTR from nucleotides 27104–27510 was carried out at 50 °C in hybridization buffer overnight. Membranes were washed extensively with stringent buffers before subjected to detection with CDPStar (Roche, Indianapolis, USA) according to the manufacturer's instructions.

2.5. SDS-PAGE and Western blot analysis

Virus infected cells were lysed with 2 \times SDS loading buffer containing 100 mM DTT and 5 mM of iodoacetamide, boiled at

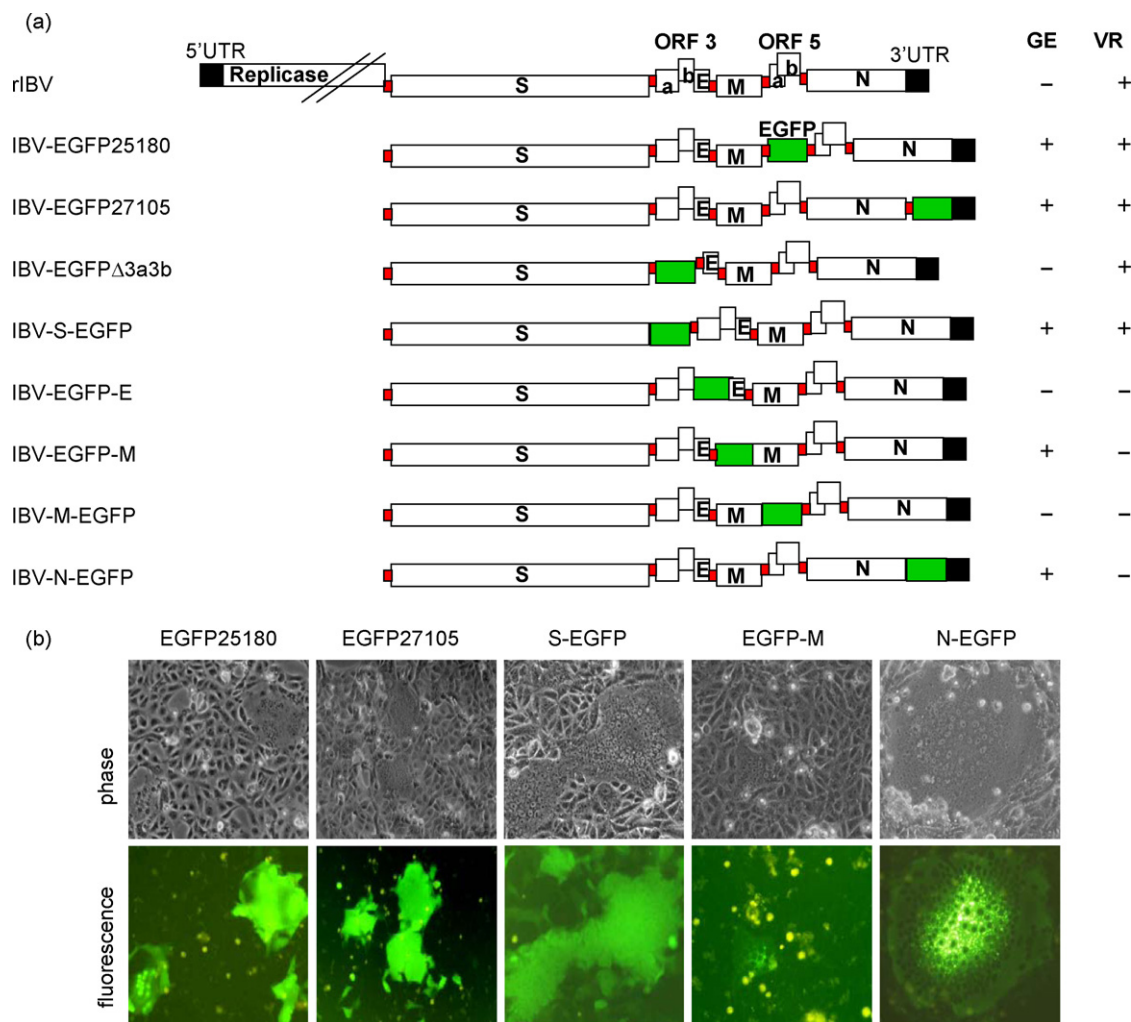


Fig. 1. Construction of recombinant IBV viruses with insertion of EGFP at different positions of the IBV genome, and analysis of EGFP expression and recovery of the infectious viruses. (a) Diagram outlining the genome organization of IBV and the positions of the inserted EGFP in each construct. Green and red boxes indicate EGFP gene and TRS sites, respectively. The EGFP gene expression (GE) in cells electroporated with different constructs and the recovery of infectious virus (VR) from each construct were listed. (b) EGFP expression observed by fluorescence microscopy in Vero cells electroporated with IBV-EGFP25180, IBV-EGFP27105, IBV-S-EGFP, IBV-EGFP-M and IBV-N-EGFP, respectively, at 3 days post-electroporation. Typical CPE and expression of EGFP were observed in cells electroporated with IBV-EGFP25180, IBV-EGFP27105, IBV-S-EGFP and IBV-N-EGFP transcripts. Less CPE was observed. The corresponding EGFP expression was also found in cells electroporated with IBV-M-EGFP transcripts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

100 °C for 5 min and clarified. Proteins were separated by SDS–PAGE and transferred to PVDF membrane (Bio-Rad, Hercules, USA). Membrane was blocked overnight at 4 °C or for 2 h at room temperature in blocking buffer (10% fat free milk powder in PBS buffer containing 0.1% Tween-20), and then incubated with 1:10,000 diluted primary antibodies in blocking buffer for 2 h at room temperature. After washing three times, membranes were incubated with 1:2000 diluted anti-rabbit-HRP before subjected to detection using a chemiluminescence detection kit (ECL, Amersham Biosciences, Sunnyvale, USA) according to the manufacturer's instructions.

2.6. Infection of chicken embryo with wild type and recombinant IBV viruses

Eleven-day-old chicken embryonated eggs were obtained from a specific pathogen free farm (Lim Chu Kang Veterinary Station, Singapore) and were used for inoculation of viruses. Approximately 1000 PFU per egg of each virus was inoculated by the allantoic route and the eggs were incubated at 37 °C for 3 days. The amniotic fluid, brain and the lung/liver mixtures of the infected eggs were harvested separately after the embryos were chilled at 4 °C overnight. All samples were stored at –80 °C and analyzed subsequently by Western blot analysis.

3. Results

3.1. Insertion of the EGFP gene at different positions of the IBV genome and recovery of infectious recombinant viruses

The ability of IBV to express foreign gene was first assessed by construction of eight recombinant viruses (Fig. 1a) with either insertion of the EGFP reporter gene at different genomic positions or replacement of group-specific genes ORF3a and 3b with EGFP, using the infectious cDNA clone system described previously (Fang et al., 2007; Tan et al., 2006). The first three virus constructs, IBV-EGFP25180, IBV-EGFP27105 and IBV-EGFPΔ3a3b, were made by inserting the EGFP gene preceded by a TRS sequence from ORF5a into nucleotide position 25180 and 25181, 27105 and 27106, respectively, or replacing the 3a and 3b ORFs with EGFP. The other five constructs, IBV-S-EGFP, IBV-EGFP-E, IBV-M-EGFP, IBV-EGFP-M and IBV-N-EGFP, were made by fusing the EGFP gene onto the C-terminus of the S gene, the N-terminus of E gene, the C- and N-terminus of the M gene, and the C-terminus of the N gene.

These constructs were used as templates for *in vitro* transcription; subsequently, the *in vitro* transcribed full-length RNAs together with the *in vitro* transcribed IBV N transcripts were electroporated into Vero cells. At 3 days post-electroporation,

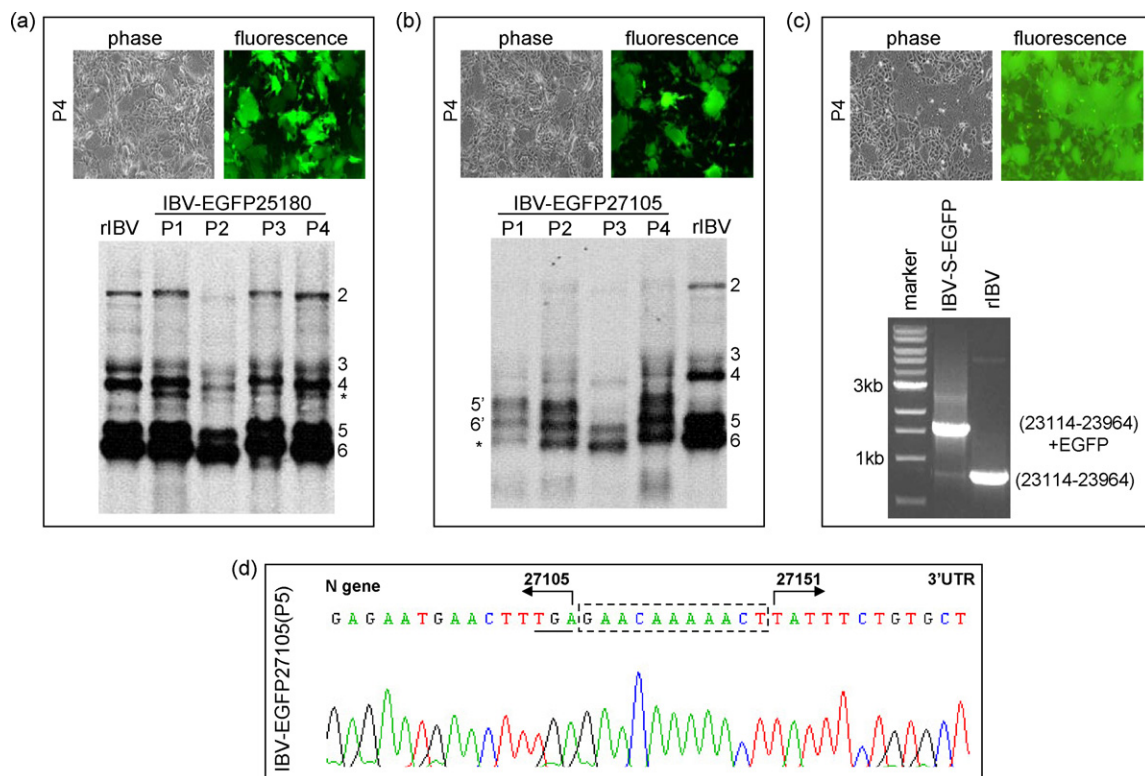


Fig. 2. Analysis of the genetic stability of three recombinant viruses IBV-EGFP25180, IBV-EGFP27105 and IBV-S-EGFP, and the efficiency of EGFP expression in cells infected with the recombinant viruses. (a) EGFP expression in Vero cells infected with IBV-EGFP25180 and Northern blot analysis of viral RNAs in cells infected with passages 1–4 of the recombinant virus. Total RNA was extracted from cells infected with wild type and passages 1–4 (P1–P4) of IBV-EGFP25180 (lower panel). Hybridization was performed with a DIG-labeled DNA probe specific for the IBV 3'-UTR. Numbers on the right indicate positions of individual sgRNA. The position of the new sgRNA-EGFP generated from IBV-EGFP25180 is indicated by an asterisk. Also shown are the phase-contrast and fluorescence images of cells infected with passage 4 of the recombinant virus (upper panels). (b) EGFP expression in Vero cells infected with IBV-EGFP27105 and Northern blot analysis of viral RNAs in cells infected with passages 1–4 of the recombinant virus. Total RNA was extracted from cells infected with wild type and passages 1–4 (P1–P4) of IBV-EGFP27105 (lower panel). Hybridization was performed with a DIG-labeled DNA probe specific for the IBV 3'-UTR. Numbers on the right indicate positions of individual sgRNA. The position of the new sgRNA-EGFP generated from IBV-EGFP27105 is indicated by an asterisk. Also shown are the phase-contrast and fluorescence images of cells infected with passage 4 of the recombinant virus (upper panels). (c) Detection of EGFP expressed in cells infected with IBV-S-EGFP and RT-PCR analysis of the recombinant virus carrying the EGFP gene. Total RNA was extracted from cells infected with passage 4 of IBV-S-EGFP (lower panel), and the presence of EGFP was shown by RT-PCR. Also shown are the phase-contrast and fluorescence images of cells infected with passage 4 of the recombinant virus (upper panels). (d) Sequencing analysis of passage 5 (P5) of IBV-EGFP27105. Cells were infected with passage 5 of the plaque-purified IBV-EGFP27105 and total RNAs were extracted. Regions flanking the insertion site were obtained by RT-PCR and sequenced. Deletion of the EGFP expression cassette together with 46 nucleotides from nucleotide position 27106–27151 in the 3'-UTR and the remaining partial TRS sequences (as shown in dashed box) are indicated.

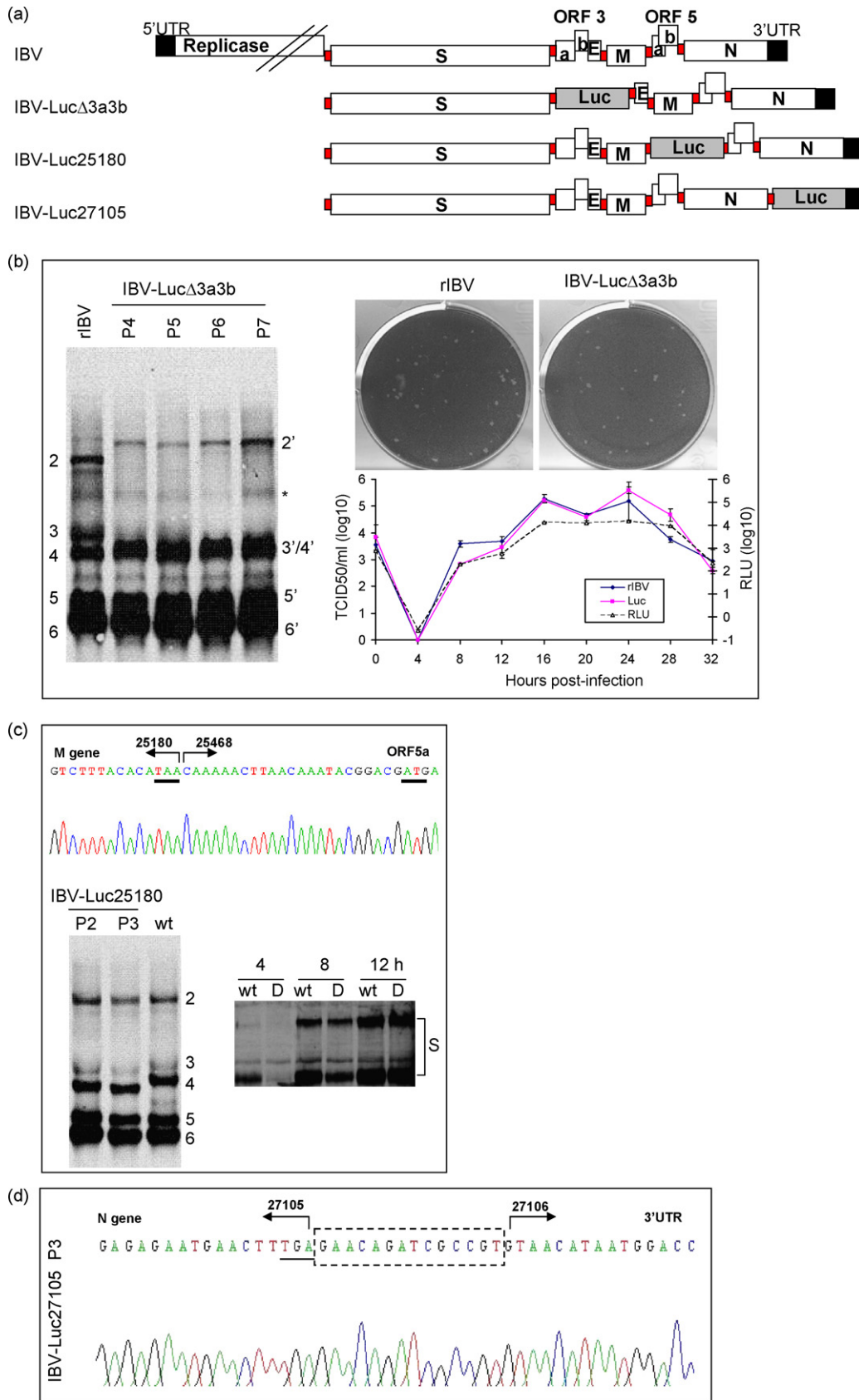


Fig. 3. Characterization of the recombinant IBV viruses expressing the firefly luciferase gene. (a) Diagram showing the genome organization of IBV and positions of the inserted luciferase gene. The luciferase expression cassette either inserted between M gene and ORF5a (IBV-Luc25180), N gene and 3'-UTR (IBV-Luc27105), or used to replace ORF3a3b (IBV-Luc Δ 3a3b) is shown. (b) Genetic stability and phenotypic characterization of the recombinant virus IBV-Luc Δ 3a3b. Total RNA was extracted from cells infected with wild type and passages 4–7 (P4–P7) of IBV-Luc Δ 3a3b (left panel). Hybridization was performed with a DIG-labeled DNA probe specific for the IBV 3'-UTR. Numbers on the left indicate positions of individual sgRNA in cells infected with wild type virus and number on the right indicate positions of sgRNAs in cells infected with the recombinant virus. Also shown are plaques of cells infected with wild

green fluorescence was observed in cells electroporated with IBV-EGFP25180, IBV-EGFP27105, IBV-S-EGFP, IBV-EGFP-M and IBV-N-EGFP, respectively (Fig. 1b). Fluorescence was not detected in cells electroporated with wild type, IBV-EGFP Δ 3a3b, IBV-EGFP-E and IBV-M-EGFP, respectively (Fig. 1a [GE]). Furthermore, infectious recombinant viruses were recovered from cells electroporated with wild type, IBV-EGFP25180, IBV-EGFP27105, IBV-S-EGFP and IBV-EGFP Δ 3a3b (Fig. 1a [VR]). Small CPE and EGFP expression could be observed in cells electroporated with IBV-EGFP-M and IBV-N-EGFP, respectively (Fig. 1b). However, no infectious viruses were recovered when the EGFP gene was fused to E, M or N gene, implying that fusion of EGFP to these three structural proteins might interfere with their proper folding or function which was pivotal for virion assembly and virus recovery.

3.2. Characterization of the recombinant IBV viruses expressing EGFP

Although no fluorescence was observed in cells infected with IBV-EGFP Δ 3a3b, Western blot analysis using anti-EGFP antibody (Sigma) recognized a ~25 kDa protein band equivalent to the full-length EGFP protein size (data not shown). This indicated that although the inserted EGFP gene was transcribed and expressed in virus infected cells. The reason for this is under investigation.

The genetic stability and growth properties of the other three recovered recombinant viruses, IBV-EGFP25180, IBV-EGFP27105 and IBV-S-EGFP, were analyzed subsequently. Propagation of the recombinant viruses in Vero cells showed EGFP expressions up to passage 4 (Fig. 2a–c). For both IBV-EGFP25180 and IBV-EGFP27105, Northern blot analysis clearly demonstrated the presence of an extra sgRNA (marked by an asterisk) initiated by the inserted artificial TRS sequences. However, EGFP expression could not be observed in cells infected with passage 5 onwards (data not shown). RT-PCR and sequencing analysis revealed that the complete EGFP coding sequence together with 46 nucleotides (from 27104 to 27150) located upstream of the 3'-UTR was lost in cells infected with passage 5 of the plaque-purified IBV-EGFP27105 (Fig. 2d). The remaining 11-nucleotide sequence (dashed box) represented the partial core sequence of the inserted TRS (with GAACAA), implying that template switching between neighboring identical TRS sequences might take place during virus propagation and resulted in deletion of the EGFP reporter gene. In cells infected with IBV-S-EGFP, the 3' region of sgRNA2 was shown to contain the EGFP gene of RT-PCR analysis, as demonstrated by a 0.7 kb increase in size when compared to wild type virus (Fig. 2c). The virus was stable at least up to passage 5.

3.3. Construction and characterization of recombinant IBV expressing luciferase

To investigate further the influence of size and nature of foreign genes on the IBV vectors, three recombinant viruses, IBV-Luc Δ 3a3b, IBV-Luc25180 and IBV-Luc27105, expressing a larger reporter gene, the firefly luciferase gene, were constructed. In IBV-Luc Δ 3a3b, the luciferase gene was used to replace the group-specific gene cluster ORF3a3b, and the ORF5a TRS was added upstream of the E gene to restore its transcription and expression after interruption

of the IRES sequence due to ORF3a3b replacement (Fig. 3a). In IBV-Luc25180 and IBV-Luc27105, the luciferase gene was placed downstream of the ORF5a TRS and inserted between nucleotides 25180 and 25181, 27105 and 27106, respectively (Fig. 3a). Recombinant viruses were recovered at 48 h post-electroporation and serial passages up to passage 15 in Vero cells were conducted subsequently.

In cells infected with IBV-Luc Δ 3a3b, typical IBV-induced CPE including syncytia formation in Vero cells was observed (data not shown). In contrast to the absence of fluorescence of IBV-EGFP Δ 3a3b, intracellular luciferase activity remained high and stable up to passage 15. To analyze the growth property and stability of this virus in detail, plaque-purified virus was passaged serially and total RNA was isolated from Vero cells infected with different passages (P4–P7) in a parallel experiment and subjected to Northern blot analysis. The expected size increases in sgRNAs 2, but not sgRNA 4–6 were observed when compared to wild type control (Fig. 3b, left panel). An extra sgRNA containing luciferase gene was detected (marked by an asterisk). The E gene under control of the inserted ORF5a TRS was also detected as an extra transcript (sgRNA3') slightly above sgRNA4' (Fig. 3b). No difference in the sgRNA species was observed within the four continuous passages (P4–7). These results demonstrate that the 1.7 kb luciferase expression cassette was incorporated successfully and maintained stably in the recombinant virus genome. Subsequent RT-PCR analysis and luciferase assay also confirmed the presence of luciferase gene cassette (data not shown).

The growth properties of IBV-Luc Δ 3a3b and the luciferase activity in cells infected with the virus were characterized. In Vero cells infected with wild type and IBV-Luc Δ 3a3b (P7) viruses, similarly sized plaques were observed at 3 days post-infection (Fig. 3b). Both viruses also showed similar growth kinetics in Vero cells, reaching peak titre at 16 h post-infection (Fig. 3b). Measurement of the luciferase activity in cells infected with the recombinant virus also showed that the activity reached its maximum at 16 h post-infection and remained stable up to 28 h post-infection (Fig. 3b), reflecting the stability of the enzyme in the cells.

In contrast to the stable expression of luciferase by IBV-Luc Δ 3a3b, luciferase activity could not be detected in cells infected by either IBV-Luc25180 or IBV-Luc27105 from P2 onwards. Northern blot analysis of cells infected with passage 2 of IBV-Luc25180 clearly demonstrated a decrease in size of sgRNA3 and sgRNA4, implying that the luciferase gene cassette was lost when the virus was propagated in cultured cells (Fig. 3c). To analyze further the genetic basis of this instability, RT-PCR and sequencing analysis of the luciferase insertion region revealed that the entire luciferase gene together with a 287-nucleotide-long IBV genomic sequence (from 25181 to 25467) was deleted while leaving the original ORF5a and its TRS sequences intact (Fig. 3c). This finding was concordant with the observation that the luciferase activity was lost in cells infected with passage 2 of IBV-luc25180, whereas the virus started to replicate to higher titers (data not shown). It also implied that the deleted IBV genomic sequence (from 25181 to 25467) was dispensable for virus replication. Western blot analysis showed similar amounts of accumulation of the S protein in cells infected with the deletion mutant virus (D) and wild type virus in a time course experiment (Fig. 3c).

type and the P7 recombinant virus (upper right panels), and the growth curves as well as the relative luciferase activity (RLU) in cells infected with wild type and P7 recombinant virus (lower right panel). (c) Genetic stability of IBV-Luc25180. RT-PCR and sequencing analysis of the luciferase insertion site confirmed the loss of the inserted luciferase gene together with 287 nucleotides downstream of the stop codon for the M gene (underlined) in cells infected with passage 3 of the recombinant virus (upper panel). The lower left panel shows Northern blot analysis of cells infected with wild type and passages 2 and 3 of the recombinant virus (P2 and P3). Numbers on the right indicate the individual sgRNAs. Also shown is Western blot analysis of IBV S protein in cells infected with wild type and the mutant virus with deletion of the luciferase cassette and 287 nucleotides in the IBV genome (D) (lower right panel). (d) Genetic stability of the recombinant virus IBV-Luc27105. Total RNA was extracted from cells infected with passage 3 of IBV-Luc27105, and regions flanking the insertion site were amplified by RT-PCR and sequenced. The loss of the inserted luciferase gene together with the remaining sequence left from the inserted luciferase cassette (dashed box) is shown.

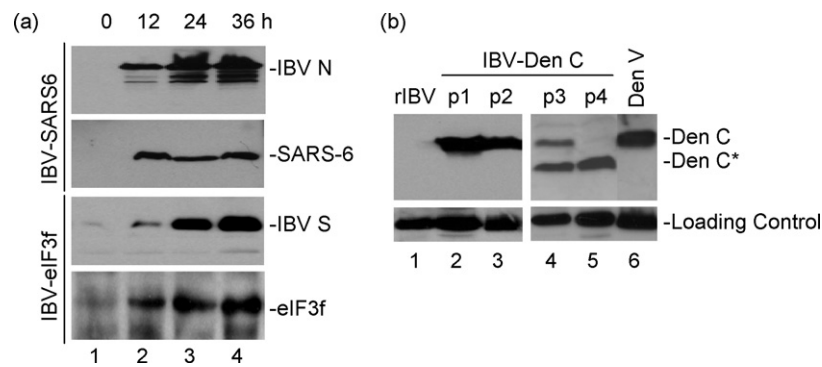


Fig. 4. Western blot analysis of the expression of host and other viral genes in cells infected with the corresponding recombinant IBV. (a) Expression of SARS-CoV gene 6 and eIF3f in cells infected IBV-SARS6 and IBV-eIF3f. Vero cells were infected with IBV-SARS6 or IBV-eIF3f at 2 PFU per cell and harvested at indicated times. Polypeptides were separated by SDS-PAGE and analyzed by Western blotting with anti-IBV N, anti-SARS-CoV gene 6, anti-IBV S and anti-eIF3f antibodies, respectively. (b) Expression of dengue virus core protein in Vero cells infected with passages 1–4 (P1–P4) of the recombinant virus IBV-DenC. Vero cells were infected with IBV-DenC at 2 PFU per cell and harvested at indicated times. Polypeptides were separated by SDS-PAGE and analyzed by Western blotting with anti-dengue virus core protein antibodies. A more rapid migrating band detected in cells infected with P3 and P4 viruses (lanes 4 and 5) is indicated (Den C*). Cells infected with wild type IBV (lane 1) and dengue virus type 1 (lane 6) was used as control.

The same RT-PCR strategy was employed to analyze IBV-Luc27105. In cells infected with passage 2 of IBV-Luc27105, the luciferase activity fell suddenly and no full-length RT-PCR fragment was amplified from cells infected with passage 3 of the same recombinant virus. Instead, a 1 kb PCR fragment, which is the same size as the RT-PCR fragment amplified from cells infected with wild type virus, was detected, suggesting that the luciferase gene was lost. Sequencing analysis of the PCR fragment confirmed that only 14 nucleotides from the inserted luciferase gene cassette were left (Fig. 3d).

3.4. Expression of cellular and viral genes using recombinant IBV

In a previous report, stable and efficient expression of SARS-CoV 8b and 8ab proteins using the recombinant IBV system were achieved (Le et al., 2007). In the current study, three more viral and host proteins, the SARS-CoV ORF6, the dengue virus 1 core protein and the eukaryotic translation initiation factor 3f (eIF3f), were expressed using the same system. The foreign genes were inserted at the position either between M and ORF 5a or downstream of the N gene, and infectious recombinant viruses were recovered after electroporation of the *in vitro* transcribed full-length RNA into Vero cells. Infection of Vero cells with recombinant IBV carrying the SARS-CoV gene 6 or eIF3f showed efficient expression of the two proteins (Fig. 4A). The two viruses were stable up to passage 5 in Vero cells (data not shown). In cells infected with the recombinant virus carrying the dengue virus core protein, efficient expression of the protein was also detected (Fig. 4b). However, the virus was much less stable when propagated in the cultured cells. In cells infected with the passage 3 virus, full-length core protein and a more rapidly migrating core protein band, probably representing a product encoded by the partially deleted core gene, were detected (Fig. 4b, lane 4). In cells infected with passage 4 of the virus, the full-length core protein was barely detected (Fig. 4b, lane 5). RT-PCR amplification and nucleotide sequencing confirmed that the C-terminal region of the core gene together with some sequence downstream of the insertion was deleted from the IBV genome (data not shown).

3.5. Infection of chicken embryo with the recombinant IBV viruses expressing EGFP and the dengue virus core protein

Two recombinant viruses, IBV-EGFP27105 and IBV-DenC, were used to infect 11-day embryonated eggs by inoculation of 1000 PFU

per egg of each virus into the amniotic cavity. Two eggs were infected with each virus. At 3 days post-inoculation, the amniotic fluid, liver and lung mixture, and brain were separately harvested and subjected to analysis by Western blot using anti-IBV N, anti-GFP and anti-Den C antibodies, respectively. In eggs inoculated with wild type virus, approximately equal amounts of the N protein were detected from the amniotic fluid, liver and lung mixture, and brain (Fig. 5). In eggs infected with IBV-EGFP27105, equal amounts

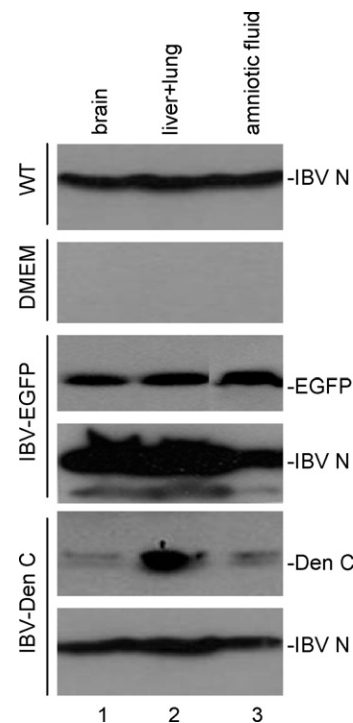


Fig. 5. Western blot analysis of the expression EGFP and dengue virus core protein in chicken embryo infected with the recombinant IBV viruses. Eleven-day-old chicken embryonated eggs were inoculated with 1000 PFU per egg of wild type and passage 2 of the recombinant viruses IBV-EGFP27105 and IBV-DenC, respectively. The brain (lane 1), the lung/liver mixtures (lane 2) and the amniotic fluid (lane 3) of the infected eggs were harvested separately at 3 days post-inoculation and analyzed by Western blot with anti-IBV N, anti-EGFP, anti-dengue virus core protein antibodies, respectively. Eggs inoculated with medium (DMEM) were included as a negative control.

of EGFP were detected in the amniotic fluid, liver and lung mixture, and brain, but slightly less IBV N protein was detected in the amniotic fluid (Fig. 5). In eggs infected with IBV-DenC, similar amounts of IBV N protein were detected in the liver/lung mixture, brain, and the amniotic fluid (Fig. 5). Probing the same membrane with anti-Den C antibodies showed that the protein was detected mainly in the liver/lung mixture; only minor amounts of the protein were detected in brain and the amniotic fluid (Fig. 5).

4. Discussion

Coronavirus possesses several attractive traits as a potential gene delivery system. First, as an RNA virus, it replicates exclusively in the cytoplasm of the infected cells without a DNA stage, making integration of viral sequence into the host genome unlikely. Second, it is the largest RNA virus with a genome size of around 30 kb and a cloning capacity of more than 6 kb is therefore expected (Enjuanes et al., 2005). Third, coronavirus displays a unique transcription process involving transcription of a nested set of 6–8 subgenomic mRNA species. Heterologous genes can be expressed simply by inserting them with an appropriate TRS into the virus genome, as demonstrated for MHV (Fischer et al., 1998), SARS-CoV (Sims et al., 2005) and IBV (Le et al., 2007; Youn et al., 2005). Fourth, coronavirus host specificity and tropism could be altered readily by exchange of the S gene between viruses of different host origin (Casais et al., 2003; Sanchez et al., 1997). Therefore, it is possible to engineer the tissue and species tropism to target expression to different organs and animal species, including humans. Fifth, it may be used to express multiple genes, as demonstrated by the possibility of constructing coronavirus-based HIV multigene vaccine (Eriksson et al., 2006; Thiel et al., 2003). Finally, the mucosal route is the natural way of coronavirus transmission. In this study, efforts were made to explore the possibility of developing coronavirus IBV as gene delivery vectors and multivalent vaccines. In this study, a number of infectious recombinant IBV carrying foreign genes with variable degrees of genetic stability were obtained by insertion of reporter genes or host and other viral genes at different positions of the IBV genome. Several of these recombinant viruses could be potential vaccine candidates and useful tools for studying the molecular and cellular biology of coronavirus.

This study demonstrated that genetic stability of recombinant IBV carrying foreign genes depends on the size and the intrinsic property of the inserted genes as well as the genomic locations at which they are inserted. It is likely that foreign genes may contain sequences that make them more susceptible to mutation or recombination during coronavirus replication. For example, instability caused by the firefly luciferase in recombinant MHV might be associated with its high GC content (46.7%) when compared to the coronavirus genome (MHV, 41.8%; FIPV, 38.1%) (de Haan et al., 2005). Indeed, it was shown for poliovirus vectors that manipulation of the G/C contents increased the genetic stability of foreign inserts (Lee et al., 2002). In contrast, the EGFP gene with a G/C content of 61–64% was stably maintained in the recombinant virus IBV-EGFP27105 genome, as shown in this study. Previous studies also described stable expression of GFP after its insertion into TGEV and MHV (Curtis et al., 2002; Fischer et al., 1998). Foreign inserts may also contain RNA secondary structures, which are prone to undergo RNA recombination, as recombination was demonstrated to occur more frequently at regions of predicted RNA secondary structure in the MHV S gene (Rowe et al., 1997). Recombination events within a foreign gene may also be dependent on the degree of base pairing between the foreign gene and the virus genome sequence (Lai, 1996). In addition, the molar ratio of the genuine

coronavirus sgRNAs and their encoded proteins could be disturbed after insertion of foreign gene (de Haan et al., 2003)

Apart from viral mutation and recombination rates, the selective advantage of variants newly emerged during propagation of the recombinant viruses is also an important determinant for the instability of the recombinant viruses. In this study, a key phenomenon observed for all unstable recombinant viruses was that loss of foreign genes was accompanied inevitably by increasing viral titre (data not shown). It was hypothesized that recombinant viruses carrying full-length foreign genes appear to have a replicative disadvantage than those with deletion or recombination, hence are less favourably selected when propagated in cultured cells.

In this study, genetic instability of recombinant IBV carrying foreign genes at the position between M gene and ORF5a was observed for both IBV-EGFP25180 and IBV-Luc25180, though high level expression of these reporter genes could be detected at early passages. Expression of the reporter genes decreased when the virus was propagated serially in cultured cells and the expression became eventually undetectable in subsequent passages. This was accompanied by gradual deletion of the inserted foreign gene and even intergenic IBV backbone sequences. When the reporter genes were inserted downstream of the N gene, expression of EGFP could be observed in serial passages for IBV-EGFP27105 in cultured cells, but the luciferase activity was decreased as early as at the second passage of IBV-Luc27105, implying that this position was at least not stable for the luciferase gene. When GFP gene was inserted into the equivalent position of the TGEV genome, a highly unstable virus was obtained (Sola et al., 2003), and attempts to insert GFP at the same position of the MHV genome did not result in a viable virus (Hsue and Masters, 1997).

Recombinant coronaviruses generated by replacement of non-essential genes were generally more stable. Curtis et al. (2002) demonstrated stable EGFP expression in TGEV replicon particles. Recombinant MHV expressing EGFP was also reported to be stable when it was used to monitor the virus spread *in vitro* as well as in the mouse central nervous system (Sarma et al., 2002). In a previous study by Sola et al. (2003), recombinant TGEV engineered by replacing ORF3a3b with GFP led to efficient ($>40 \mu\text{g}/10^6$ cells) and stable (>20 passages) expression of GFP. A stable recombinant FIPV expressing the *Renilla* luciferase was also made by replacing the nonessential group-specific gene cluster, ORF3abc (de Haan et al., 2005). Although the functions of IBV ORF3a/3b encoded proteins remain to be elucidated, studies based on IBV and other coronaviruses demonstrated that deletion of such group-specific ORFs did not influence dramatically the replication efficiency or viral RNA synthesis in cell culture, and may even play little role in *in vivo* replication (Casais et al., 2005; Haijiema et al., 2004; Hodgeson et al., 2006; Ortego et al., 2003; Yount et al., 2005). This possibly explains the stable expression of luciferase observed in cells infected by IBV-Luc Δ 3a3b.

A potentially attractive application of this IBV system would be the development of multivalent vaccines against avian and other zoonotic diseases transmitted through avian species. Since insertion of large genes into the IBV genome results usually in the generation of unstable recombinant viruses, a practical approach would be to develop recombinant viruses expressing the major antigenic domain of a protein. For example, it would be interesting to construct a recombinant IBV expressing the major antigenic domain of the hemagglutinin protein of H5N1 influenza virus, and explore the immune response and protection of chicks infected with the virus. This possibility is under investigation.

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