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Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection

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Abstract

A reverse genetics system for the avian coronavirus infectious bronchitis virus (IBV) has been described in which a full-length cDNA, corresponding to the IBV (Beaudette-CK) genome, was inserted into the vaccinia virus genome following in vitro assembly of three contiguous cDNAs [Casais, R., Thiel, V., Siddell, S.G., Cavanagh, D., Britton, P., 2001. Reverse genetics system for the avian coronavirus infectious bronchitis virus. J. Virol. 75, 12359–12369]. The method has subsequently been used to generate a recombinant IBV expressing a chimaeric S gene [Casais, R., Dove, B., Cavanagh, D., Britton, P., 2003. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77, 9084–9089]. Use of vaccinia virus as a vector for the full-length cDNA of the IBV genome has the advantage that modifications can be made to the IBV cDNA using homologous recombination, a method frequently used to insert and delete sequences from the vaccinia virus genome. We describe the use of homologous recombination as a method for modifying the Beaudette full-length cDNA, within the vaccinia virus genome, without the requirement for in vitro assembly of the IBV cDNA. To demonstrate the feasibility of the method we exchanged the ectodomain of the Beaudette spike gene for the corresponding region from IBV M41 and generated two recombinant infectious bronchitis viruses (rIBVs) expressing the chimaeric S protein, validating the method as an alternative way for generating rIBVs.

Keywords: Coronavirus; Avian; IBV; Transient dominant selection; Infectious bronchitis; Vaccinia virus

1. Introduction

Avian infectious bronchitis virus (IBV), a group three member of the genus Coronavirus (order Nidovirales, family Coronaviridae), is a highly infectious pathogen of domestic fowl that replicates primarily in the respiratory tract but also in epithelial cells of the gut, kidney and oviduct (Cavanagh, 2001; Cavanagh and Naqi, 2003; Cook et al., 2001). Genetically very similar coronaviruses cause disease in turkeys and pheasants (Cavanagh et al., 2001, 2002). Coronaviruses are enveloped viruses that replicate in the cell cytoplasm and contain an unsegmented, 5′-capped and 3′-polyadenylated, single-stranded, positive-sense RNA genome of 28–32 kb (de Vries et al., 1997; Lai and Cavanagh, 1997; Siddell, 1995). All coronavirus envelopes contain at least three membrane proteins, the spike glycoprotein (S), a small membrane protein (E) and an integral membrane protein (M). In addition, the coronavirus virion also contains a nucleocapsid protein (N) that interacts with the gRNA.

Molecular analysis of the structure and the role of individual genes in pathogenesis of RNA viruses has been advanced by the availability of full-length cDNAs, for the generation of infectious RNA transcripts that can replicate and result in infectious viruses from permissive cell lines (Boyer and Haenni, 1994). Such reverse genetics systems have resulted in the recovery of a number of infectious positive-stranded RNA viruses including, picornaviruses, caliciviruses, alphaviruses, flaviviruses, arteriviruses and Closterovirus, whose RNA genomes range in size from 7 to 20 kb in length (Agapov

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et al., 1998; Davis et al., 1989; Racaniello and Baltimore, 1981; Rice et al., 1987, 1989; Satyanarayana et al., 2001; Sosnovtsev and Green, 1995; Sumiyoshi et al., 1992; van Dinten et al., 1997). Recently full-length cDNAs capable of producing infectious RNA transcripts for several coronaviruses, viruses with the largest RNA genomes, Transmissible gastroenteritis virus (TGEV; (Almazán et al., 2000; Yount et al., 2000)), Human coronavirus 229E (HCoV; (Thiel et al., 2001)), IBV (Casais et al., 2001), *Murine hepatitis* virus (MHV; (Yount et al., 2002)), and severe acute respiratory syndrome coronavirus (SARS-CoV; Yount et al., 2003) have been produced.

The assembly of full-length cDNAs corresponding to the coronavirus genomes was hampered due to some cDNAs, derived from regions of the replicase gene, being unstable in bacteria. However, despite this, three methods have been described for the assembly of full-length cDNAs of the genomic RNA of coronaviruses. The first method involved the assembly of a TGEV full-length cDNA in a bacterial artificial chromosome (BAC), immediately downstream of a viral RNA polymerase II promoter. Transfection of the BAC DNA into susceptible cells resulted in synthesis of infectious RNA and the recovery of infectious virus (Almazán et al., 2000). The second system involved the in vitro assembly of full-length cDNAs using a series of contiguous cDNAs with engineered specific restriction sites. Bacteriophage T7-RNA polymerase-derived RNA transcripts of the cDNAs were used to generate infectious virus (Yount et al., 2000, 2002, 2003). The third strategy involved the in vitro assembly of contiguous cDNAs followed by direct cloning into the genome of vaccinia virus. Infectious RNA transcripts were synthesised in vitro from the vaccinia virus DNA, using bacteriophage T7 RNA polymerase, and transfected into cells resulting in the recovery of infectious virus (Thiel et al., 2001). In an alternative strategy infectious IBV was recovered following transfection of restricted vaccinia virus DNA, containing the IBV full-length cDNA, into cells infected with recombinant fowlpox virus expressing T7 RNA polymerase (Casais et al., 2001). The vaccinia virus system has the advantage that the coronavirus full-length cDNA is present in a non-bacterial system and offers the opportunity of modifying the coronavirus cDNA by homologous recombination.

Falkner and Moss (1990) devised a general method for modifying the vaccinia virus genome, involving insertions, deletions and specific mutations, termed transient dominant selection (TDS). The method relies on a two-step procedure. In the first step, a complete plasmid sequence is introduced into the vaccinia virus genome as a result of a single cross-over event involving homologous recombination between vaccinia virus sequences in the plasmid DNA and virus genome. The plasmid sequence contains the sequence being modified and a dominant selectable marker gene, *Escherichia coli* guanine Xanthine phosphoribosyltransferase (*Ecogpt*) (Mulligan and Berg, 1981), under the control of the vaccinia virus $P_{7.5K}$ promoter. Recombinant viruses expressing the *gpt* gene are selected for resistance against mycophenolic acid (MPA) in the presence of xanthine and hypoxanthine. In the second step, the MPA-resistant viruses are grown in the absence of selection, resulting in loss of the *Ecogpt* gene due to a single homologous recombination event between duplicated sequences, present as a result of the integration of the plasmid DNA. The second step results in one of two recombination events; return to the original input virus sequence, without modification to the virus or introduction of the modified sequences into the vaccinia virus genome. Both events should occur with equivalent efficiencies if the homology regions are of equal length.

We have utilised the in vitro ligation and direct cloning into the vaccinia virus genome method of generating rIBVs for the recovery of a rIBV expressing a chimaeric S gene (Casais et al., 2003). The rIBV expressed an S gene in which the ectodomain of the protein was derived from a different strain of IBV. Having demonstrated that it was possible to produce a rIBV expressing a modified S gene we decided to investigate an alternative, quicker and simpler, procedure for modifying the full-length IBV cDNA within the vaccinia virus genome for the production of rIBVs containing the chimaeric S gene. In this paper, we describe the generation of two homologous rIBVs, rIBV-M41S-A and rIBV-M41S-B, in which the ectodomain sequence of the Beaudette S gene in vaccinia virus vNotI-IBV_{FL} (Casais et al., 2001) was exchanged with the corresponding region from the M41 strain of IBV using the TDS method. Our results demonstrated that rIBVs can be generated following direct modification of the full-length IBV cDNA by homologous recombination within the vaccinia virus genome.

2. Materials and methods

2.1. Cells and viruses

The growth of IBV in either 11-day-old embryonated domestic fowl eggs or chick kidney (CK) cells was as described previously (Pénzes et al., 1994, 1996; Stirrups et al., 2000). The IBV isolates used were: (1) Beaudette-CK (Beau-CK; (Cavanagh et al., 1986)), a virus adapted for growth in CK cells that can grow on, but has not been adapted for growth on, Vero cells an African green monkey cell line; (2) Beau-R, a recombinant IBV (rIBV) produced from an infectious RNA transcribed from a full-length cDNA of Beau-CK (Casais et al., 2001); (3) M41-CK, a virus adapted from M41 (Darbyshire et al., 1979) for growth on CK cells but not Vero cells; and (4) BeauR-M41(S), a rIBV expressing a M41-CK/Beau-CK chimaeric S gene, produced following in vitro assembly of a full-length IBV cDNA in vaccinia virus, that grows on CK but not Vero cells (Casais et al., 2003). Beaudette and M41 belong to the same, Massachusetts, serotype. Vaccinia viruses were titrated on monkey kidney fibroblast cells (CV-1) grown in Dulbecco's-modified Eagle medium (D-MEM) supplemented with 0.37% (w/v) sodium bicarbonate, L-glutamine, 10% foetal calf serum and an-

tibiotics. Baby hamster kidney cells (BHK-21) cells were grown in Glasgow medium supplemented with 0.37% (w/v) sodium bicarbonate, tryptose phosphate broth, L-glutamine, 10% foetal calf serum and antibiotics and used for the propagation of vaccinia viruses for isolation of virus DNA. Recombinant fowlpox virus rFPV-T7 (fpEFLT7pol; Britton et al., 1996), expressing the bacteriophage T7 RNA polymerase under the direction of the vaccinia virus $P_{7.5}$ early/late promoter, was grown in chick embryo fibroblast (CEF) cells (Casais et al., 2001).

2.2. Recombinant DNA techniques

Recombinant DNA techniques used standard procedures (Ausubel et al., 1987; Sambrook et al., 1989) or were used according to the manufacturers' instructions. All IBV-related nucleotide and amino acid residue numbers refer to the positions in the IBV Beau-R genome (Casais et al., 2001), accession no. AJ311317.

2.3. Plasmid constructions

The complete Beau-CK S gene, within a 5.6 kb *Stu*I and *Bam*HI fragment, was removed from pFRAG-3 (Casais et al., 2001) and inserted into *Hinc*II and *Bam*HI digested pGPT-NEB193 (a gift from Dr. M. Skinner IAH) (Boulanger et al., 1998), resulting in pGPT-IBV-*Stu*I-*Bam*HI (Fig. 1). Plasmid pGPTNEB193 contains the *E. coli* guanine xanthine phosphoribosyltransferase (*Ecogpt*) gene under the control of the vaccinia virus $P_{7.5}$ early/late promoter and a separate multiple cloning site. Most (94%) of the Beau-CK S gene was removed from pGPT-IBV-*Stu*I-*Bam*HI by digestion with *Pac*I and *Sty*I followed by end repair of the DNA and ligation, resulting in pGPT-IBV-ΔS (Fig. 1).

The M41-CK/Beau-CK chimaeric S gene, within a 4.5 kb *Pac*I and *Bam*HI fragment, was removed from pFRAG-3-M41S (Casais et al., 2003) and used to replace the Beau-CK S gene sequence in pGPT-IBV-*Stu*I-*Bam*HI, resulting in pGPT-M41S (Fig. 2).

2.4. Modification of IBV cDNA sequences within vaccinia virus genomes

Recombinant vaccinia viruses were generated as a result of transient dominant selection (TDS; (Falkner and Moss, 1990)) using the *Ecogpt* gene as the transient selectable marker. CV-1 cells were infected with vaccinia virus vNotI-IBV_{FL}, containing the full-length IBV cDNA derived from Beau-CK, at a multiplicity of infection (MOI) equivalent to 0.2 plaque-forming units (PFU) per cell, and subsequently transfected with pGPT-IBV-ΔS in the presence of lipofectin (Invitrogen). Recombinant viruses expressing GPT, from the *Ecogpt* gene, were selected by three rounds of plaque purification in CV-1 cells in the presence of selection medium, CV-1 growth medium containing 25 μg/ml MPA, 250 μg/ml xanthine and 15 μg/ml hypoxanthine (Fig. 3). Several MPA-resistant vaccinia viruses were subsequently plaque purified (X3) in the absence of MPA resulting in the spontaneous loss of the *Ecogpt* gene (Fig. 3). Several recombinant vaccinia viruses that had a GPT-negative phenotype were analysed by PCR and found not only to lack the *Ecogpt* gene sequence but also, as expected, to have the Beau-CK S gene sequence deleted from the IBV cDNA. One such recombinant vaccinia virus, vNotI-IBV-ΔS_{FL}, was used in subsequent experiments.

The TDS method was used to insert the M41-CK/Beau-R chimaeric S gene into the IBV cDNA within vNotI-IBV-ΔS_{FL} using pGPT-M41S and involving *Ecogpt* selection in the presence of MPA followed by loss of the *Ecogpt* gene in the absence of MPA (Fig. 4). Recombinant vaccinia viruses that had a GPT-negative phenotype were analysed by PCR and found to lack the *Ecogpt* gene sequence. Further analysis identified a recombinant vaccinia virus, vNotI-TDSR-$M41S_{FL}$, that contained the M41-CK/Beaudette-CK chimaeric S gene sequence within the full-length IBV cDNA.

2.5. Isolation of vaccinia virus DNA

Vaccinia virus vNotI-TDSR-$M41S_{FL}$ was grown in 13 T150 flasks of BHK-21 cells until a CPE was evident. The infected cells were harvested, resuspended in 10 mM Tris–HCl pH 9.0, 1 mM EDTA lysed by three cycles of freeze thawing, sonicated and the cell nuclei pelleted by centrifugation at $750 \times g$ for 5 min at 4 °C. Virus particles were partially purified by centrifugation, through a cushion of 30% sucrose in 10 mM Tris–HCl, pH 9.0, at 14,000 rpm for 1 h at 4 °C, using a SW28C rotor in a Sorvall OTD65B ultracentrifuge and resuspended in 10 mM Tris–HCl, pH 9.0, 1 mM EDTA. The semi-purified virus was incubated in 100 mM Tris–HCl pH 7.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl containing 0.1 mg/ml proteinase K, incubated at 50 °C for 2 h, extracted with phenol/chloroform and the DNA ethanol precipitated. The vaccinia virus DNA was digested with *Asc*I (1 U/μg of DNA) and analysed by pulse field gel electrophoresis using 1% agarose gels (Casais et al., 2001).

2.6. Recovery of infectious recombinant IBV

CK cells, grown to 50% confluence (approximately 2×10^6 cells) in 60 mm diameter plates, were infected with rFPV-T7 (Britton et al., 1996) at an MOI of 10. After 45–60 min, the cells were transfected with 10 μg of *Asc*I-restricted vNotI-TDSR-$M41S_{FL}$ DNA and 5 μg of pCi-Nuc (Hiscox et al., 2001) using 30 μg of lipofectin (Invitrogen) (Casais et al., 2001, 2003). The transfected cells (P_0 cells) were incubated at 37 °C for 16 h after which the transfection medium was replaced with fresh maintenance medium (Pénzes et al., 1994). At 2.5 days post transfection, the culture medium, potentially containing rIBV (V_1), was removed, centrifuged for 3 min at 2500 rpm, filtered through a 0.22 μm filter, to remove any rFPV-T7 (Evans et al., 2000), and used for serial passage on CK cells. Two independently rescued

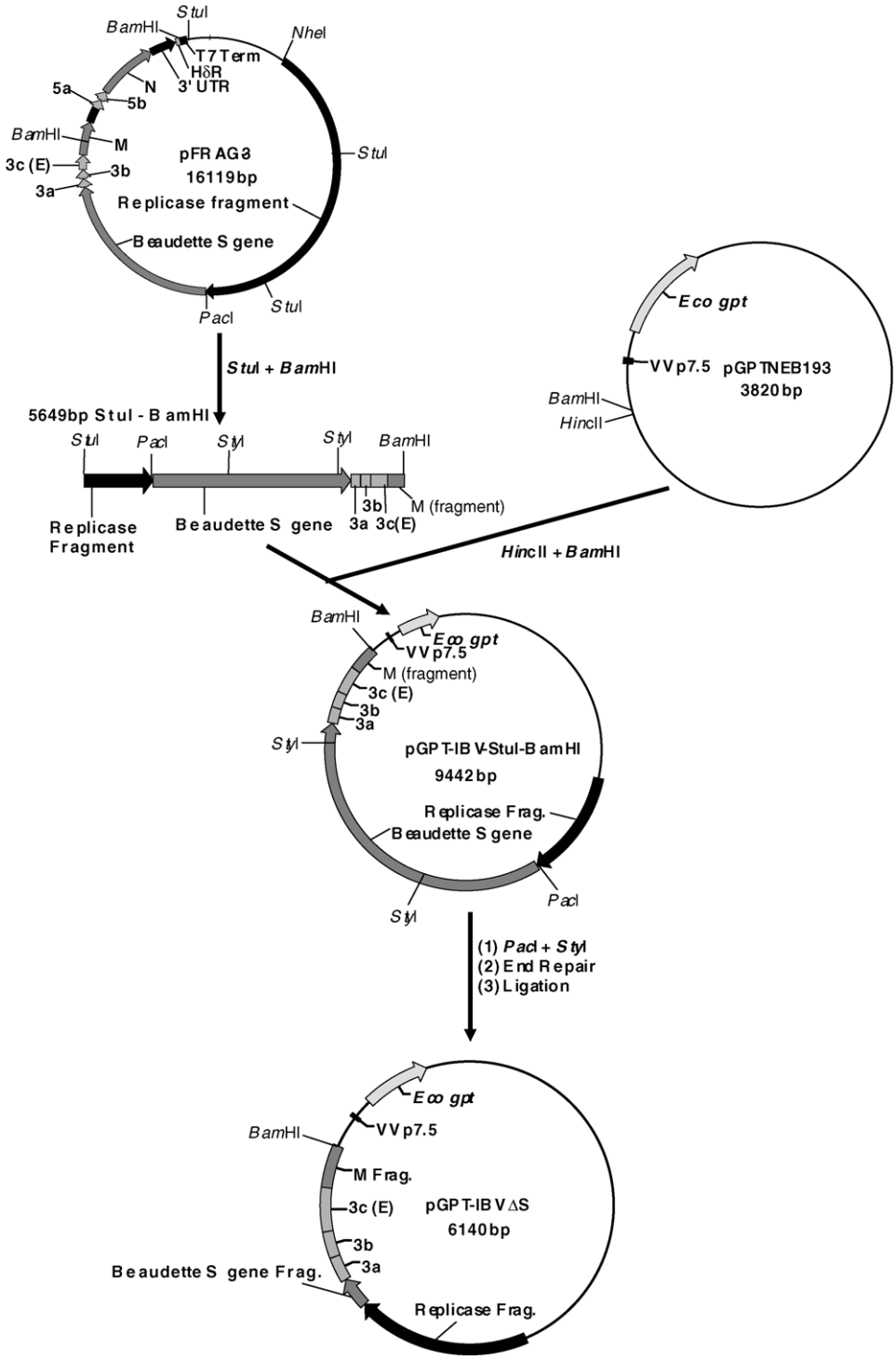

Fig. 1. Schematic diagram for the construction of the recombination vector for deleting the IBV S gene from the full-length cDNA in the vaccinia virus genome. The recombination vector is based on a GPT expressing plasmid, pGPTNEB193, in which a segment of the IBV genome, corresponding to the IBV S gene and flanking sequences of approximately equal length, was introduced. The majority of the S gene was then removed, leaving only the transmembrane and C-terminal domains within the recombination vector, pGPT-IBV-ΔS. The flanking regions were included to allow homologous recombination events to occur between the IBV sequences in pGPT-IBV-ΔS and the corresponding sequences in the IBV full-length cDNA in the vaccinia virus (vNotI-IBV$_{FL}$) genome. The flanking regions were of approximate equal length to allow unbiased homologous recombination events. The various IBV gene sequences and relevant restriction sites are indicated; S represents the spike glycoprotein gene, 3a, 3b, 3c (E) represent the IBV gene 3 products; M represents the integral membrane protein; 5a, 5b represent the IBV gene 5 products; N represents the nucleocapsid protein; 3′ UTR represents the IBV 3′ untranslated region; HδR represents the hepatitis delta ribozyme sequence; T7 term represents the T7 termination sequence; *Ecogpt* represents the *E. coli* GPT gene and VV $P_{7.5}$ represents the vaccinia virus early/late $P_{7.5}$ promoter.

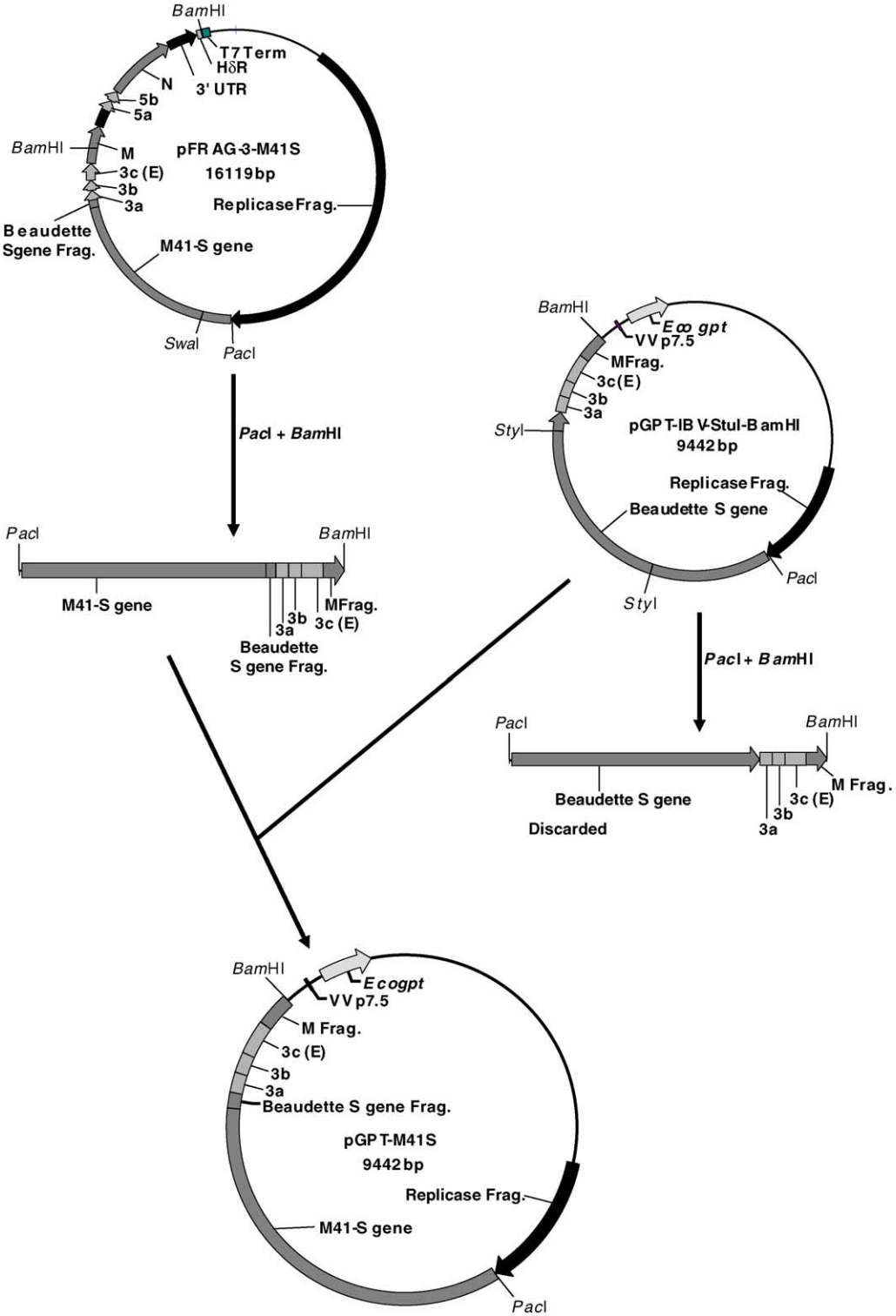

Fig. 2. Schematic diagram for the construction of the recombination vector for inserting the chimaeric S gene into the IBV cDNA lacking an S gene sequence in the vaccinia virus genome. The signal sequence, ectodomain and transmembrane domain of the M41-CK S gene sequence was used to replace the corresponding Beau-CK sequence in pGPT-IBV-*Stu*I-*Bam*HI. The transmembrane domains from the two viruses are identical, therefore the chimaeric S gene has essentially the signal sequence and ectodomain sequences derived from M41-CK and transmembrane and C-terminal domain sequences from Beau-CK in pGPT-M41S. The flanking regions of the chimaeric S gene were as described in the legend for Fig. 1 and were present to allow integration of the chimaeric S gene by homologous recombination events into the IBV cDNA lacking an S gene sequence in vNotI-IBV-ΔS_{FL}. The various IBV gene sequences are as described in the legend for Fig. 1 and the relevant restriction sites are shown.

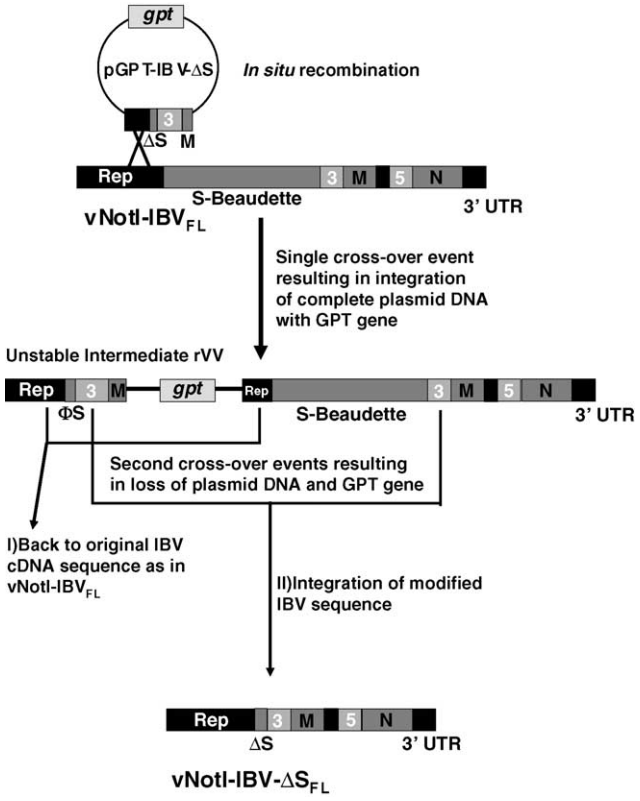

Fig. 3. Schematic diagram demonstrating the TDS method for removing the S gene sequence from the full-length IBV cDNA in vNotI-IBV$_{FL}$. The diagram represents a potential recombination event between the replicase sequence in one of the flanking regions in pGPT-IBV-ΔS and the corresponding region in the IBV cDNA in vNotI-IBV$_{FL}$. The single step recombination event results in the integration of the complete plasmid sequence into the IBV cDNA in the vaccinia virus genome. Resultant recombinant vaccinia viruses are selected in the presence of MPA due to incorporation of the GPT gene under the control of a vaccinia virus promoter. The integrated sequences result in tandem repeat sequences within the IBV cDNA. Such resultant vaccinia viruses are relatively unstable and are only able to maintain the duplicate sequences in the presence of MPA. In the absence of MPA one of the duplicated sequences is lost as a result of spontaneous recombination events. In the absence of MPA, two types of potential recombination events, I and II, are possible resulting in the loss of the GPT gene. Recombination event (I) results in retention of the S gene sequence, such recombinant vaccinia viruses have the same sequence as the original input vaccinia virus; (II) results in the loss of most of the S gene sequence, the desired modification. Both types of recombination events have an equal chance of occurring. The various IBV genes are as described in the legend to Fig. 1 Rep represents the end of the IBV replicase gene, ΔS indicates that the signal sequence and ectodomain are deleted with the transmembrane and C-terminal domain sequences remaining in the partial S gene sequence in the IBV cDNA; 3 and 5 represent the IBV gene 3 and 5 sequences, respectively.

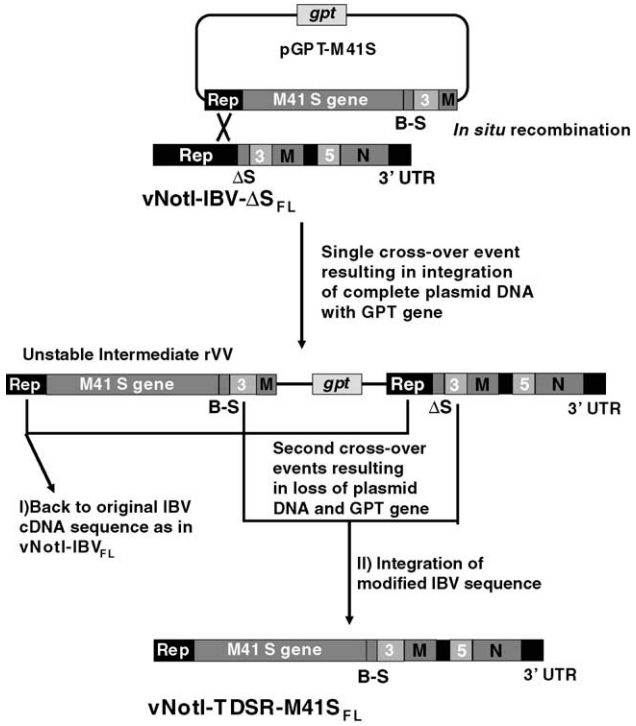

Fig. 4. Schematic diagram demonstrating the TDS method for inserting the chimaeric S gene sequence into the IBV cDNA lacking an S gene sequence in vNotI-IBV-ΔS$_{FL}$. The diagram represents two potential recombination events, the first involving the integration of the complete pGPT-M41S plasmid into the IBV cDNA sequence in vNotI-IBV-ΔS$_{FL}$ with concomitant selection in the presence of MPA. The second type of recombination events occur in the absence of MPA, resulting in the loss of the GPT gene and resulting in recombinant vaccinia viruses with either (I) the original sequence of the input vaccinia virus, with an IBV cDNA sequence lacking the S gene sequence, or (II) insertion of the chimaeric S gene sequence, the desired modification. The various IBV genes are as described in the legend to Fig. 1 B-S indicates that the transmembrane and C-terminal domain are derived from Beau-CK.

rIBVs, rIBV-M41S-A and rIBV-M41S-B, were obtained and characterised.

2.7. Analysis of rVV DNA and rIBV RNA

Oligonucleotides GPT-FORW (5′-ATGAGCGAAAAATACATCGTC-3′) and GPT-REV1 (5′-TTAGCGACCGGAGATTGG-3′) were used to determine the presence and absence of the *Ecogpt* gene sequence in the intermediary vaccinia virus recombinants and in the vaccinia virus recombinants, potentially containing the modified IBV sequences; a 459 bp product was indicative of the *Ecogpt* gene. Oligonucleotides BG-44 (5′-^{20941}GCTGGTGGACCTATAACT^{20958}-3′) and BG-134 (5′-^{1417}AGCAATTGAAACTGAAAGTG^{21398}-3′) were used to analyse vNotI-IBV-ΔS$_{FL}$ and vNotI-TDSR-M41S$_{FL}$ DNA to determine the absence of the Beau-R or presence of the M41-CK S gene sequences, respectively; a 476 bp product was indicative of the M41-CK S gene sequence. Sequence analysis was used to confirm that the PCR products were derived from the M41-CK S gene sequence.

Total cellular RNA was extracted from CK cells infected with the rIBVs, rIBV-M41S-A and rIBV-M41S-B, by the RNeasy method (Qiagen) and analysed by RT-PCRs, using Ready-To-Go™ RT-PCR beads (Amersham Pharmacia Biotech) and a variety of oligonucleotide pairs. The RT-PCR products were sequenced to confirm the sequence of the S gene present in the two rIBVs.

2.8. Virus growth curves

Confluent monolayers of CK and Vero cells in 60 mm dishes were infected with 7.5×10^4 PFU of M41-CK, BeauR-M41(S), rIBV-M41S-A and rIBV-M41S-B or 1×10^4 PFU of Beau-R. A lower multiplicity of infection for Beau-R was used to further enhance the difference of growth on Vero cells of IBVs expressing either a Beaudette-derived or M41-derived S protein ectodomain. Following adsorption, for 1 h at 37 °C, the cells were washed three times with phosphate-buffered saline (PBS) to remove residual virus and incubated at 37 °C in 5 ml of CK media. Samples of media were, at selected times over a 48 h period, analysed in triplicate for progeny virus by plaque assay in CK cells.

2.9. Sequence analysis

Sequence analysis of plasmid DNA, PCR products from the IBV cDNA sequences within vNotI-IBV-ΔS_{FL} and vNotI-TDSR-M41S_{FL} and from RT-PCR products generated from RNA isolated from rIBV-M41S-A and rIBV-M41S-B infected CK cells, was done using an ABI prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) or CEQ™ DTCS quick start kit (Beckman Coulter). Oligonucleotide primers used for the sequencing reactions were derived from the Beau-CK sequence (Boursnell et al., 1987). Sequences were determined on an Applied Biosystems 377 DNA sequencer or a CEQ™ 8000 capillary sequencer. PREGAP4 and GP4 of the Staden Sequence Software Programs (Bonfield et al., 1995) were used for sequence entry, assembly and editing.

3. Results and discussion

3.1. Modification of the Beau-CK full-length cDNA within the vaccinia virus genome by TDS

We have developed a reverse genetics system for the avian coronavirus IBV in which the full-length IBV cDNA was assembled by in vitro ligation followed by insertion into the vaccinia virus genome (Casais et al., 2001). We decided to investigate an alternative procedure, utilising homologous recombination by TDS (Falkner and Moss, 1990), for modifying the full-length IBV cDNA within the vaccinia virus genome to circumvent the necessity of assembling the IBV cDNA in vitro and subsequent insertion into the vaccinia virus genome.

To test the TDS-based method we proposed to replace the ectodomain of the Beau-CK S gene, within the full-length IBV cDNA in vNotI-IBV$_{FL}$ (Casais et al., 2001), with the ectodomain of the M41-CK S gene. However, to replace the 3.4 kb Beau-CK S sequence with the corresponding M41-CK S sequence an intermediary recombinant vaccinia virus, vNotI-IBV-ΔS_{FL}, containing the Beau-CK cDNA with the S gene deleted had to be produced. This was to avoid recombination events occurring within the S gene sequence if attempts were made to directly exchange the two sequences. Plasmid pGPT-IBV-ΔS (Fig. 1) with 94% of the Beau-CK S gene sequence deleted and Beaudette-derived sequences, 1188 bp proximal and 1155 bp distal to the deleted region of the S gene sequence, for homologous recombination with the IBV cDNA in vNotI-IBV$_{FL}$, was used for the TDS process for generation of vNotI-IBV-ΔS_{FL} (Fig. 3). PCR and sequence analysis of the IBV cDNA within vNotI-IBV-ΔS_{FL} confirmed that the Beau-CK S gene had been deleted (data not shown).

The M41-CK/Beau-CK chimaeric S gene sequence for insertion into the IBV cDNA within vNotI-IBV-ΔS_{FL} was identical to the sequence we had previously used for the generation of rIBV BeauR-M41(S) (Casais et al., 2003). The chimaeric S gene consisted of the signal sequence, ectodomain and transmembrane regions derived from IBV M41-CK and the cytoplasmic tail domain from Beau-CK. The transmembrane domains are identical between the two sequences but the cytoplasmic tail domain of the M41-CK S protein is truncated by nine amino acids when compared to the Beau-CK S protein. We had previously indicated that we retained the Beau-CK cytoplasmic tail domain because this region of the S protein has been demonstrated to interact with other virus proteins (Godeke et al., 2000). The chimaeric S gene sequence, from pFRAG-3-M41S (Casais et al., 2003), was used to replace the corresponding Beau-CK S gene sequence in pGPT-IBV-*Stu*I-*Bam*HI, resulting in pGPT-M41S (Fig. 2). Plasmid pGPT-M41S, contained the chimaeric S gene with Beaudette-derived sequences, 1188 bp proximal and 1155 bp distal to the chimaeric S sequence, for homologous recombination with the Beau-CK-derived IBV cDNA in vNotI-IBV-ΔS_{FL}, to insert the chimaeric S gene sequence into vNotI-IBV-ΔS_{FL} (Fig. 4). A recombinant vaccinia virus, vNotI-TDSR-M41S_{FL}, was isolated and PCR and sequence analysis confirmed that the IBV cDNA within the vaccinia virus genome contained the chimaeric S gene sequence.

3.2. Recovery of infectious IBV from vNotI-TDSR-M41S_{FL}

Two rIBVs, rIBV-M41S-A and rIBV-M41S-B, were independently recovered from CK cells, previously infected with rFPV/T7, to provide T7 RNA polymerase, and co-transfected with *Asc*I-restricted vNotI-TDSR-M41S_{FL} DNA and pCi-Nuc (Casais et al., 2001, 2003). The vNotI-TDSR-M41S_{FL} DNA was prepared from semi-purified vaccinia virus and pCi-Nuc, a plasmid expressing the IBV N protein under control of both the T7 and CMV promoters, was required for the successful recovery of rIBV (Casais et al., 2001). The transfected CK cells (P_0) were incubated for 2.5 days post transfection, until they showed a cytopathic effect (CPE), the medium was filtered to remove any rFPV/T7 and any potential IBV passaged on fresh CK cells (P_1). The two independently rescued rIBVs, isolated from the P_1 cells, were partially analysed and found to contain the chimaeric S gene

and subsequently virus RNA derived from P_3 CK cells, was sequenced to confirm the presence of the chimaeric S gene.

3.3. Characterisation of the rIBVs

The IBV strains, Beau-CK and M41-CK, have different cell tropisms, both viruses replicate to similar titres in CK cells but only Beau-CK produces infectious virus on Vero cells (Casais et al., 2003). Previous results using BeauR-M41(S), containing the chimaeric S gene following assembly of the full-length IBV cDNA in the vaccinia virus genome, showed that the rIBV had a phenotype like that of M41-CK rather than Beau-CK for growth on Vero cells (Casais et al., 2003). Therefore, we analysed the growth characteristics of rIBV-M41S-A and rIBV-M41S-B, containing the chimaeric S gene generated as a result of the TDS method, on CK and Vero cells for comparison with BeauR-M41(S).

Beau-R, M41-CK, BeauR-M41(S), rIBV-M41S-A and rIBV-M41S-B were used to infect CK and Vero cells and the titre of progeny virus determined over a 48 h period. All five viruses displayed similar growth profiles on CK cells; progeny viruses were detectable 8 h post infection with peak titres between 5×10^7 and 8×10^8 PFU/ml 30 h post infection (Fig. 5A). All the viruses caused observable CPE within 24 h on the CK cells. Analysis of the growth profiles of the five viruses on Vero cells (Fig. 5B) showed that only Beau-R replicated, with a maximum titre of 10^5 PFU/ml by 48 h post infection. In contrast, both rIBV-M41S-A and rIBV-M41S-B together with M41-CK and BeauR-M41(S) showed significantly lower titres on Vero cells in comparison to Beau-R (Fig. 5B). Overall, our results showed that rIBV-M41S-A and rIBV-M41S-B had similar phenotypes to those observed for BeauR-M41(S), indicating that all three rIBVs, expressing the chimaeric S glycoprotein, were similar irrespective of the method used to modify the IBV cDNA used to generate the rIBVs.

The results presented in this paper demonstrate the feasibility of producing rIBVs from IBV cDNAs within the vaccinia virus DNA that had been modified by homologous recombination. Our results affirmed that the TDS method is a suitable method for modifying the IBV full-length cDNA within the vaccinia virus genome, resulting in isogenic variants that differ only in the introduced sequences. This method for generating rIBVs will be useful for generating isogenic rIBVs for determining the effect of the modifications on gene functions. We also demonstrated that an intermediary form of the IBV cDNA in the vaccinia virus, containing a deleted region of the IBV genome, can be used for swapping large regions of the IBV genome avoiding the generation of chimaeric sequences.

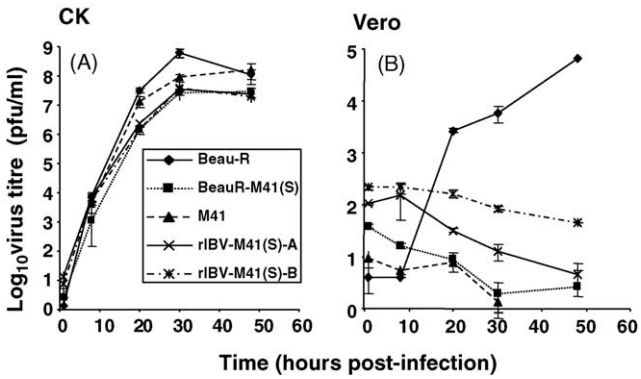

Fig. 5. Growth profiles of the rIBVs on (A) CK and (B) Vero cells. The cells were infected with Beau-R, M41-CK, BeauR-M41(S), rIBV-M41S-A and rIBV-M41S-B. Cell medium from the infected cells was analysed, for progeny virus, by plaque titration assay on CK cells 1–48 h post infection.

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