Utilizing fowlpox virus recombinants to generate defective RNAs of the coronavirus infectious bronchitis virus

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Coronavirus defective RNAs (D-RNAs) have been used as RNA vectors for the expression of heterologous genes and as vehicles for reverse genetics by modifying coronavirus genomes by targetted recombination. D-RNAs based on the avian coronavirus infectious bronchitis virus (IBV) D-RNA CD-61 have been rescued (replicated and packaged into virions) in a helper virus-dependent manner following electroporation of in vitro-generated T7 transcripts into IBV-infected cells. In order to increase the efficiency of rescue of IBV D-RNAs, cDNAs based on CD-61, under the control of a T7 promoter, were integrated into the fowlpox virus (FPV) genome. The 3′-UTR of the D-RNAs was flanked by a hepatitis delta antigenomic ribozyme and T7 terminator sequence to generate suitable 3′ ends for rescue by helper IBV. Cells were co-infected simultaneously with IBV, the recombinant FPV (rFPV) containing the D-RNA sequence and a second rFPV expressing T7 RNA polymerase for the initial expression of the D-RNA transcript, subsequently rescued by helper IBV. Rescue of rFPV-derived CD-61 occurred earlier and with higher efficiency than demonstrated previously for electroporation of in vitro T7-generated RNA transcripts in avian cells. Rescue of CD-61 was also demonstrated for the first time in mammalian cells. The rescue of rFPV-derived CD-61 by M41 helper IBV resulted in leader switching, in which the Beaudette-type leader sequence on CD-61 was replaced with the M41 leader sequence, confirming that helper IBV virus replicated the rFPV-derived D-RNA. An rFPV-derived D-RNA containing the luciferase gene under the control of an IBV transcription-associated sequence was also rescued and expressed luciferase on serial passage.

Introduction

Coronaviruses (order Nidovirales, family Coronaviridae, genus Coronavirus) comprise a group of enveloped viruses that replicate in the cell cytoplasm and contain a non-segmented, 5′-capped and 3′-polyadenylated single-stranded, positive-sense RNA genome of 27–32 kb (de Vries et al., 1997; Lai & Cavanagh, 1997; Siddell, 1995). The avian coronavirus infectious bronchitis virus (IBV) has a genome of 27608 nt (Boursnell et al., 1987) and produces five subgenomic (sg) mRNAs in IBV-infected cells that possess a 64 nt leader sequence, derived from the 5′ end of the genomic RNA, at their 5′ ends. IBV is an economically important pathogen of the domestic fowl, affecting respiratory, kidney and oviduct tissues, resulting in respiratory disease, retarded growth and reduced egg production (Cavanagh & Naqi, 1997).

Coronaviruses have the largest genomes of any known RNA virus and defective RNAs (D-RNAs), which contain the necessary sequences for replication and packaging into virions in the presence of helper virus, have been used as RNA vectors for modifying coronavirus genomes by targetted recombination (Masters, 1999). As a result, recombinant viruses have been obtained for the coronaviruses murine hepatitis virus (MHV) (Fischer et al., 1997, 1998; Hsue & Masters, 1997, 1999; Koetzner et al., 1992; Kuo et al., 2000; Liao & Lai, 1992; Masters et al., 1994; Peng et al., 1995a, b; Phillips et al., 1999; van der Most et al., 1992) and transmissible gastroenteritis virus (TGEV) (Sanchez et al., 1999).

We have been developing an IBV-based D-RNA system as an RNA vector for both expression of genes and targetted recombination. The IBV system is based on D-RNA CD-61, which contains the sequences necessary for replication and for packaging into virus particles and can therefore be rescued (replicated and packaged into virions) in a helper IBV-dependent manner (Penzes et al., 1994, 1996). Previous work
demonstrated that CD-61 can be rescued by heterologous strains of IBV (Stirrups et al., 2000a), resulting in the phenomenon of leader-switching (Makino & Lai, 1989), and can be used for the expression of heterologous genes (Stirrups et al., 2000b). Expression of reporter genes from D-RNAs has been accomplished for a variety of viruses including poliovirus (Barclay et al., 1998), arteriviruses (Molenkamp et al., 2000) and coronaviruses (Izeta et al., 1999; Liao & Lai, 1994; Lin et al., 1994; Stirrups et al., 2000b). The expression of a reporter gene from a D-RNA provides a convenient, highly sensitive and established way of monitoring replication and rescue of the D-RNAs.

Previous work demonstrated the rescue of IBV D-RNAs following electroporation of in vitro T7-transcribed D-RNAs into IBV-infected cells (Pénzes et al., 1996; Stirrups et al., 2000a). Analysis of the amounts of D-RNAs detected by Northern blot analysis (Pénzes et al., 1994, 1996; Stirrups et al., 2000a) or following expression of a reporter gene (Stirrups et al., 2000b) showed that the efficiency of initial rescue of D-RNAs was low, probably resulting from the small number of cells that were both infected with IBV and electroporated with T7-transcribed RNA. A recombinant fowlpox virus (rFPV)-based system was devised for delivery and initial transcription of D-RNAs, thereby increasing the number of cells potentially containing the D-RNA in order to increase the initial efficiency of rescue by helper IBV. An FPV system was chosen because avian cells are readily infected by FPV and an rFPV is available that expresses T7 RNA polymerase to initiate the in situ synthesis of the D-RNA (Britton et al., 1996).

Methods

Cells and viruses. The growth of IBV in embryonated domestic fowl eggs and chick kidney (CK) cells was as described previously (Pénzes et al., 1994, 1996; Stirrups et al., 2000a, b). FPV strain FP9 (Mockett et al., 1992; Somogyi et al., 1993) was used for the generation of rFPV. The recombinant rFPV-T7 (rFPV-T7pol; Britton et al., 1996) was derived from FP9 and expresses bacteriophage T7 RNA polymerase under the direction of the vaccinia virus Pφ late promoter. All FPVs were grown in chick embryo fibroblast (CEF) cells in medium 199 (M199) supplemented with 2% newborn calf serum. Vero cells, an African green monkey kidney cell line, were maintained in the same way as CK cells.

Recombinant DNA techniques. Recombinant DNA techniques used standard procedures (Ausubel et al., 1987; Sambrook et al., 1989) or the manufacturers’ instructions.

Construction of plasmids containing modified CD-61. The hepatitis delta ribozyme (HδR) and T7 RNA polymerase termination signal (T7φ) sequences were isolated as a discrete restriction fragment from plasmid p2.0 (Patnaik et al., 1992) and inserted into pZSL1190 (Pénzes et al., 1996), resulting in pZSLHφR6. IBV CD-61 cDNA, under the control of the T7 promoter (T7φ), was removed from pCD-61 (Pénzes et al., 1996) as an EcoRI–NotI fragment, in which the NotI site had been end-filled and fused to the Smal site of the HδR in EcoRI/Smal-digested pZSHδR6. The resulting construct, pIBV-Vec, contained the modified CD-61 sequence T7φ–CD-61–HδR–T7φ. The luciferase (Luc) gene, under the control of the Beaudette gene 5 transcription-associated sequence (TAS; Hiscox et al., 1995), was inserted into the PmaCl site of pIBV-Vec, as described by Stirrups et al. (2000b), to produce pIBV-Vec-Luc. The T7φ–CD-61–HδR–T7φ and T7φ–CD-61–Luc–HδR–T7φ cassettes were excised from pIBV-Vec and pIBV-Vec-Luc, respectively, and inserted into the ScaI site of the FPV transfer vector pEFL10 (Qingzhong et al., 1994). Sequence analysis of the resulting plasmids, pEF-CD-61 and pEF-CD-61-Luc, was used to confirm the orientation of each insert.

Rescue of D-RNAs by IBV Beaudette. In vitro T7-transcribed D-RNAs, corresponding to CD-61 and CD-61–Luc, synthesized from 2 µg of pEF-CD-61 and pEF-CD-61–Luc were electroporated into IBV-infected CK cells (Pp0) as described by Stirrups et al. (2000a). Progeny virus (Vp) from 1 ml of the Pp0 supernatants was serially passaged on CK cells for up to six passages (Pp1–Pp6). DNA (2 µg) from pEF-CD-61 and pEF-CD-61–Luc was electroporated, as for RNA electroporation, into CK cells co-infected with IBV and rFPV-T7 for the rescue of transiently transcribed CD-61 and CD-61–Luc. Supernatants were filtered (0.2 µm) to remove the FPV and progeny IBV was passaged on CK cells.

Isolation of rFPVs containing IBV D-RNAs. The FPV transfer vector pEFL10 (Qingzhong et al., 1994) contains sequences from the termini of FPV ORF 1, flanking the ScaI cloning site, for the integration of cloned sequences into the FPV genome. The β-galactosidase (Luc) gene, under the control of the FPV Pφ promoter, is present in pEFL10 for detection of potential rFPVs. DNA (5–10 µg) from pEF-CD-61 and pEF-CD-61–Luc was transfected into FPV rFPV-infected CEF cells by using 20 µl lipofectin (1 µg/µl; Gibco BRL) or 25 µl Effectene (1 µg/µl; Qiagen) as described by Qingzhong et al. (1994). Potential recombinant viruses were titrated on CEF cells in the presence of X-Gal and blue-staining plaques were purified three times (Boursnell et al., 1990).

To confirm that the CD-61 and CD-61–Luc sequences were within the rFPV genomes, FPV genomic DNA was extracted from rFPV-infected CEF cells. A modified version of the method described by Black et al. (1986) was used to isolate the rFPV genomic DNA. Briefly, CEF cells in 25 cm² tissue culture flasks (Falcon), infected with rFPV and grown for 4 days, were washed with 10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 5 mM EDTA, followed by 300 µl 10 mM Tris–HCl (pH 8.0), 10 mM KCl and 5 mM EDTA. The cells were cooled on ice for 10 min and lysed by the addition of 2.5 µl β-mercaptoethanol and 100 µl 10% (v/v) Triton X-100. The lysed cells were centrifuged at 2000 r.p.m. for 5 min at 4°C to remove the nuclei and the supernatants were centrifuged at 13,000 r.p.m. for 1 h at 4°C. The viral cores were resuspended in 80 µl cold 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (TE buffer) followed by 1.5 µl β-mercaptoethanol, 5 µl protease K (10 mg/ml) and 20 µl 20% (w/v) Sarkosyl and incubated at 4°C for 30 min. Following digestion, 140 µl 54% (w/v) sucrose was added to the samples, which were incubated at 55°C for 2 h, after which 40 µl 5 M NaCl was added. The rFPV DNA was phenol extracted twice, ethanol precipitated and redissolved in TE buffer.

Rescue of CD-61 D-RNAs expressed from rFPV. CK or Vero cells (Pp0) were co-infected simultaneously with either rFPV-CD-61 or rFPV-CD-61–Luc, rFPV-T7 and IBV (Beaudette or M41) at 10³ p.f.u. for each virus. After 24 h, the supernatants were filtered (0.2 µm) and progeny virus (Vp) was serially passaged on CK or Vero cells.

Isolation and analysis of RNAs from infected cells. Total cellular RNA was extracted from infected cells by the RNasy method (Qiagen), electrophoresed in denaturing 1% agarose–2 M formaldehyde gels (Sambrook et al., 1989) and Northern blotted onto Hybond-
C extra 0.45 μm nitrocellulose membranes (Amersham). IBV-derived RNAs were detected by hybridization with a 32P-labelled 590 bp 3′-UTR probe (Stirrups et al., 2000a). Luc-containing RNAs were detected with a 1664 bp Luc-specific probe, produced by PCR with oligonucleotides corresponding to the 5′ and 3′ ends of the Luc gene and labelled with [32P]dCTP by using the random oligonucleotide-primed synthesis method (Feinberg & Vogelstein, 1983). Alternatively, IBV-derived RNAs were detected non-isotopically following transfer onto Hybrid XL nylon membranes (Amersham) by using a 309 bp IBV 3′-UTR probe corresponding to the last 309 nt at the 3′ end of the IBV genome. The probe was labelled covalently with psoralen–biotin (BrightStar, Ambion), hybridized to the IBV-derived RNAs at 42 °C for 16 h and detected with streptavidin–alkaline phosphatase and an alkaline phosphatase 1,2-dioxetane chemiluminescent substrate (CDP-star, BrightStar, BioDetect, Ambion) by exposure to film at 20 °C for 2 h. The presence of FPV in infected cell lysates was analysed by RT–PCR from total cellular RNA with oligonucleotides MASH-48 and MASH-49. Production of a 1.4 kb DNA fragment, derived from the FPV 39K protein gene, was indicative of FPV (Boulanger et al., 1998).

### Analysis of reporter gene expression.

Cells (approximately 2 × 10⁶) were centrifuged at 2500 r.p.m. and lysed by using 0.5 ml lysis buffer (Promega). Equal volumes (50 μl) of cell lysate and luciferase assay reagent (Promega) were mixed and analysed for luciferase activity with a luminometer (Labtech, model Jade 1253).

### Results

#### Rescue of modified CD-61 by IBV

**In vitro** T7-transcribed D-RNAs with either two or three extra G residues at the 5′ end have been rescued successfully by the coronaviruses MHV (Koetzner et al., 1992; van der Most et al., 1991), IBV (Pénzes et al., 1994, 1996; Stirrups et al., 2000a, b) and TGEV (Izeta et al., 1999). The extra non-viral G residues present on the D-RNAs are a consequence of the T7 promoter sequences used to increase T7 RNA polymerase transcription levels (Martin & Coleman, 1987; Milligan et al., 1987). In addition to the extra nucleotides on the 5′ end, the IBV T7-transcribed D-RNAs also contained seven extra nucleotides distal to the 27 nt poly(A) tail on their 3′ end. These extra nucleotides resulted from a 5 nt linker sequence and the remains of the NotI site used to linearize the D-RNA-containing plasmids. The presence of the extra 5′ and 3′ nucleotides did not prevent replication and subsequent rescue of the *in vitro* T7-transcribed IBV D-RNAs (Pénzes et al., 1996; Stirrups et al., 2000a, b). We have demonstrated recently that leader switching occurs following rescue of an IBV D-RNA with a heterologous strain of IBV (Stirrups et al., 2000a). The replacement of the Beaudette leader sequence on the D-RNA with that of the helper virus leads to the loss of the extra nucleotides present on the 5′ end of the input *in vitro* T7-transcribed D-RNA. We concluded that leader switching may occur during replication of any coronavirus D-RNA and, as a consequence, the 5′ end of the input D-RNA would be altered.

Intracellular (in situ) expression of an IBV D-RNA from an rFPV, under the control of the T7 promoter, requires the presence of a T7φ sequence at the 3′ end to terminate RNA synthesis. However, addition of the 128 nt T7φ sequence would potentially affect replication of the D-RNA transcript by the IBV RNA-dependent RNA polymerase. Therefore, the HφR sequence was included between the end of the IBV D-RNA and the T7φ sequence to allow self-cleavage of the HφR–T7φ sequence. The end-filled NotI site of the CD-61 cDNA sequence from pCD-61 (Pénzes et al., 1996) was ligated to the Smal-digested HφR–T7φ sequence, resulting in pIBV-Vec. Synthesis of T7-transcribed RNAs followed by self-cleavage of the HφR–T7φ sequence would result in a CD-61-type D-RNA with 11 extra residues, four more than on CD-61 derived from NotI-linearized pCD-61.

In order to determine whether modified CD-61 could be rescued, *in vitro* T7-transcribed RNAs from pIBV-Vec were electroporated into IBV-infected cells and progeny virus was serially passaged. Northern blot analysis of RNA extracted from the cells confirmed rescue of pIBV-Vec-derived CD-61 (data not shown), demonstrating that the additional HφR–T7φ sequence had not affected rescue of CD-61 adversely, presumably resulting from self-cleavage of the sequence by the HφR.

The Luc reporter gene, under the control of an IBV TAS (Stirrups et al., 2000b), was inserted into the PnaCl site of pIBV-Vec, resulting in pIBV-Vec-Luc. Expression of luciferase was used to monitor replication in Pα cells and the subsequent rescue of CD-61–Luc in a convenient and sensitive manner and to determine whether expression of an IBV D-RNA from an rFPV would result in expression of a heterologous gene. Rescue of *in vitro* T7-transcribed CD-61–Luc from rIBV-Vec-Luc was observed following electroporation of the RNA into IBV-infected CK cells and was confirmed by luciferase expression (data not shown).

#### Rescue of CD-61 from FPV recombination vector

The T7φ–CD-61–HφR–T7φ and T7φ–CD-61–Luc–HφR–T7φ cassettes from pIBV-Vec and pIBV-Vec-Luc, respectively, were removed using Sall and, following end repair, were ligated into the SalI site of the FPV recombination vector pEFl10, thus generating pEF-CD-61 and pEF-CD-61-Luc. The FPV recombination vector pEFl10 contains FPV sequences for the integration of heterologous sequences into the FPV genome via homologous recombination but does not contain an FPV promoter at the SalI site. Plasmids pEF-CD-61 and pEF-CD-61-Luc were electroporated into CK cells co-infected with rFPV-T7 and IBV and progeny virus was serially passaged on CK cells. Cell lysates were assessed for luciferase activity and the presence of D-RNA. D-RNA CD-61 was detected by Northern blot analysis from Pα cells (data not shown) demonstrating that transiently transfected CD-61 was capable of being rescued by helper IBV. Transient transcription of CD-61–Luc resulted in rescue of the D-RNA and expression of luciferase.
Fig. 1. Northern blot analysis of IBV-specific RNAs following rescue of D-RNA CD-61 initially transcribed from rFPV-CD-61 in CK and Vero cells. (A) CK cells (P₀) were co-infected simultaneously with (panel 1) rFPV-T7 and IBV, and IBV-derived RNAs were analysed from P₀–P₃ cells, or (panel 2) rFPV-CD-61, rFPV-T7 and IBV, and IBV-derived RNAs were analysed from P₁–P₆ cells. (B) Vero cells (P₀) were co-infected simultaneously with rFPV-CD-61, rFPV-T7 and IBV and IBV-derived RNAs were analysed from P₀–P₅ cells. Progeny virus (V₁) and any potentially packaged CD-61 from P₀ supernatants were filtered and serially passaged on either CK or Vero cells. Total cellular RNA was extracted from the infected cells, electrophoresed in denaturing formaldehyde–agarose gels and Northern blotted and IBV-derived RNAs were hybridized with ³²P-labelled IBV 3'-UTR probe. The arrows indicate the position of D-RNA CD-61, detected by the IBV 3'-UTR probe, in RNA samples derived from infected cells. The positions of the IBV sg mRNAs, genomic RNA (gRNA) and D-RNA CD-61 are indicated. The IBV sg mRNAs marked S, E, M and N express the virion proteins spike, envelope, membrane and nucleoprotein, respectively. The RNAs detected between sg mRNAs 4 and 5 are observed routinely from all strains of IBV, as identified originally by Stern & Kennedy (1980), and are of unknown origin.

**Generation of rFPVs containing CD-61 and CD-61–Luc cDNAs**

Plasmids pEF-CD-61 and pEF-CD-61-Luc were transfected into CEF cells infected with FPV FP9 and resulting rFPVs, potentially containing the IBV D-RNA cDNA sequences, were identified by a blue plaque phenotype. DNA from plaque-purified rFPVs was analysed by dot-blot analysis with the ³²P-labelled 590 bp IBV-specific 3’-UTR probe (Stirrups et al., 2000a). The rFPVs rFPV-CD-61 and rFPV-CD-61-Luc, containing CD-61 and CD-61–Luc, were identified.

**Rescue of IBV D-RNA CD-61 from rFPV-CD-61**

CK cells were co-infected simultaneously with rFPV-CD-61, rFPV-T7 and IBV. After incubation at 37 °C for 24 h, supernatants were filtered to remove FPV and progeny IBV and any packaged D-RNA was serially passaged on CK cells. RNA extracted from the infected cells was analysed for the presence of IBV-derived RNAs by using the ³²P-labelled 590 bp IBV-specific 3’-UTR probe (Fig. 1A). CD-61 was detected initially at P₁ and was observed to increase in quantity following serial passage (Fig. 1A), confirming rescue of rFPV-CD-61-derided CD-61.

Since the initial transcription of CD-61 was by T7 RNA polymerase expressed from rFPV-T7, it was important to prevent subsequent passage of the rFPVs, as this would lead to the continued synthesis of T7-derived CD-61. T7-transcribed RNA would be indistinguishable from CD-61 replicated by helper IBV. No CD-61 was detected following serial passage of progeny virus from filtered supernatants following co-infection of cells with FPV-CD-61 and rFPV-T7 in the absence of helper IBV (data not shown), indicating that detection of CD-61 in the presence of IBV resulted from rescue by helper IBV. This suggests that filtration of the P₀-derived supernatants was sufficient to prevent serial passage of the rFPVs.

In order to establish further that helper IBV rescued the rFPV-CD-61-derided CD-61, the initial co-infection (P₀) was carried out in Vero cells. Vero cells, being of mammalian origin, do not support a productive FPV infection. Progeny virus in supernatants from P₀ was filtered to remove any FPV that may have been released into the tissue culture medium through cell
disruption and then serially passaged on CK cells. Northern blot analysis detected CD-61 in RNA isolated from the cells (data not shown). The experiment was repeated except that serial passage of $P_6$-derived progeny virus and any potentially packaged CD-61 was carried out in Vero cells rather than CK cells. Analysis of RNA isolated from the Vero cells showed that CD-61 was rescued from $P_6$-infected cells (Fig. 1B). This confirmed the previous observation that the helper IBV rescued CD-61 and demonstrated that Vero cells were capable of supporting the rescue of an IBV D-RNA. Overall, our results showed that rescue of rFPV-CD-61 derived CD-61 was more efficient than the rescue of an IBV D-RNA. Overall, our results showed that rescue of rFPV-CD-61-derived CD-61 rescued with either M41 or II to nucleotide 23 at the 5' end of the D-RNA was replaced by a consequence of leader-switching transcribed CD-61 RNA by M41 resulted in leader-switching of the CD-61 Beaudette-derived leader sequence with that described in Fig. 1, except that the M41-derived sg mRNAs are slightly smaller than the Beaudette-derived RNAs due to a 184 nt deletion in the M41 3'-UTR.

In order to confirm that FPV was not passaged beyond $P_0$, total RNA was extracted from $P_0$-infected cells and analysed by RT–PCR with oligonucleotides MASH-48 and -49, which result in a product of 1-4 kb, specific for the FPV 39K protein gene (Boulanger et al., 1998), in the presence of FPV. RT–PCR products of 1-4 kb were only amplified from RNA isolated from $P_0$ cells (Fig. 2). No 1-4 kb RT–PCR product was amplified from RNA isolated from cells after $P_0$, confirming that FPV was not being transferred following serial passage of progeny virus in $P_0$ supernatants.

Previous work had demonstrated that heterologous strains of IBV were able to rescue electroporated in vitro T7-transcribed CD-61 (Stirrups et al., 2000a). A consequence of the rescue of CD-61 by a heterologous helper IBV was that the leader sequence at the 5' end of the D-RNA was replaced by the leader sequence of the helper virus (Stirrups et al., 2000a). In order to confirm further that helper IBV replicated rFPV-CD-61-derived CD-61, the D-RNA was rescued by using IBV M41. CK cells were infected simultaneously with rFPV-CD-61, rFPV-T7 and IBV M41, the $P_0$-derived supernatants were filtered and progeny virus was serially passaged on CK cells. Northern blot analysis of RNA isolated from the infected cells with the IBV 3'-UTR probe showed that CD-61 was rescued in increasing amounts from $P_2$-infected cells (Fig. 3) by IBV M41 in a manner analogous to that observed with Beaudette as helper virus.

RT–PCR analysis with oligonucleotides 93/106 and 93/136 (Stirrups et al., 2000a) of RNA isolated from cells containing rFPV-CD-61-derived CD-61 rescued by M41 resulted in a product of 910 bp. The oligonucleotides span the domain I/II junction of CD-61 and production of a 910 bp DNA fragment is characteristic of the presence of CD-61 (Pénzes et al., 1994). Rescue of electroporated in vitro T7-transcribed CD-61 RNA by M41 resulted in leader-switching of the CD-61 Beaudette-derived leader sequence with that from the M41 genomic RNA, as shown by the Beaudette-to-M41 nucleotide substitutions T → C (nucleotides 30) and TT → CC (nucleotides 53–54) (Stirrups et al., 2000a). RT–PCR analysis to identify the leader sequence present on the M41-recused rFPV-CD-61-derived CD-61 was carried out with oligonucleotides 43 and 93/136 (Stirrups et al., 2000a), which resulted in a product of 16 kb, extending from within domain II to nucleotide 23 at the 5' end of CD-61. The leader sequence of rFPV-CD-61-derived CD-61 rescued with either M41 or...
Fig. 4. Sequence analysis of the leader sequence on CD-61 following rescue of the D-RNA with IBV M41. (A) Sequences corresponding to the leader sequence on D-RNAs derived from rFPV-CD-61 and rescued with either M41 (upper sequence) or Beaudette (lower sequence) IBV helper viruses. The sequences were determined directly from RT–PCR products generated from within domain II to nucleotide 23 at the 5′ end of D-RNA CD-61 by using the dye termination sequencing thermosequenase kit (Amersham) on an Applied Biosystems 373 DNA sequencer. Both sequences represent the negative strand and nucleotide differences between the leader sequences present on the rescued D-RNAs are marked with arrows. The chromatograms were viewed by using Gap4 of the Staden sequence software (Bonfield et al., 1995). (B) Comparison of leader sequences derived from rescued CD-61 (FPV-CD61Bea and FPV-CD61M41 rescued with Beaudette or M41 helper IBV, respectively), the helper virus genomes Beaudette (Boursnell et al., 1987) and M41 (Stirrups et al., 2000a) and CD-61 generated from rFPV-CD-61 by using rFPV-T7 (T7-CD61). The single line denotes the IBV leader sequence and the leader junction sequence is boxed. Nucleotide differences derived from the Beaudette and M41 sequences are shaded in grey and white, respectively. Identical nucleotides are outlined in black.

Beaudette (Fig. 4A) or from in vitro T7-transcribed CD-61 RNA was determined from the 1–6 kb RT–PCR products by using oligonucleotide K5UTR-1 (Stirrups et al., 2000a). Comparison of the D-RNA-derived leader sequences with the Beaudette and M41 genomic leader sequences showed that rFPV-CD-61-derived CD-61 rescued by M41 contained a leader sequence acquired from the M41 genomic RNA (Fig. 4B). The sequence distal to the leader junction site on the rescued D-RNAs corresponded to the Beaudette sequence, confirming that leader-switching had occurred during rescue of rFPV-CD-61-derived CD-61 by M41 and providing direct evidence that IBV helper virus was responsible for replication and subsequent rescue of the rFPV-derived D-RNAs.

Rescue of IBV D-RNA CD-61–Luc from rFPV-CD-61-Luc

Having demonstrated that helper IBV rescued rFPV-CD-61-derived CD-61 efficiently, we assessed the ability of the rFPV system to express a reporter gene from CD-61. Previous studies had demonstrated through analysis of luciferase activity that expression of luciferase from rescued CD-61–Luc was possible but that the D-RNA was not detectable by Northern blot analysis and appeared unstable (Stirrups et al., 2000b).

A derivative of rFPV-CD-61, rFPV-CD-61-Luc, was produced, in which the Luc gene, under the control of an IBV TAS,
within the PnuCl site of domain III in CD-61 was integrated into the FPV genome, in order to evaluate the ability of the rFPV system to generate a D-RNA expressing a reporter gene. Following co-infection of CK cells with rFPV-CD-61-Luc, rFPV-T7 and IBV Beaudette. Progeny virus (V\textsubscript{p}) and any potentially packaged CD-61 were filtered and serially passaged on CK cells. Cell lysates derived from P\textsubscript{0}–P\textsubscript{6} infected cells were assayed for luciferase activity. Bars represent mean luciferase activities ± SEM (n = 9), determined for each passage from nine experiments. The luciferase activities were observed to vary between experiments, possibly because of differences in the CK cells. Background relative light unit (RLU) levels were 0.001.

Fig. 5. Detection of luciferase activity in CK cell lysates following serial passage of CD-61–Luc initially transcribed from rFPV-CD-61-Luc in CK cells. CK cells (P\textsubscript{0}) were co-infected simultaneously with rFPV-CD-61, rFPV-T7 and IBV Beaudette. Progeny virus (V\textsubscript{p}) and any potentially packaged CD-61 were filtered and serially passaged on CK cells. Cell lysates derived from P\textsubscript{0}–P\textsubscript{6} infected cells were assayed for luciferase activity. Bars represent mean luciferase activities ± SEM (n = 9), determined for each passage from nine experiments. The luciferase activities were observed to vary between experiments, possibly because of differences in the CK cells. Background relative light unit (RLU) levels were 0.001.

RT–PCR analysis with oligonucleotides 43 (Stirrups et al., 2000a) and Kluc-3 (complementary to nucleotides 357–376 of the Luc gene) was carried out on RNA isolated from cells exhibiting luciferase activity following rescue of rFPV-CD-61-derived CD-61–Luc. An RT–PCR product of 449 bp was generated, indicative of the 5’ end of a D-RNA-derived Luc mRNA, since oligonucleotide 43 corresponded to the 5’ end of the IBV leader sequence. Sequence analysis of the 449 bp RT–PCR product, generated from RNA isolated from luciferase-positive P\textsubscript{3} cells, identified the IBV leader sequence fused to the canonical TAS-2 site of the Beaudette gene 5 TAS proximal to the Luc gene (Fig. 6B). This confirmed the presence of an IBV-transcribed Luc-specific mRNA in luciferase-positive cell lysates following rescue of rFPV-CD-61-derived CD-61–Luc, which supported results of the Northern blot analysis, which initially identified such an mRNA. The result corroborated our previous observation that the canonical TAS-2 site of the Beaudette gene 5 TAS is utilized preferentially for acquisition of the leader sequence during synthesis of a D-RNA-derived mRNA (Stirrups et al., 2000b).

Discussion

This study reports, for the first time, the in situ synthesis of a coronavirus D-RNA directly from an rFPV that was subsequently rescued in a helper IBV-dependent manner. Previous studies have demonstrated helper virus-dependent
rescue of electroporated in vitro T7-transcribed IBV D-RNAs (Pénzes et al., 1996; Stirrups et al., 2000a). However, the initial efficiencies of rescue of the electroporated D-RNAs varied. The amounts of rescued D-RNA observed were always smaller in early passages and increased significantly on serial passage, as determined by Northern blot analysis (Pénzes et al., 1994, 1996; Stirrups et al., 2000a, b). The expression of some D-RNA-derived reporter genes increased on serial passage of the reporter-containing D-RNA, supporting the observation that the amount of D-RNA increased on serial passage (Stirrups et al., 2000b). Most strains of IBV only infect primary cells (CK cells), with the proportion of cells initially infected being below 10%. This, coupled with low electroporation efficiencies, potentially resulted in a small number of cells being both
infected with IBV and electroporated with in vitro T7-transcribed D-RNA, resulting in small amounts of the D-RNA being rescued in early passages. In addition to infecting CK cells, IBV Beaudette is able to infect Vero cells, a mammalian cell line. However, we were unable to demonstrate the rescue of an IBV D-RNA in Vero cells in previous studies, possibly because of insufficient initial rescue of the D-RNA by helper IBV (Pénzes et al., 1996). To circumvent the low efficiency of rescue of an IBV D-RNA in early passages, we postulated that increasing the number of cells either initially infected by IBV or containing the D-RNA would increase the overall efficiency of rescue. Therefore, we decided to investigate an alternative method for the introduction of an IBV D-RNA into cells.

The vaccinia virus (VV) T7 RNA polymerase system (Fuerst et al., 1986, 1987) was used to express an MHV-derived D-RNA transiently, from transfected DNA, which was subsequently replicated by co-infected MHV helper virus (van der Most et al., 1992). D-RNAs from the coronavirus TGEV have been rescued successfully by using a two-step amplification system that involves pol II transcription in the nucleus followed by TGEV helper virus replication in the cytoplasm (Izeta et al., 1999). However, in this system, there is the possibility that cryptic splice sites may be present in the D-RNA sequences, although no obvious splicing was observed following the pol II amplification step with the TGEV D-RNA sequences. The MHV and TGEV results, using transfected DNA to introduce the D-RNA into cells, demonstrated that in situ transcription of a coronavirus D-RNA either by T7 RNA polymerase or pol II resulted in rescue of the D-RNA by a helper coronavirus. The transfection of cells with DNA rather than RNA has been reported to be more efficient, although transfection efficiencies vary depending on cell type.

We chose to use a poxvirus-based method as an alternative delivery system to introduce IBV D-RNAs into primary cells, followed by in situ synthesis of the D-RNA, with the aim of increasing the efficiency of rescue. Poxviruses have been employed successfully for the expression of various genes following insertion of the gene into the poxvirus genome via homologous recombination (Carroll & Moss, 1997; Moss, 1992, 1996). The expression of the gene can be either direct from a poxvirus promoter or under the control of a T7 promoter, which requires the co-infection of cells with a second recombinant virus expressing T7 RNA polymerase. The most widely used poxvirus system is based on VV: however, an important alternative to VV is FPV. Infectious FPV is only produced from avian cells, making it a more appropriate system for use with IBV. FPV replication is abortive in mammalian cells, with no production of infectious virus (Somogyi et al., 1993). Cytopathic effects associated with FPV infection occur later in the replication cycle than those observed for VV, potentially decreasing any interference by FPV with the replication of other co-infecting viruses. We elected to use an FPV-based system in which cDNAs corresponding to the IBV D-RNAs are inserted into the FPV genome under the control of a T7 promoter. The system has three steps: (i) introduction of the D-RNA sequence into cells by infection with rFPV, potentially involving 100% of cells; (ii) in situ transcription of the D-RNA by T7 RNA polymerase expressed as a result of co-infection of cells with an rFPV expressing T7 RNA polymerase (Britton et al., 1996); and (iii) rescue of the D-RNA by co-infecting the FPV-infected cells with helper IBV.

Co-infection of cells with rFPV-T7, IBV and either rFPV-CD-61 or rFPV-CD-61–Luc resulted in the rescue of the D-RNAs CD-61 and CD-61–Luc. The D-RNAs were transcribed initially under the control of T7 RNA polymerase and then rescued in a helper IBV-dependent manner, indicating that FPV did not interfere significantly with replication of IBV in co-infected cells. Rescue of rFPV-derived D-RNAs was more efficient than the rescue of electroporated in vitro T7-transcribed D-RNAs or transiently expressed in situ T7-transcribed D-RNAs following transfection of DNA. The rFPV-derived D-RNAs were detected both in larger amounts and in cells at earlier passages and were rescued successfully in Vero cells. Work by Stirrups et al. (2000b) showed that electroporation of in vitro-transcribed CD-61–Luc resulted in rescue of the D-RNA, as shown by the expression of luciferase. However, the levels of luciferase activity detected on serial passage never attained the levels observed in P3 cells. No CD-61–Luc D-RNA was detected by Northern blot analysis in cells expressing luciferase, although RNAs smaller than CD-61–Luc were detectable. In contrast, serial passage of the rFPV-CD-61–Luc-derived D-RNA resulted in observable amounts of the D-RNA, luciferase activities that routinely increased from P5 to P4 and the detection of a Luc-specific mRNA synthesized from CD-61–Luc. The observation that the luciferase activities increased on serial passage was similar to previous observations of the expression of CAT protein following rescue of electroporated in vitro T7-transcribed CD-61–CAT (Stirrups et al., 2000b). Although our previous results demonstrated that D-RNA CD-61–CAT was detectable on serial passage, no D-RNA-derived CAT-specific mRNA was detected by Northern blot analysis. However, RT–PCR analysis detected the CAT-specific mRNA, indicating that the mRNA was either unstable or produced in small amounts. As observed previously with the expression of CAT from CD-61–CAT and for the expression of heterologous genes from other coronavirus D-RNAs, expression of luciferase from rFPV-CD-61–Luc-derived CD-61–Luc was eventually lost over continued serial passage. The observation that luciferase activity peaked at P4, with gradual loss on further passage, was concordant with the identification of smaller RNAs, detectable with the Luc probe, in RNA isolated from P3 onwards (Fig. 6A, panel 2).

Overall, our results demonstrate that expression of IBV D-RNAs from an rFPV provides an efficient and alternative method for the rescue of coronavirus D-RNAs. Increasing the number of cells potentially containing the D-RNA increased the efficiency of rescue of a D-RNA by helper IBV.
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