Use of an Infectious Bronchitis Virus D-RNA as an RNA Vector

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1. INTRODUCTION

In the absence of a complete infectious IBV cDNA we have been developing an alternative strategy for the production of recombinant IBVs, utilising a defective RNA (D-RNA), CD-61. Coronavirus D-RNAs function like a minigenome and are useful as RNA vectors for the expression of heterologous genes and for targeted recombination. IBV D-RNA CD-61 (Pénzes *et al.*, 1996) was derived by deletion mutagenesis from a natural D-RNA, CD-91, produced by multiple passage of high titre IBV Beaudette in chick kidney (CK) cells (Pénzes *et al.*, 1994). CD-61 lacks internal parts of the genome but contains the sequences required for replication and for packaging into virus particles and can therefore be replicated and packaged (rescued) in a helper virus-dependent manner.

2. RESULTS AND DISCUSSION

We have developed two systems for the rescue of IBV D-RNA CD-61. The first system relies on the electroporation of *in vitro* T7-generated CD-61 transcripts into IBV-infected cells. The second system involves co-infection of IBV-infected cells with two recombinant fowlpox viruses (rFPV), one

The Nidoviruses (Coronaviruses and Arteriviruses).
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containing the cDNA corresponding to CD-61 (rFPV-CD-61) and the second rFPV expressing T7 RNA polymerase. Following co-infection of the IBV-infected cells with the two rFPVs CD-61 RNA is initially transcribed *in situ* using T7 RNA polymerase and then replicated and rescued on serial passage by the helper IBV (Fig. 1).

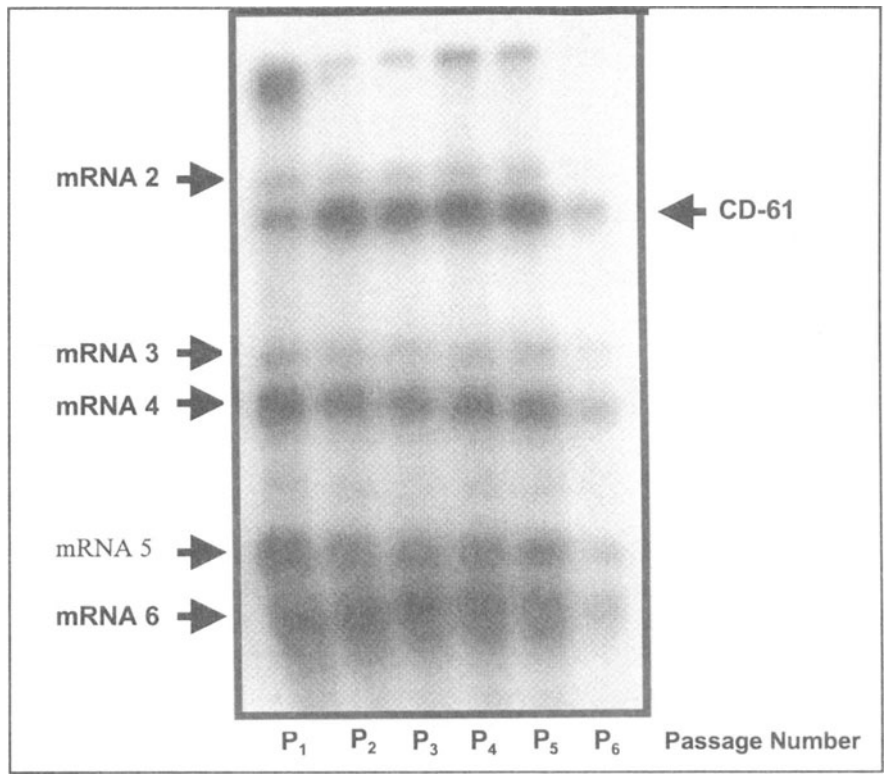

Figure 1. Northern blot analysis of IBV-derived RNAs showing rescue of CD-61 on serial passage (P_1-P_6) in IBV-infected CK cells. The IBV-derived RNAs, following electrophoresis in denaturing formaldehyde-agarose gels and transfer to nitrocellulose membrane, were detected by hybridisation with a ^{32}P-labelled IBV 3' UTR probe. CD-61 was derived from rFPV-CD-61.

IBV D-RNA CD-61 was investigated as a potential RNA vector for the expression of heterologous genes, using the luciferase (Luc) and chloramphenicol acetyltransferase (CAT) reporter genes. Expression of the genes was under the control of a transcription-associated sequence (TAS; Hiscox *et al.*, 1995) derived from the Beaudette gene 5, responsible for transcription of subgenomic mRNA 5 (Stirrups *et al.*, 2000b). However, following electroporation and subsequent rescue of *in vitro* T7-transcribed D-RNA CD-61-Luc (CD-61 containing the luc gene) the D-RNA was found to be unstable and incapable of expressing luciferase to the activity initially

detected in the electroporated cells. In contrast, following rescue of D-RNA CD-61-CAT (CD-61 expressing the CAT gene) the D-RNA was shown to be capable of producing CAT protein in larger amounts than initially detected in the electroporated cells. This observation indicated that CD-61-CAT was more stable than CD-61-Luc. In some cases the amount of CAT protein detected following rescue of CD-61-CAT reached 1.6 □g/10^6 cells. The reporter genes were expressed from two different sites within CD-61, *Sna*BI in Domain II which interrupted the CD-61 specific ORF and *Pma*CI in Domain III which did not interrupt the CD-61 specific ORF (Pénzes *et al.*, 1996). The rescue of CD-61-CAT and expression of CAT was not affected by interruption of the CD-61-specific ORF (Fig. 2).

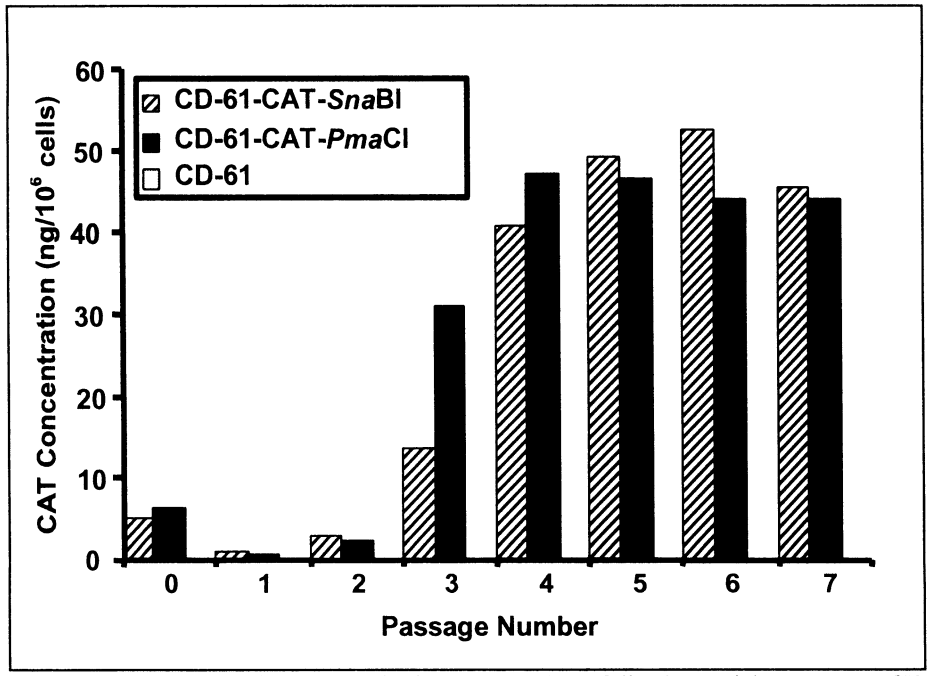

Figure 2. Expression of CAT protein from CD-61-CAT following serial passage on CK cells. The CAT gene was inserted in two different sites, *Sna*BI and *Pma*CI, with in the D-RNA. ELISA was used to detect CAT protein in cell lysates derived from CK cells infected with IBV and CD-61-CAT.

The IBV gene 5 TAS is composed of two tandem repeats of the IBV canonical consensus sequence CTTAACAA (Stirrups *et al.*, 2000b). We have demonstrated that only one sequence is required for expression of an mRNA, although both sequences can function as acceptor sites for acquisition of the leader sequence (Stirrups *et al.*, 2000b).

To determine whether CD-61-CAT could be rescued *in vivo*, cell supernatants containing helper IBV and CD-61-CAT were used to infect 11 day old embryonated eggs. Allantoic fluid from the infected embryonated

eggs was then used to infect CK cells and cell lysates were analysed for the presence of CAT protein. Analysis of CK cell lysates, following infection with virus in allantoic fluid derived from the infected eggs, showed that CD-61-CAT was replicated *in ovo* (Fig. 3).

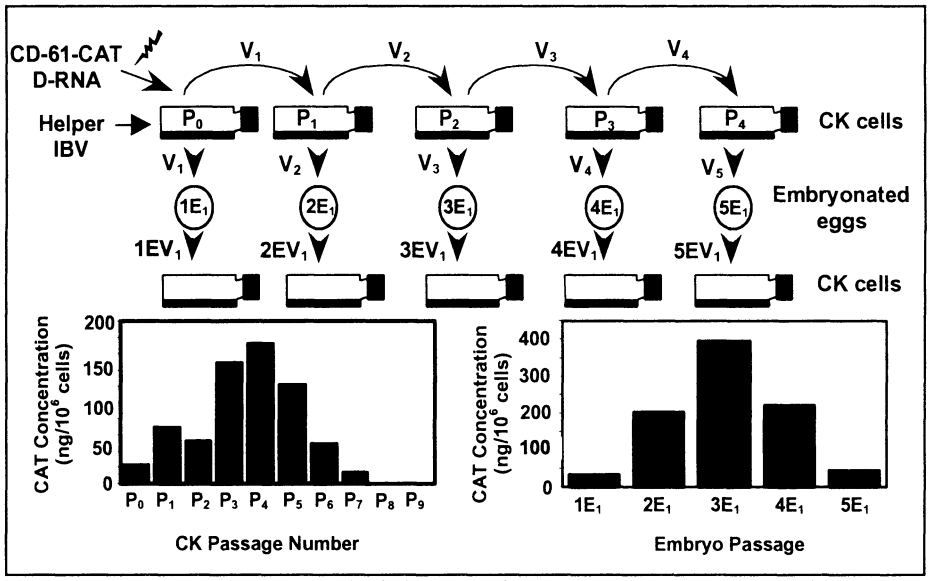

Figure 3. Analysis of CAT protein following infection of embryonated eggs with D-RNA CD-61-CAT. *In vitro* T7-transcribed CD-61-CAT was electroporated into IBV-infected CK cells. The rescued D-RNA was serially passaged on CK cells and cell lysates analysed for the presence of CAT protein. Virus (V_1-V_5) was either used to infect CK cells or 11 day old embryonated eggs. Virus ($1EV_1$-$5EV_1$) derived from the embryonated eggs, $1E_1$-$5E_1$ was used to infect CK cells to detect the presence of D-RNA CD-61-CAT via the expression of CAT protein.

However, serial passage of CD-61-CAT in embryonated eggs resulted in loss of the D-RNA (Fig. 4). Previous experiments had shown that IBV-derived D-RNAs containing heterologous genes eventually lost the ability to express the foreign gene (Stirrups *et al.*, 2000b). Therefore the loss of CAT expression from CD-61-CAT was not unexpected. Overall our results demonstrated that an IBV D-RNA containing a foreign gene was replicated *in ovo*.

We have demonstrated that CD-61 can be rescued by heterologous strains of IBV (Stirrups *et al.*, 2000a). The 5' ends of the heterologous IBV genomes were analysed and found to contain a variety of nucleotide substitutions when compared to each other and the Beaudette sequence from which CD-61 was derived. Analysis of the 5' ends of CD-61 rescued by the heterologous helper IBV identified that the Beaudette-derived leader sequence initially present on the electroporated *in vitro* T7-generated D-RNA transcript had been replaced with the leader sequence corresponding to

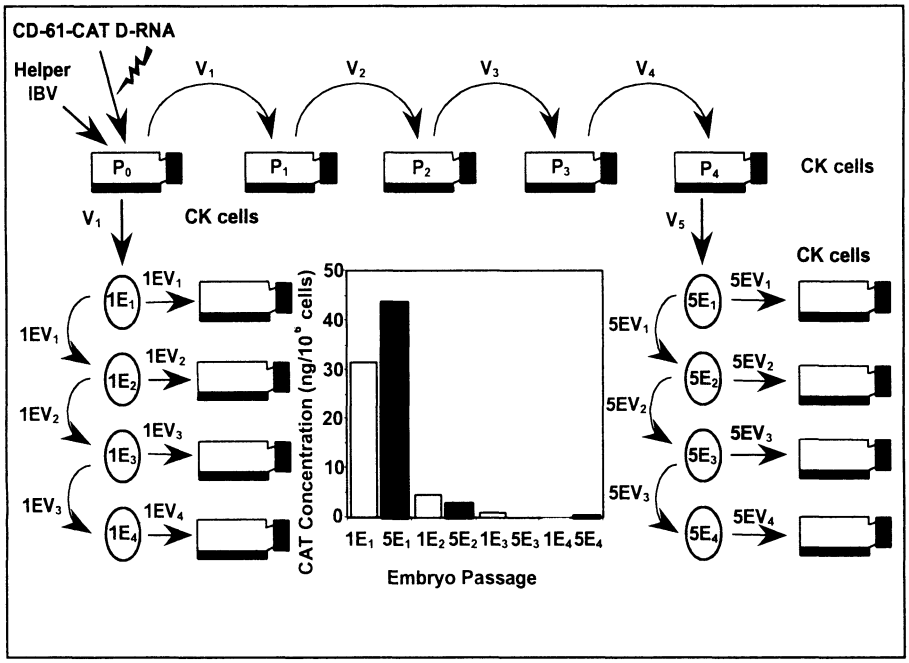

Figure 4. Analysis of CAT protein following serial passage of CD-61-CAT in embryonated eggs. IBV containing CD-61-CAT (V_1 and V_5) derived from CK cells was used to infect embryonated eggs. Virus ($1EV_1$ and $5EV_1$) derived from the allantoic fluid of infected eggs ($1E_1$ and $5E_1$) was serially passaged in eggs, $1E_1$-$1E_4$ and $5E_1$-$5E_4$. Virus derived ($1EV_1$-$1EV_4$ and $5EV_1$-$5EV_4$) from the infected eggs was used to infect CK cells to detect the presence of D-RNA CD-61-CAT via the expression of CAT protein.

the 5' end of the helper virus genome. In contrast, the adjacent 5' UTR sequence corresponded to the original CD-61 Beaudette sequence demonstrating that rescue of the unmodified CD-61 resulted in the phenomenon of leader switching (Makino & Lai, 1989). Three predicted stem-loop structures were identified within the 5' UTR on the various IBV strains (Stirrups *et al.*, 2000a). Stem-loop I showed a high degree of covariance amongst the IBV strains providing phylogenetic evidence that this structure exists and is potentially involved in replication (Stirrups *et al.*, 2000a), supporting previous observations that a bovine coronavirus (BCoV) stem-loop homologue was essential for replication of BCoV D-RNAs (Chang *et al.*, 1994).

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