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RESCUE OF IBV D-RNA BY HETEROLOGOUS HELPER VIRUS STRAINS

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1. ABSTRACT

Coronavirus defective RNA (D-RNA) vectors could be developed to deliver selected genes for the production of recombinant coronavirus vaccines. An IBV D-RNA, CD-61, derived from a naturally occurring IBV Beaudette D-RNA, CD-91, is being developed as a D-RNA vector for IBV. In order to use CD-61 as a vector it will require rescue by heterologous strains in addition to Beaudette. Rescue will be determined by recognition of replication and packaging signals within the D-RNA by the helper virus. The 5' and 3' UTRs are believed to contain sequences involved in replication and transcription. The 5' and 3' UTRs of six strains of IBV have been sequenced and experiments performed using six strains of helper virus for rescue of CD-61 to determine whether rescue correlates with sequence conservation within the 5' and 3' UTRs. Results indicate that all strains of helper virus rescued the D-RNA to varying degrees. Sequence comparisons show a high degree of sequence identity in the UTRs, but enough strain differences exist to be used as markers. The 5' and 3' UTRs of the D-RNAs rescued by the heterologous strains were also sequenced and leader switching between the helper virus and the Beaudette leader on the D-RNAs was observed.

2. INTRODUCTION

Coronaviruses have large non-segmented RNA genomes making the production of a cDNA clone difficult, hence targeted recombination is a potential method for the introduction

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
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of site-specific mutations into the coronavirus genome. Recombination has been shown to occur in MHV in vitro and in vivo (Lai, 1990) and in IBV following a mixed infection (Kottier *et al.,* 1995). It should therefore be possible to utilise recombination between genomic and replicating defective-RNA (D-RNA) to create IBV targeted recombinants, as demonstrated recently for MHV (Zhang *et al.,* 1997). The D-RNA vector available for IBV is CD-61 derived from the naturally occurring D-RNA CD-91 from the Beaudette strain (Penzes *et al.,* 1996) (Fig. 1). One possible use of recombination would be to replace the spike gene of a given strain of IBV with one of a strain with different properties such as nephropathogenicity and determine the effect on pathogenicity, antigenicity and tissue tropism. Alternatively, the spike gene of the current vaccine strain could be exchanged with that predominant in the field. The Beaudette strain grows atypically in chickens and therefore it is desirable to use another strain of IBV in the recombination experiments. The D-RNA vector, CD-61 can be rescued (replicated and packaged) by the parental strain, Beaudette, but for use as a potential vector for recombination it will require rescue by heterologous strains of IBV. Rescue of the D-RNA should occur if replication and packaging signals within the D-RNA are recognised by the helper strain of IBV. The 5' and 3' UTRs are believed to contain sequences involved in the replication of coronavirus RNAs and the leader sequence is required for the discontinuous transcription of coronavirus subgenomic mRNAs.

The rescue of CD-61 has been investigated using six different heterologous helper strains to determine which may be suitable for rescue of CD-61. The six strains were chosen because they were either used as vaccines, capable of being differentiated by the use of neutralising monoclonal antibodies, consisted of different spike serotypes which may indicate sequence differences in the 5' and 3' UTRs or because of known deletions in the 3' UTR. Both the 5' and 3' UTRs of all seven strains were sequenced and analysed to determine the level of sequence conservation, resulting in potential differences between the replication and transcription signals and to look for nucleotide differences to act as markers for identification of any recombination between the D-RNA and helper virus.

In this paper, we describe that heterologous virus can rescue the D-RNAs derived from another strain and when this occurred leader switching was also observed.

3. MATERIALS AND METHODS

Virus stocks were grown in chick kidney (CK) cells and embryonated fowl eggs.

Rescue of T7-transcribed CD-61 RNA in IBV-infected CK cells and isolation and analysis of IBV-derived RNA as described previously by Penzes *et al.* (1996).

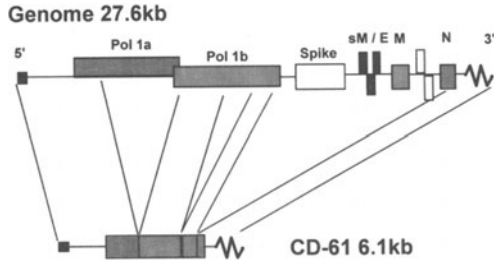

Figure 1. Comparison of the IBV genome with the IBV D-RNA CD-61.

PCR products from either the 5' or 3' UTR were generated by RT-PCR using Gibco Reverse transcriptase and Pharmacia Taq DNA polymerase. Oligonucleotides were designed using published IBV Beaudette sequence (Boursnell *et al.*, 1987).

The PCR-derived DNAs were blunt-ended and ligated into the EcoRV site of pBluescript II SK(+) (Stratagene). Dideoxy sequencing was carried out using a USB Sequenase version 2.0 DNA sequencing kit. Sequence data was compiled and analysed using the Staden package.

DNA fragments specific for CD-61 D-RNA were produced by RT-PCR using an oligonucleotide complementary to a region within the IBV polymerase 1b sequence present in CD-61 and an oligonucleotide corresponding to the first 23 bases of the Beaudette genome sequence. Sequencing of the PCR products was carried out using the USB 70170 sequenase PCR product sequencing kit.

4. RESULTS

4.1. Rescue of CD-61 D-RNA by Heterologous Strains

Rescue of the D-RNA CD-61 occurred with all six heterologous strains of IBV in chicken kidney cells (CKs). This has been demonstrated from Northern blot analysis of RNA extracted from the cells and PCR analysis looking for CD-61 specific junction regions and a missing A residue at base 749, a feature of CD-61. As can be seen from Fig. 2 although rescue occurs with all six heterologous strains it did not always occur with the same efficiency as observed by the passage number or by the amount detected when compared to the mRNAs.

4.2. Sequence Comparisons of 5' and 3' UTRs of the Seven IBV Strains

Sequence analysis of the 5' and 3' UTRs of seven strains of IBV shows a high level of conservation when compared to average level of conservation of other regions of the

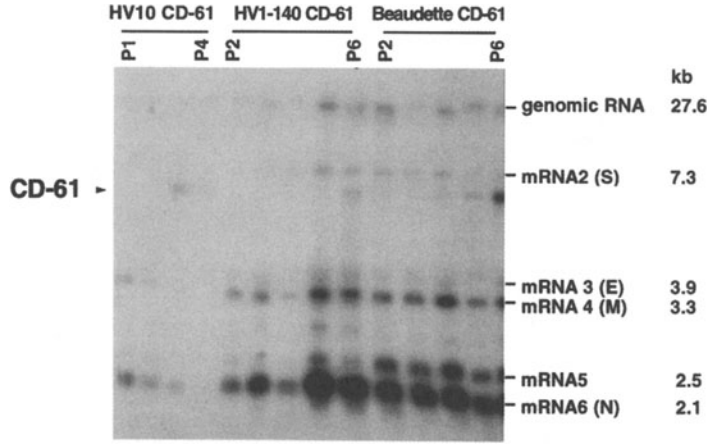

Figure 2. Analysis of IBV RNAs. RNA was extracted from CK cells infected with three different strains (HV10, HV1-140 and Beaudette) of IBV and electroporated with D-RNA CD-61. The passage number in the CKs from which the RNA was extracted is mentioned. The IBV derived RNAs were detected by northern blot analysis using ^{32}P-labelled PCR products from the N gene and 3' UTR.

Table 1. Average percentage nucleotide differences between the six heterologous IBV strains and Beaudette genomic sequence for the 5' and 3' UTRs

5' UTR			
Whole UTR 529 bases	Leader only 68 bases	UTR without leader 461 bases	First 350 bases of polymerase
2.64%	10.4%	2.0%	5.7%

3' UTR			
Whole UTR	Bases 1-206*	Bases 207 – 517	Nucleocapsid gene[1]
6.1%	13.6%	0.7%	5.9%

*Bases 1-188 of the 3'UTR are missing from M41 strain

[1]Data from Zwaagstra *et al.*, (1991) - % nucleotide differences between 20 strains of IBV compared to strain D1466 for a 360 base region of the nucleocapsid gene

genome. However, there are a greater number of changes in the leader and first 206 bases of the 3' UTR than the rest of the UTRs.

4.3. Leader Switching in D-RNAs Rescued by Heterologous Strains

Any evidence of sequence changes in the UTRs of CD-61 rescued by heterologous helper strains of IBV was investigated. This was done by a junction specific PCR of CD-61 and subsequent sequence analysis of the product. Results of the analysis revealed that the leader sequence of the rescued D-RNA has been preferentially derived from the helper virus genome rather than from the input D-RNA (Fig. 3).

Figure 3. Sequence comparison of the 5' UTRs. The sequence of the 5' UTRs (nucleotides 1–245) derived from CD-61, HV10 genome and CD-61 rescued by HV10 (HCD-61) were determined and aligned. The sequence differences are boxed and the leader junction site is underlined.

5. DISCUSSION

5.1. Rescue of CD-61 D-RNA by Heterologous Strains

The IBV D-RNA CD-61 was rescued by all six heterologous strains of virus but to varying degrees. There are a number of potential reasons for the observed differences in rescue. The heterologous strains can only be propagated in the primary cell line of CKs a heterogeneous and variable mix of cells which can affect the passage number of rescue of the CD-61. Alternatively rescue of CD-61 could be affected by differences in the ability of each helper virus to replicate in CKs, some strains may not replicate as well as others in the 24 hour span allowed for each passage. Although the observed levels of mRNAs detected for each virus does not seem to vary significantly by northern blot analysis. Also the level of input virus may differ and future work will include a determination of virus titre of each strain to determine if this is the case. A final reason could be due to slight incompatibility of replication, or packaging signals between strains affecting rescue of CD-61.

5.2. Sequence Comparisons of 5' and 3' UTRs of the Seven IBV Strains

No striking sequence differences were observed between the UTRs of the strains that could be assigned to potential differences in replication signals. Our data agrees with that of Williams *et al.* (1993) on the comparison of the 3' UTRs showing that nucleotides 1–206 of the 3' UTR are hypervariable. One of the strains, M41, is lacking this region of the 3'UTR. M41 is able to rescue CD-61 indicating that any signals involved in rescue are unlikely to be located in this region. In contrast, nucleotides 207–517 of the 3'UTR are highly conserved, with only the occasional base difference occurring. The 5' UTR is also well conserved, though a greater percentage of the differences occur in the 68 base leader sequence. The data therefore suggests that if there are constraints on any potential changes throughout the 5' and conserved part of the 3' UTRs that are involved in recognition by the polymerase they occur at very specific nucleotides. Enough strain differences were found to identify the six strains from each other.

5.3. Leader Switching in D-RNAs Rescued by Heterologous Strains

The phenomenon of leader switching for D-RNAs rescued by heterologous helper viruses of MHV and BCV has previously been demonstrated (Makino and Lai, 1989; Chang *et al.,* 1996). Therefore, any evidence of sequence changes in the UTRs of CD-61 rescued by heterologous helper strains of IBV were investigated. This was done by a junction specific PCR of CD-61 and subsequent sequence analysis of the product. Results of the analysis revealed that the leader sequence of the rescued D-RNA has been preferentially derived from the helper virus genome rather than from the input D-RNA. This indicated a recombination event, leader switching had occurred analogous to the discontinuous mechanism involved in the transcription of subgenomic mRNAs. No sequence differences derived from the genome of the helper virus were found in the 3' UTR of the D-RNA. Our results indicated that the alternative mechanism for replication of D-RNAs, involving the direct copying and subsequent amplification of the D-RNA in an analogous manner to the replication of the genomic RNA does not take place. Our results also indicate an intimate interaction between the replicating D-RNA and genomic RNA, an essential requirement for recombination, and that CD-61 can be used with heterologous strains of IBV for the production of recombinant viruses.

REFERENCES

Boursnell, M.E.G., Brown, T.D.K., Foulds, I.J., Green, P.F., Tomley, F.M. and Binns, M.M., 1987, Completion of the sequence of the genome of the coronavirus infectious bronchitis virus, *J. Gen. Virol.* **68**: 57–77.

Chang, R.-Y., Krishnan, R. and Brian, D.A., 1996, The UCUAAAC Promoter motif is not required for high efficiency leader recombination in bovine coronavirus defective interfering RNA, *J. Virol.* **70**: 2720–2729.

Kottier, S.A., Cavanagh, D. and Britton, P., 1995, Experimental evidence of recombination in coronavirus infectious bronchitis virus, *Virology* **213**: 569–580.

Lai, M.M.C., 1990, Coronavirus: Organization, replication and expression of genome, *Ann. Rev. Microbiol.* **44**: 303–333.

Makino, S. and Lai M.M.C., 1989, High frequency leader switching during coronavirus defective interfering RNA replication, *J. Virol.* **63**: 5285–5292.

Penzes, Z., Tibbles, K., Shaw, K., Britton, P., Brown, T.D.K. and Cavanagh, D., 1994, Characterization of a replicating and packaged defective RNA of Avian coronavirus Infectious Bronchitis Virus, *Virology.* **203**: 286–293.

Penzes, Z., Wroe, C., Brown, T.D.K., Britton, P. and Cavanagh, D., 1996, Replication and packaging of coronavirus infectious bronchitis virus defective RNAs lacking a long open reading frame, *J. Virol.* **70**: 8660–8668.

Williams, A.K., Wang, L., Sneed, L.W. and Collisson, E.W., 1993, Analysis of a hypervariable region in the 3' non-coding region of the infectious bronchitis virus genome, *Virus Res.* **28**: 19–27.

Zhang, L., Homberger, F., Spaan, W. and Luytjes, W., 1997, Recombinant genomic RNA of Coronavirus MHV-A59 after coreplication with a DI RNA containing the MHV-RI spike gene, *Virology* **230**: 93–102.

Zwaagstra, K. A., Van Der Zeijst, B. A. M. and Kusters J.G., 1991, Rapid detection and identification of avian infectious bronchitis virus, *J. Clin. Microbiol.* **30**: 70–84.