A DNA-launched reverse genetics system for rabbit hemorrhagic disease virus reveals that the VP2 protein is not essential for virus infectivity

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Rabbit hemorrhagic disease virus (RHDV), a member of the family Caliciviridae comprising positive-stranded RNA viruses, is a highly virulent pathogen of rabbits. Until recently, studies into the molecular mechanisms of RHDV replication and pathogenesis have been hindered by the lack of an in vitro culture system and reverse genetics. This study describes the generation of a DNA-based reverse genetics system for RHDV and the subsequent investigation of the biological role of the RHDV VP2 protein. The full-length RHDV genome was assembled as a single cDNA clone and placed under the control of the eukaryotic human cytomegalovirus promoter. Transfection of cells with the DNA clone resulted in a clear cytopathic effect and the generation of infectious progeny virions. The reconstituted virus was stable and grew to titres similar to that of the parental virus. Although previous reports have suggested that the minor structural protein (VP2) of other caliciviruses is essential for the production of infectious virions, using the DNA-launch-based RHDV reverse genetics system described here it was demonstrated that VP2 is not essential for RHDV infectivity. Transfection of cells with a cDNA clone of RHDV lacking VP2 resulted in the generation of infectious virions. These studies indicate that the presence of VP2 could reduce the replication of RHDV, suggesting that it may play a regulatory role in the life cycle of RHDV.

INTRODUCTION

Rabbit hemorrhagic disease virus (RHDV) is an emerging disease in rabbits and is considered the single most economically important disease of rabbits worldwide. The disease was first recognized in China (Liu et al., 1984), but was subsequently isolated from other areas of Asia, various European countries, Mexico and elsewhere (Gregg et al., 1991; Nowotny et al., 1997). In 1995, the virus reached mainland Australia after escaping from an island where it had been kept for experimental purposes (Meyers et al., 1991). Two years later, rabbit haemorrhagic disease was also observed in New Zealand (Ohlinger et al., 1990). The aetiological agent was identified as a calicivirus (Mitro & Krauss, 1993), a positive-sense, single-stranded RNA virus that is antigenically related to European brown hare syndrome virus (Wirblich et al., 1994; Mutze et al., 1998). The complete genome of the virus has been elucidated for the German isolate (Meyers et al., 1991) and shown to comprise a genome of 7437 nt. The genome contains two open reading frames, the first of which, ORF1, contains 2344 codons and encodes a large polyprotein containing the viral non-structural proteins as well as the viral coat protein at the C terminus. The genome also has a virus-encoded protein, VPg, attached covalently to the 5' end (Gregg et al., 1991) and is polyadenylated at the 3' end (Morales et al., 2004).

In RHDV-infected cells, a 2.2 kb subgenomic mRNA is transcribed that is collinear with the 3' third of the genomic RNA (Meyers et al., 1991). This mRNA is thought to represent the major source of the RHDV capsid protein, VP60; however, VP60 is also generated via cleavage of the ORF1-encoded polyprotein (Parra et al., 1993; Wirblich et al., 1995; Boga et al., 1999). A second ORF, ORF2, is located at the extreme 3' end of the genomic and subgenomic RNAs. The start codon for ORF2 is located at nt 7025 and shares a 17 nt overlap with ORF1, but has a −1 frame shift relative to the capsid ORF. RHDV ORF2 is 117 aa and encodes a polypeptide of 12.7 kDa (VP2), which is considered a component of RHDV virions (Wirblich et al., 1996; Meyers et al., 2000; Meyers, 2003). VP2 is conserved throughout the caliciviruses, suggesting that it may play a role in virus replication or assembly. However, the precise biological function of RHDV VP2 is not clear, and the inability to grow RHDV together with a
lack of reagents to detect ORF2 has hampered studies of the VP2 protein. Studies on other members of the calivirus family have demonstrated that the minor capsid protein, VP2, is critical for infectivity, as interruption of the feline calivirus (FCV) VP2 ORF results in a loss of infectivity (Sosnovtsev et al., 2005).

Recently, an infectious cDNA clone of RHDV was constructed. The rescued virus is adapted to growth in rabbit RK13 cells, providing a useful platform for the study of RHDV (Liu et al., 2006). In the present paper, we describe a DNA-launched reverse genetics system for RHDV and the application of this system to investigate the biological relevance of VP2. The entire ORF2 of RHDV was deleted to generate a cDNA clone lacking ORF2. The role of the VP2 protein for virus infectivity was then investigated in vivo using the reconstructed genomic clone. Our results indicated that VP2 is not essential for the infectivity of RHDV.

**METHODS**

**Cells, virus antibody and plasmids.** The rabbit kidney cell line RK13 was obtained from the China Center for Type Culture Collection. Construction of an infectious cDNA clone was based on the CHA/JX/97 strain of RHDV. CHA/JX/97 is a virulent strain of RHDV isolated in 1997 from an outbreak of rabbit haemorrhagic disease in Jiaxing, China. Monoclonal antibodies (mAbs) specific for VP60 and VP10 were prepared in our laboratory. Plasmid pcDNA3.1(+)(Invitrogen) was used for the assembly of a full-length cDNA clone of RHDV. The core sequence of self-cleaving ribozyme of the hepatitis delta virus (HDV) was kindly provided by Haixue Zheng (Lanzhou Veterinary Institute, Gansu, China).

**Mutagenesis and reconstruction of pcDNA3.1.** To ensure that the transcripts had a precise 5′-terminal sequence, 2 nt downstream of the human cytomegalovirus (HCMV) promoter were mutated (A833→G and A835→T) using a QuikChange Site-directed Mutagenesis kit according to the manufacturer’s protocol (Stratagene) to introduce a KpnI site near the putative transcriptional start site of pcDNA3.1. Subsequently, pcDNA3.1 was digested with KpnI and an 80 bp DNA fragment was released. Finally, the core sequence of self-cleaving ribozyme of HDV was subcloned into the above plasmid and the resulting plasmid was designated pCMV-HDV.

**Assembly of a full-length RHDV cDNA clone.** A full-length cDNA clone of CHA/JX/97 was assembled by following a multistep strategy. First, the pcCMV-HDV plasmid was digested with KpnI and EcoRV to allow insertion of the CD fragment from the RHDV genome (nt 1–2909), previously amplified from pRHDV using the specific primers F1 (5′-ACTGTTAACCCTGAAAATTTATGCCCGCTATG-3′; KpnI site underlined) and F2 (5′-GTTGCAAGTTGTTGGGCACACAG-3′). This yielded a recombinant plasmid, pCMV-CD, containing nt 1–2909, previously amplified from pRHDV using the specific primers F1 (5′-GAAAAGCGATTGTCGCGCCCCAGGTGAAC-3′), F2 (5′-GCTGACCGCTGGCTCGTATG-3′) and R1 (5′-TCTGAGGCTGCTGCTGCTGAGG-3′), the DNA fragment was inserted into pCMV-CD, which had been cleaved by the same enzymes. The recombinant plasmid was named pRHDV.

**Generation of a mutant RHDV cDNA clone without the VP2-coding region.** To delete the VP2-coding region (ORF2) from the RHDV genome, two Nael cleavage sites were introduced on either side of ORF2. The first mutation was introduced at nt 7013–7018, where 2 nt were mutated (G7015→C and A7018→C) to generate a Nael site. The second Nael site was introduced at nt 7361–7366 by two point mutations (T7365AG→T366, C→G). Four specific primers were used during the mutagenesis: Nael315 (sense primer: 5′-GTTGCAACCTGGGAGGGGCCACCAATTGTTTTTCT-3′), Nael315 (antisense primer: 5′-GAAAAAGCGATTGTCGCGCCCCAGGTGAAC-3′), Nael315 (sense primer: 5′-TACCACTGGGAGGGCTCAGCTGA-3′) and Nael315 (antisense primer: 5′-TACACCTGGGAGGGCTACGTGA-3′). The mutations were carried out using a QuikChange Site-directed Mutagenesis kit. Subsequently, the mutated recombinant plasmid was digested with NaelI and the VP2-coding region was released. The resulting plasmid was designated pRHDVAVF2.

**Transfection of RK13 cells.** RK13 cells were grown to 80% confluency and transfected for 4 h at 37 °C with a mixture of 2–5 μg pRHDV or pRHDVAVF2 and 10 μL Lipofectamine 2000 (Invitrogen), according the supplier’s instructions.

**RT-PCR for RHDV genome and sequencing.** RNA from the recombinant and parental viruses was purified using an RNAeasy Extraction kit (Qiagen). A 443 bp fragment that included the genetic marker was then amplified by RT-PCR using the primers JD′ (5′-CCACTGGCAACTTTACATTCCG-3′) and JD (5′-TGAACATGAAGGGTCTGGTGGG-3′). The RT-PCR products were digested with EcoRV and analysed on a 1.5% agarose gel.

**Immunofluorescence assay (IFA).** Indirect IFAs were used to detect viral protein expression in RK13 cells. Cells were fixed in 3.7% paraformaldehyde in PBS (pH 7.5) at room temperature for 30 min and permeabilized by incubation in −20 °C methanol for 30 min. The fixed cells were washed with PBS and stained with a mAb specific for VP60 (1:500 dilution), followed by goat anti-mouse-immunoglobulin G conjugated to fluorescein isothiocyanate. Finally, the samples were observed under a fluorescence microscope equipped with a video documentation system.

**Detection of VP2 expression.** Lysates were analysed by Western blot analysis using a mouse hyperimmune serum to VP2 after separation by SDS-PAGE and transfer to nitrocellulose membranes (Hybond-C; Amersham Life Sciences).

**Virus titration.** To compare the growth kinetics of the mutant and parental virus, RK13 cells were cultured in 96-well plates and then infected at an m.o.i. of 0.003 TCID50 per cell with stocks of virus generated from four passages on RK13 cells. After 2 h of incubation, the cells were washed twice and fresh growth medium was added (time 0). The cells were incubated at 37 °C in a humidified 5% CO2 atmosphere and observed daily for the appearance of cytopathic effect (CPE). From the onset of CPE, the titres of rescued viruses were determined as TCID50 values at 24, 36, 48, 60 and 72 h post-infection.

**Quantification of RHDV genome RNA levels.** A comparative analysis of the increase in genome copies of recombinant RHDV between virus recovered from pRHDV and from pRHDVAVF2 was carried out. Two pairs of PCR primers were designed. The first pair, F1 (5′-AGGCAAAAAGCAATATAAGGAAGA-3′) and R1 (5′-GGTGGCAGTATGGGAAACACAC-3′), were used to quantify the RHDV genomic RNA levels, whilst the second pair, F2 (5′-TGCGGTGGTTGAGTATGTTTG-3′) and R2 (5′-GATTGGGGCTGGATTGTGGTT-3′), were used to detect RHDV subgenomic RNA levels. Viral RNA was extracted from lysates of infected cells using a RNeasy Mini kit and reverse-transcribed into cDNA using the specific primers. Subsequently, the cDNA was amplified by real-time PCR using a SYBR Green PCR mix (TaKaRa). PCR amplification was performed using a program of 10 min at 95 °C followed by 40 cycles of 1 min at
94 °C, 1 min at 60 °C and 1 min at 72 °C. Each reaction was performed in triplicate in a Perkin Elmer ABI Prism 7700 Sequence Detection system (TaKaRa). Standards to establish genome equivalents were synthetic RNAs transcribed from a clone of the full-length cDNA of RHDV. The RNA was quantified by absorbance and 10-fold serial dilutions were prepared from 10^6 to 10^1 copies.

RESULTS

Construction of a full-length RHDV cDNA clone and ORF2 knockout

cDNA fragments of RHDV were assembled into a single full-length clone from two overlapping fragments using the restriction sites NotI, EcoRV and KpnI derived from the plasmid. The full-length genomic cDNA was placed under the control of the HCMV promoter of pcDNA3.1, engineered to eliminate the addition of non-viral nucleotides at the 5’ extremities of viral transcripts. To ensure that the final transcripts generated had the correct 3’ end, the core sequence of HDV ribozyme was placed immediately downstream of the viral poly(A) tract. Thus, the RNA transcripts generated from the plasmid contained a 16 nt poly(A) tract followed by an additional GC. The final plasmid was designated pRHDV. To knock out the ORF2 from the full-length genome, two NarI sites were introduced on either side of ORF2 in pRHDV. In the newly derived plasmid, the ORF1 sequence and 3’-end non-translated region were bordered by NarI restriction sites and ORF2 was deleted from pRHDV by NarI digestion and subsequent religation. The resulting plasmid was designated pRHDVΔVP2.

Infectivity of cDNA clones in cell culture

Transfection of RK13 cells with pRHDV resulted in the appearance of CPE at 12 h post-transfection, which became more prominent at 48 h post-transfection. The generation of viral RNA and antigen was subsequently confirmed by RT-PCR followed by sequencing, as well as by IFA using a VP60-specific mAb. At 2 days post-transfection, VP60, the major capsid protein, was clearly detected in the cytoplasm of cells transfected with pRHDV, whereas untransfected cells failed to cross-react with the VP60 antiserum (Fig. 1).

To rule out the possibility of contamination with the parental virus, RT-PCR and sequencing were performed for virus recovered from DNA transfection. The reconstituted virus derived from pRHDV retained the genetic marker (EcoRV site) located at nt 2907, as indicated by the ability of EcoRV to digest the RT-PCR-amplified region. These results confirmed that the infectivity in transfected cells was derived from the DNA construct.

No VP2 expression in the mutant virus

The results of Western blot analysis demonstrated that VP2 was expressed by the virus rescued using either pRHDV or in vitro-synthesized transcripts (Fig. 2). As expected, the VP2 protein was not detected in cells infected with the virus rescued from pRHDVΔVP2 (Fig. 2), indicating that the recombinant virus lacked VP2.

Infectivity of pRHDVΔVP2

The mutant cDNA clone containing a deletion of the entire VP2-coding region was transfected into RK13 cells and the appearance of CPE was monitored. At 12 h post-transfection, visible CPE was observed, and when stained with a VP60-specific mAb, cells showed bright fluorescence, indicating the presence of the VP60 protein in the cytoplasm (Fig. 1). In contrast, transfection of pcDNA3.1 did not produce any visible CPE for up to 72 h post-transfection, suggesting that VP60 can be expressed in the absence of VP2.
To examine further the infectivity of pRHDVΔVP2, RT-PCR was conducted from passage 3 viruses and a specific fragment (nt 2729–3171) was RT-PCR amplified. As anticipated, a 443 bp product was amplified from the culture cells (data not shown), and sequencing results confirmed the authenticity of the amplified product. These results confirmed that the mutant virus was infectious in the absence of VP2.

Growth kinetics
The growth curve of the mutant virus was analysed by determination of TCID_{50} values in comparison with the wild-type parental virus. The results showed that the titres of the rescued virus from pRHDVΔVP2 were slightly lower than the titres obtained for the parental virus, but both viruses reached a similar maximum titre of 1 \times 10^{4.32} and 1 \times 10^{4.68} TCID_{50} ml^{-1} by 3 days post-infection, respectively (Fig. 3). These data indicated that, unlike previous observations with FCV, deletion of RHDV VP2 did not result in complete loss of RHDV replication ability, although there was a slight decrease in infectivity.

Quantification of genome replication by quantitative (q)RT-PCR
To evaluate the effect of VP2 deletion on RHDV replication, qRT-PCR was used to quantify genomic and subgenomic RNA levels. A pair of primers (F1/R1) located in the coding region of the non-structural protein p16 was used to detect replication of RHDV genomic RNA, whilst a pair of primers (F^{+}/R^{-}) targeting the VP60-coding region was used to detect levels of subgenomic RNA. The results demonstrated that the level of the viral subgenomic RNA was decreased up to 60 h post-infection as result of VP2 deletion (Fig. 4a), although it showed an increase at 72 h post-infection. The replication level of the VP2 deletion mutant was reproducibly higher than that of the parental virus (Fig. 4b).
DISCUSSION

It is well known that the use of reverse genetics systems is a useful tool for studying RNA virus replication, pathogenesis and in vivo function of individual viral proteins, as well as for developing new vaccines. For caliciviruses, several infectious cDNA clones have been reported for FCV (Thumfart & Meyers, 2002; Sosnovtsev et al., 2005), RHDV (Liu et al., 2006), murine norovirus (Chaudhry et al., 2007) and porcine enteric calicivirus (Chang et al., 2005). These systems have the common feature that the full-length cDNA molecule is placed downstream of the T7 or SP6 promoter, and the entire genomic RNA is either synthesized in vitro and the transcripts introduced into host cells in order to recover infectious virus or the RNA is synthesized in vivo by the use of a T7 RNA polymerase-expressing helper virus. In the present study, we placed the full-length cDNA of RHDV under the control of the HCMV immediate-early eukaryotic promoter to generate genomic RNA after transfection into cells and rescue infectious RHDV. To ensure the authenticity of the 5′ end of the virus genome derived from the HCMV promoter, the nucleotides between the HCMV promoter and genome start site were deleted, so that transcription by RNA polymerase II began at or very near the 5′ end of the viral genome. Similarly, to ensure a precise 3′ end of the viral genome, a DNA fragment containing the core sequence of the self-cleaving ribozyme of HDV was inserted between the 3′ end of the genome and the bovine growth hormone poly(A) sequence. In the reverse genetics system described here, plasmid DNA was used for direct transfection of RHDV-susceptible cells, which makes the manipulation of the RHDV genome technically easier and more consistent than RNA transfection.

All caliciviruses are known to encode the VP2 protein in a separate ORF near the 3′ end of the genome (ORF2 for sapoviruses and lagoviruses and ORF3 for noroviruses and vesiviruses). Although the VP2 protein was proposed to be a minor structural protein, the biological function of VP2 remains to be fully elucidated. In 2003, the function of Norwalk virus VP2 was studied by the Estes group (Bertolotti-Ciarlet et al., 2003; Glass et al., 2003); their results showed that the protein could interact with capsid protein and regulate the expression and stability of the viral capsid protein VP1. Two years later, Sosnovtsev et al. (2005) showed that FCV VP2 was essential for the production of infectious virions, as deletion of VP2 resulted in complete loss of infectivity. In addition, Kaiser et al. (2006) reported that the FCV VP2 protein can interact with capsid protein and possibly the RNA polymerase in a yeast two-hybrid system. For RHDV, the function of VP2 is still unknown. In the present study, we utilized a DNA-launch-based RHDV reverse genetics system to investigate the role of VP2 in viral replication. We demonstrated that the VP2 protein is not essential for the production of infectious RHDV virions, which is in contrast to previous suggestions that VP2 is involved in the maturation and assembly of calicivirus particles (Wirblich et al., 1995; Glass et al., 2000; Sosnovtsev & Green, 2000; Oehmig et al., 2003).

We have systematically studied the effects of the VP2 deletion on viral replication, capsid expression and RHDV infectivity. The results clearly demonstrated that RHDV retains its infectivity in the absence of VP2, but the titre of the mutant virus is slightly lower than that of the parental virus. We hypothesize that RHDV VP2 might not participate in the production of progeny virions but may in fact represent an important virulence gene. In agreement with this, we have found that the RHDV VP2 protein can induce apoptosis when transfected into host cells (data not shown) and that deletion of the protein decreases the ability of RHDV to induce apoptosis and cell death in tissue culture cells (data not shown). Of course, to determine more precisely the role of VP2 in the RHDV life cycle, additional experimental evidence is required and will be the focus of our future research.

Our results also showed that deletion of VP2 decreased the levels of the RHDV subgenomic RNA up to 60 h post-transfection, but expression of the RHDV mutant increased gradually in line with culture time and its expression exceeded that of parental virus at 72 h post-infection. Quantification of the RHDV genome during virus replication by qRT-PCR demonstrated that the replication level of the VP2 deletion mutant was reproducibly higher than that of the parental virus. This increased level of genome replication in the absence of VP2 is in agreement with our hypothesis that VP2 may be involved in the induction of apoptosis during infection, as VP2 expression would result in cell death at an earlier stage of the virus life cycle.

In summary, we have constructed a convenient and robust system to allow manipulation of the RHDV genome and utilized this system to investigate the effects of VP2 deletion on viral replication. These data and the reverse genetics system described herein will undoubtedly aid in the study of the molecular mechanisms of RHDV replication and pathogenesis.

ACKNOWLEDGEMENTS

We would like to thank Dr Ian G. Goodfellow, Department of Virology, Faculty of Medicine, Imperial College London, UK, for critical reading of the manuscript. This work was funded by grants from the National Basic Research (973) Program (2006CB708209), the Zhejiang Natural Sciences Foundation (Y305047 and Y307582), the Chinese Natural Sciences Foundation (30670074) and the Graveness Technology Program of the Science and Technology Department of Zhejiang Province (2007C12G401009).

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