DNA-dependent RNA polymerases and RNA-dependent RNA polymerases (RdRps) synthesize RNA and, consequently, are essential for life forms of all kingdoms. Among RNA viruses, two classes of polymerases are distinguished, namely reverse transcriptases (e.g. from *Human immunodeficiency virus 1*; *Jaeger et al.*, 1998) and RdRps from several families of single- and double-stranded RNA viruses, including human *Hepatitis C virus* (*Ago et al.*, 1999; *Bressanelli et al.*, 1999; *Lesburg et al.*, 1999), calicivirus (*Ng et al.*, 2002), *Poliovirus* (*Hansen et al.*, 1997), F6 (*Butcher et al.*, 2001) and reovirus (*Tao et al.*, 2002), have been determined. In spite of a low sequence identity (*Koonin*, 1991), the overall structure of plus-strand RNA virus RdRp is conserved and resembles in shape a right hand with fingers, thumb and a palm domain. Regardless of the initiation mode for RNA synthesis, the thumb and finger domains of RdRps form a channel through which template and product are moved along towards the catalytic centre, represented by a Gly–Asp–Asp (GDD) motif. The latter is located within the palm region, which shows the greatest three-dimensional conservation of all domains (*Choi et al.*, 2004).

Pestiviruses are important livestock pathogens and belong to the family *Flaviviridae*; the latter also contains the closely related human *Hepatitis C virus*, as well as *Yellow fever virus* and *West Nile virus*. Pestiviruses possess a single-stranded RNA genome of positive polarity with a length of about 12–3 kb. The viral genomic RNA contains one large open reading frame (ORF) that is flanked by 5′ and 3′ non-translated regions. The ORF encodes a polyprotein of approximately 3900 aa that is processed co- and post-translationally by cellular and viral proteases, which generate the mature viral proteins. The 5′-terminal one-third of the viral genome encodes a viral autoprotease, Npro, and the structural proteins, namely the capsid protein C and the glycoproteins E′ns (RNase, secreted), E1 and E2. The remaining part of the polyprotein is processed to the non-structural proteins (NS) p7, NS2–3 (NS2, NS3), NS4A, NS4B, NS5A and NS5B (Fig. 1b). Together with putative cellular cofactors, NS3–NS5B are part of the virus replication complex, where NS5B represents the viral RdRp (reviewed by *Lindenberg & Rice*, 2001; *Thiel et al.*, 1996; and references therein).

Recently, we established a cell-culture system to study molecular aspects of RNA recombination (*Gallei et al.*, 2004, 2005b). This system allowed the efficient generation of recombinant pestiviral strains after the transfection of non-infected cells with pairs of synthetic subgenomic and non-replicable transcripts. These synthetic RNAs were derived from the infectious cDNA clone of *Bovine viral diarrhea virus* (BVDV), strain CP7-5A (*Becher et al.*, 2000), and overlapped in the region encoding the viral RdRp. Demonstrating the existence of a virus replication-independent mechanism of RNA recombination, we obtained a unique set of 11 cytopathogenic (cp) recombinant BVDVs that resulted from non-homologous RNA recombination.

The three-dimensional structure of RNA-dependent RNA polymerases (RdRps) is highly conserved among RNA viruses. In a previous study, a unique set of mutant strains of *Bovine viral diarrhea virus* was obtained, encompassing either a genomic deletion of six codons or duplications of between 1 and 45 codons; these mutations affect different parts of the palm region, the most conserved part of RdRps containing the catalytic centre. In the present study, a detailed characterization of the RdRp mutant viruses was performed, demonstrating different degrees of a small-plaque phenotype in cell culture, correlating with significantly reduced viral RNA synthesis and delayed virus replication. Taken together, the results of this study demonstrate a surprising flexibility within the palm region of a plus-strand RNA virus RdRp, resulting in viral attenuation *in vitro*. This interesting insight into an essential viral protein may have implications for the development of vaccines and attenuated viral vectors.

Mutations in the palm region of a plus-strand RNA virus polymerase result in attenuated phenotype

Andreas Gallei, Simone Widauer, Heinz-Jürgen Thiel and Paul Becher

Institut für Virologie (FB Veterinärmedizin), Justus-Liebig-Universität, Frankfurter Straße 107, D-35392 Giessen, Germany

Supporting material and a supplementary figure showing RT-PCR analyses of total RNA derived from cell-culture passages of the RdRp mutant strains are available in JGV Online.
These mutant viruses carry either a deletion of six codons (mutant strain R2) or duplications of between 1 and 45 codons in the viral RdRp gene (mutant strains R3–R12) (Gallei et al., 2004). The crystal structure of the BVDV RdRp has been reported for strain NADL (Choi et al., 2004) (Fig. 1a); NADL is related closely to BVDV strain CP7-5A and their deduced amino acid sequences share a high degree of similarity (Fig. 1b; data shown for a section of the viral RdRp). When compared with the polymerase of BVDV strain NADL, the alterations in the RdRps of the CP7-5A-derived mutant viruses can be allocated to structural elements of the palm region (Fig. 1b; data shown for mutant strains R2, R7, R8, R9, R11 and R12). With regard to the high conservation of this RdRp section, these alterations represent a surprising finding that prompted us to investigate their impact on viral growth and RNA replication efficiency.

To study viral growth characteristics, focus-forming assays were performed on Madin–Darby bovine kidney (MDBK) cells with each of the RdRp mutant viruses and with reference strain CP7-5A as described previously (Becher...
When compared with CP7-5A, all mutant viruses showed a moderate (R3–R11) to strong (R2, R12) reduction of focus size (Fig. 2, left; data not shown). To examine whether the mutated RdRp genes were actually responsible for the observed small-plaque phenotypes, we selected a set of six RdRp mutant viruses (R2, R7, R8, R9, R11 and R12) on the basis of the distinct reductions in viral growth; this set also reflects the spectrum of different kinds and extents of mutation present in the mutated RdRp genes. For that purpose, we introduced specific cDNA fragments encompassing the alterations present in the different RdRp genes into the infectious cDNA clone of CP7-5A. Directly after transfection of synthetic transcripts as described previously (Gallei et al., 2004), focus-forming assays of the resulting BVDV strains CP7-R2, CP7-R7, CP7-R8, CP7-R9, CP7-R11 and CP7-R12 were performed, which confirmed that viral growth was affected similarly as was observed for the original RdRp mutant viruses (Fig. 2, right). This demonstrated that the mutations introduced into the viral RdRp genes were responsible for the observed small-plaque phenotypes.

To characterize further the reconstructed viral mutant strains, the effect of the mutations located in the RdRp gene on kinetics of viral growth was investigated; the experiment was performed as described previously (Gallei et al., 2005a). Up to 24 h after infection with transcript-derived virus, the recombinant BVDV strains showed a moderate (CP7-R7, CP7-R8, CP7-R9 and CP7-R12) to strong (CP7-R2 and CP7-R11) reduction of viral titres compared with reference strain CP7-5A; for CP7-R2, the titre of released infectious virus was reduced by almost 1000-fold at 24 h post-infection (p.i.) (Fig. 3a). At later time points of infection, however, the mutant strains reached peak titres comparable to those of parental virus CP7-5A determined at 36 h p.i. [10<sup>5.5</sup>–50% tissue culture infective dose (TCID<sub>50</sub>)] ml<sup>−1</sup>]. Titres obtained from infection with strongly attenuated strain CP7-R2 did not peak before 72 h p.i. (10<sup>5</sup>–5 TCID<sub>50</sub> ml<sup>−1</sup>; Fig. 3a). Accordingly, for the RdRp mutant viruses, delays in viral growth correlated with the previously observed reduction of focus size.

The mutant viruses with alterations in the RdRp palm region displayed distinct reductions of viral growth. To examine the question of whether this observation can be attributed to an influence on the efficiency of viral RNA synthesis, a BVDV-specific real-time RT-PCR analysis was performed. For comparative quantification of viral genomic RNAs, MDBK cells were infected with transcript-derived virus of BVDV strains CP7-5A and CP7-R2 to CP7-R12 at an m.o.i. of 0.5. Total cellular RNA was prepared at 12 h.p.i. Subsequently, spectrophotometric analyses were performed in order to determine the RNA concentration for each sample. One microlitre of each sample (containing 0.7–1.4 µg RNA) was then subjected to real-time RT-PCR analysis as reported previously (Lackner et al., 2005). For the recombinant viruses, the calculated amounts of accumulated viral RNA were reduced to about 3% (CP7-R2), 5% (CP7-R12), 8% (CP7-R11), 11% (CP7-R7), 25% (CP7-R8) and 40% (CP7-R9) compared with CP7-5A (Fig. 3b). Moreover, the relative amounts of viral genomic RNA correlated with the calculated relative mean focus areas (Fig. 3c).

Recombinant cp BVDV genomes with duplications of viral sequences can undergo secondary genetic changes by RNA
recombination during cell-culture passage (Baroth et al., 2000; Becher et al., 2001; Gallei et al., 2005a). Accordingly, the genetic stability of the reconstructed BVDV RdRp mutants CP7-R7, CP7-R8, CP7-R9, CP7-R11 and CP7-R12 carrying genomic duplications was examined; in addition, deletion mutant CP7-R2 was included in this analysis. To monitor the emergence of genomic alterations, consecutive cell-culture passages were performed for all mutant strains. For each passage, cell-culture supernatants were harvested after appearance of cytopathic effect (2–3 days p.i.), diluted with fresh medium at a ratio of 1:10 and used for subsequent infection of cells. Total RNAs prepared from the first, third and tenth passages of transcript-derived viruses were investigated by RT-PCR analyses specific for the 3′-terminal half of the RdRp gene (corresponding to nt 11003–12074 of CP7-5A). In addition, RT-PCR analyses from the tenth passages specific for the 5′-terminal part of the NS5B gene (nt 9738–11124) were performed. Shifts in size of the investigated PCR products specific for the 3′-terminal half of the RdRp gene (corresponding to nt 11003–12074 of CP7-5A). In addition, RT-PCR analyses from the tenth passages specific for the 5′-terminal part of the NS5B gene (nt 9738–11124) were performed. The obtained PCR products were subjected to agarose-gel electrophoresis and cloned into a bacterial vector. Shifts in size of the investigated PCR products specific for the 3′-terminal half of the RdRp genes, together with comparative sequence analyses, revealed that the mutant strain CP7-R11 reverted after a single passage, whilst the other viruses with genomic duplications showed a reversion to wild-type sequences after three (CP7-R7 and CP7-R9) to 10 (CP7-R8 and CP7-R12) passages (see supporting online material, available in JGV Online). Our RT-PCR analyses showed no evidence for the presence of appreciable levels of revertants at the first passage, with the exception of CP7-R11. The sensitivity of the analysis does not exclude the possibility that a low level of reversion had occurred at this passage; however, it is unlikely that this level is higher than 5–10% (see supporting online material, available in JGV Online). After one passage of CP7-R11 and after three passages of CP7-R7 and CP7-R9, the original mutants encompassing the respective duplicated sequence were still present, in addition to the revertants (data not shown); after RT-PCR analysis of RNAs from the tenth passages of CP7-R7, CP7-R8, CP7-R9, CP7-R11 and CP7-R12, only products with a size corresponding to that of the parental virus CP7-5A could be detected, and nucleotide sequence analysis indicated that the duplicated sequences were removed. In contrast, the strongly attenuated deletion mutant CP7-R2 was genetically stable during cell-culture passages. Moreover, when consensus sequences of two to three clones per PCR product were compared with corresponding sequences of the parental virus CP7-5A, point mutations leading to amino acid changes in NS5B could not be detected for any of the reconstructed RdRp mutant viruses from the investigated passages (data not shown).

Taken together, we discovered that a viral RdRp allows virus replication, despite major rearrangements within its most conserved structural compartment, the palm region. Depending on type, location and extent of the introduced
mutations, the efficiency of viral RNA synthesis was reduced moderately to strongly. Moreover, the described alterations caused small-plaque phenotypes and delays in viral growth kinetics that correlated with reduced efficiencies in viral RNA replication. Hence, it can be concluded that viral growth depends directly on the RNA replication efficiency of the viral RdRp and, consequently, that the different degrees of virus attenuation observed in cell culture actually resulted from the mutations introduced into the NS5B gene.

It can be speculated that the described mutations altered the enzymic activity of the viral polymerases. This may be due to either a steric dislocation of the catalytic centre or an altered diameter of the tunnel formed for template and nascent RNAs to pass through. Alternatively, as the described mutations are located in a long, flexible surface loop at the back of the polymerase (Fig. 1a), a disruption of essential interactions with other components of the virus replication complex also appears possible. Future studies aiming at elucidating the structure of these unique mutant RdRps, ideally in complex with a suitable substrate, will shed more light on the structure–function relationship of viral RdRps.

Several reports on different members of the family Flaviviridae demonstrated that viruses with an attenuated phenotype in vitro displayed reduced virulence in vivo (Kuhn et al., 1992; Makoschey et al., 2004; Mandl et al., 1998; Men et al., 1996). Accordingly, it is to be considered that the BVDV RdRp mutant viruses are also attenuated in vivo. Among these, z-helix 17 deletion mutant CP7-R2 turned out to be a well-suited candidate for vaccine development, as it showed the strongest attenuation in vitro and proved to be genetically stable during cell-culture passages.

With regard to other plus-strand RNA viruses and also double-stranded RNA viruses, the results of the present study suggest that the palm region of viral RdRps represents an attractive target for the attenuation of RNA viruses. Our RNA recombination system for pestiviruses (Gallei et al., 2004, 2005b) can be applied to other viral systems, with the goal to generate a variety of different recombinant viral genomes encompassing mutated RdRp genes; the latter can then be screened for both viral viability and the desired attenuated phenotype. Taken together, this report may have implications for the directed generation of attenuated RNA virus live vaccines and vectors in general.

**Note added in proof**

The crystal structure of the RdRp of mutant CP7-R12 has recently been determined (Choi et al., 2006).

**Acknowledgements**

This study was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 535 (‘Invasionsmechanismen und Replikationsstrategien von Krankheitserregern’), project B8 (‘RNA-Rekombination bei Pestiviren’). We thank Matthias König and Christine Förster (Institut für Virologie, Giessen, Germany) for introduction to real-time PCR.

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