Infections with bovine viral diarrhea virus (BVDV) elicit various diseases in cattle. For example, vertical infections may lead to persistently infected (PI) animals, which may die of mucosal disease (MD) when cytopathic (cp) BVDV biotypes emerge by mutation during virus replication (Tautz et al., 1998).

Within the family Flaviviridae, BVDV belongs to the genus Pestivirus, together with Classical swine fever virus (CSFV). The approximately 12 kb positive-strand RNA genome consists of a long open reading frame (ORF) and 5′ and 3′ non-translated regions (NTRs). Translation initiates at an internal ribosomal entry site (IRES) involving the 5′NTR and the 5′ portion of the ORF. The resulting polyprotein is processed by cellular and viral proteases to yield the viral structural and non-structural (NS) proteins (Lindenbach et al., 2007). In concert with cellular factors, the proteins NS3, NS4A, NS4B, NS5A and NS5B form viral replication complexes (RC) that catalyse the synthesis of progeny positive-strand viral RNAs via negative-strand RNA intermediates (Behrens & Isken, 2006).

Studies on the molecular mechanisms of BVDV RNA replication were facilitated significantly by functional DNA copies of the viral genome. One of the first cDNAs that enabled in vitro transcription of infectious viral RNA was generated with BVDV CP7 (Meyers et al., 1996), a cp virus isolate from a PI animal that died of MD (Tautz et al., 1994). Considering its key role during viral RNA replication, the viral 3′NTR has been particularly well-studied. As with all pestiviruses, the BVDV 3′NTR consists of a variable region (3′V) downstream of the translational stop codon, and a conserved region (3′C) at the 3′ end (Deng & Brock, 1993) (Fig. 1). 3′C contains inalterable elements that are assumed to compose the negative-strand promoter of the initial RC (Yu et al., 1999). Conversely, 3′V displays a remarkable heterogeneity in size and nucleotide composition between different virus subtypes, and certain mutations within 3′V are well-tolerated (Isken et al., 2004; Pankraz et al., 2005). Still, 3′V also encodes conserved features, such as the so-called ‘pseudo-stop codons’, i.e. nucleotide triplets within the 3′NTR that resemble translational stop codons and that are present in translational frame with the viral ORF. The presence of these ‘pseudo-stops’, as well as proper folding of the SLstop stem–loop structure in 3′V and association of the cellular NFAR proteins with SLstop were shown to be important for accurate termination of translation at the stop codon of the viral ORF. Efficient termination of translation is required for efficient RNA replication (Isken et al., 2004) (Fig. 1a).

This study was aimed mainly at establishing variants of a defined BVDV strain that should be easily distinguishable by heterologous sequence inserts. The strived-for BVDV recombinants should replicate at maximum titres and they should display a certain genetic stability.

Following these outlines, we applied a BVDV non-CP7 (NCP7) cDNA version where the CP7-specific 27 nt insert (Tautz et al., 1994) was removed from the NS2-coding region of the earlier-described BVDV CP7-5A cDNA (Becher et al., 2000; cloning procedure supplied on request). Importantly, standard transfection/reinfection procedures with in vitro transcripts of this NCP7 cDNA yielded high titres of infectious virus. That is, approximately $3 \times 10^6$ Marbin–Darby bovine kidney (MDBK) cells (ATCC) were transfected with 3 μg NCP7 in vitro...
transcript by electroporation [see Pankraz et al. (2005) for conditions of cell growth and electroporation]. At day 3 post-transfection, the culture supernatant was titrated and applied for reinfection of MDBK cells at an m.o.i. of 1. Titration was performed by end-point dilution and monitored by immunofluorescence (IF) staining of NS3 in infected cells (Behrens et al., 1998). Thus, at day 3 post-reinfection (p.i.), we measured peak titres of $5 \times 10^7$–$5 \times 10^8$ TCID$_{50}$ (ml culture medium)$^{-1}$ (Fig. 2).

As a heterologous insert, we decided to apply the 5’NTR of hepatitis C virus (HCV). HCV, also belonging to the family Flaviviridae, resembles BVDV in important aspects such as virion and genome organization. The HCV 5’NTR is used routinely for detection and quantification of this important human pathogen in clinical diagnostic assays by quantitative (q) RT-PCR (Nolte et al., 2003). As the region immediately downstream of the SL$_{\text{stop}}$ motif in the BVDV 3’NTR (Fig. 1a) was indicated as being particularly permissive to genetic changes (also see below), it was applied as the insertion site (IS).

First, we inserted two unique restriction sites into the NCP7 cDNA [Fig. 1b; cloning procedure supplied as Supplementary Methods (available in JGV Online); final clone termed NCP7SnaB/Pac]. Interestingly, transfection/reinfection experiments with in vitro transcripts of NCP7SnaB/Pac yielded infectious virus particles at titres that resembled those of the parental NCP7, i.e. $10^7$–$10^8$ TCID$_{50}$ (ml culture medium)$^{-1}$ (not shown). Encouraged by this finding, we next introduced the HCV 5’NTR into the IS. That is, an HCV 5’NTR cDNA fragment (Con1 isolate, subtype 1b; Lohmann et al., 1999) containing the appropriate restriction sites was introduced via SnaBI/PacI into the NCP7SnaB/Pac cDNA. As the HCV 5’NTR encodes a major part of the HCV IRES, the insert was placed such that several nucleotide triplets could take on the function of ‘pseudo-stop codons’ (see Fig. 1b and Supplementary Methods; final construct termed NCP7HCV5’). NCP7HCV5’ RNA transcripts were also found to be replicative and infectious, as indicated by IF staining of transfected and reinfected MDBK cells (Fig. 2a).
Most importantly, the resultant virus titres were repeatedly (i.e. through more than five parallel transfection/reinfec-
tion experiments) found to be indistinguishable from those obtained with the original NCP7 virus, i.e. up to $5 \times 10^8$ TCID$_{50}$ ml$^{-1}$ (Fig. 2c).

We next compared the replication capacity of the two recombinant strains NCP7SnaB/Pac and NCP7HCV5' with that of the original NCP7 BVDV, performing side-by-side growth curves (standard protocol). Notably, the growth characteristics of the recombinant viruses were indistin-
guishable from those of the parent virus (Fig. 2c; data of NCP7SnaB/Pac not shown).

Whilst these data suggest that the recombinant viruses display a similar stability to the parental virus, we provided evidence for this hypothesis on the molecular level. BVDV NCP7HCV5' was grown side by side with the NCP7 parent for seven passages. The 3'NTRs of RNA species were copied/amplified from the culture supernatants by RT-
PCR using the protocol of Grassmann et al. (1999) and primers 5' GGATCCGCTGTCGGTGCCACGACT-3' (forward) and 5' TCTAGACAGCTAAAGTGCTGTGTG-3' (reverse). Following analysis by electrophoresis, the BVDV NCP7HCV5' RT-PCR products were cloned via BamHI/XbaI into pBluescript (Stratagene) and five clones were sequenced (Eurofins MWG Operon). As shown in Fig. 2(b), RT-PCR of RNA species of BVDV NCP7HCV5' yielded one major product. This had the expected size of approximately 500 bp (versus approx. 170 bp for NCP7), indicating that the HCV insert remained stably introduced in the BVDV 3'NTR. In fact, when cloned and sequenced, only clones with a complete HCV insert were obtained, and the number of mutations that occurred in the foreign insert after seven passages was essentially in the same range as in the flanking BVDV 3'NTR sequence (i.e. approx. one mutation per 500 nt; Fig. 2d).

Having a stable recombinant BVDV with an HCV insert, we finally tested whether this virus could be detected specifically and quantified by qRT-PCR measuring the HCV 5'NTR. Hence, BVDV NCP7HCV5' was analysed with Roche TaqMan HCV MasterMix (ASR) and with the AcroMetrix OptiQuant HCV panel (intact HCV) as a calibrator (Barbeau et al., 2004). As explained in Fig. 3, the recombinant BVDV NCP7HCV5' behaved as wild-type HCV in the HCV-monitoring assay.

Insertion of heterologous sequence elements into the genomes of RNA viruses may dramatically affect RNA replication, genome packaging, virion stability and virus infectivity. Hence, it was interesting that even a complex RNA structure such as the HCV 5'NTR was well-tolerated when inserted into a defined segment in the 3'V region of the BVDV 3'NTR. The choice of this IS was based on earlier genetic studies that revealed mainly two elements in...
other BVDV chimeras carrying the 5’NTRs of other HCV subtypes (data not shown). Similar to earlier studies with CSFV (Stettler et al., 2002), experiments are currently under way to test whether a second ORF may be introduced downstream of the HCV IRES to generate stable ‘bicistronic’ BVDV genomes.

After passaging the BVDV recombinants, we did not sequence entire species genomes. Nevertheless, the unchanged replication kinetics of the recombinant viruses, as well as the fact that through seven rounds of infection, only a few mutations occurred in the insert and in the flanking 3’NTR sequence, strongly support the notion that no reversions or pseudoreversions occurred. Hence, the characteristics of our constructs differ from those of constructs described by Frolov et al. (1998) and Moser et al. (2001), where additional sequences were introduced into the BVDV or CSFV 5’NTR, and also from BVDV 3’NTR variants that were described by Pankraz et al. (2005). In the latter study, individual stem–loop structures of the BVDV 3’NTR were deleted and these mutants were tested for infectivity. Thus, deletion of SL\textsubscript{stop} still yielded infectious virus, which, however, displayed an evident delay of replication with respect to the parent. Whilst in this case it seems obvious that pseudorevertants emerged throughout transfection and reinfection, this was not further analysed by Pankraz et al. (2005).

The fact that a stable, highly replicating BVDV with an HCV 5’NTR insert was obtained is relevant, as this virus may serve as a standard in HCV diagnostic tests. Moreover, this study highlights the potential use of attenuated versions of such recombinant pestiviruses as live marker vaccines that can be discriminated easily from field virus by a routine qRT-PCR assay (Houe et al., 2006).

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References


