Trans-complementation of autonomously replicating Bovine viral diarrhea virus replications with deletions in the E2 coding region

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Received 30 July 2002; returned to author for revision 9 September 2002; accepted 27 October 2002

Abstract

Autonomously replicating Bovine viral diarrhea virus (BVDV) genomes (replicons) were constructed from the full-length BVDV cDNA clone pA/BVDV/Ins-H11002 (G. Meyers et al., J. Virol. 70, 8606–8613, 1996). The sequences coding for envelope protein E2, for E2 without the C-terminal transmembrane region, or for E2 and nonstructural protein p7 were deleted, and the resulting mutants were tested for their ability to replicate after transfection. All deletion mutants were able to replicate and to express the inserted green fluorescent protein but did not produce infectious progeny virus in bovine kidney PT cells. The replicons were also tested for their ability to be trans-complemented in the bovine cell line PT_805, which constitutively expresses BVDV structural proteins. E2-negative BVDV mutants were complemented and 10^6 infectious units were obtained at 24 h after transfection. Complementing PT_805 cells could only inefficiently be infected using trans-complemented virions, however, and low levels of virus production were observed when complemented BVDV was passaged using PT_805 cells. Similarly, infection of PT_805 cells with BVDV was highly inefficient, but transfection of full-length BVDV NCP7 RNA into PT_805 resulted in 10,000-fold higher virus titers when compared to those obtained 24 h after transfection of parental PT cells. We concluded that self-replicating E2-deleted BVDV RNAs can be efficiently trans-complemented by constitutively expressed E2, and that expression of BVDV structural proteins markedly influences susceptibility of cells to BVDV infection as well as BVDV titers after transfection of full-length BVDV RNA.

Keywords: Bovine viral diarrhea virus; Pestivirus; Replicon; Trans-complementation

Introduction

Bovine viral diarrhea virus (BVDV), a member of the genus Pestivirus within the family Flaviviridae (van Regenmortel et al., 2000) is the causative agent of bovine viral diarrhea, an economically important disease of cattle worldwide (Brownlie et al., 1984; Houe, 1995). The major economic losses caused by BVDV infections are reduced fertility, abortions, and the generation of persistently infected calves, which can develop fatal “mucosal disease” (Baker, 1995; Bolin, 1995; Moennig and Liess, 1995).

Genetically and structurally closely related virus species are classical swine fever virus (CSFV) and the ovine border disease virus (BDV) (van Regenmortel et al., 2000). Pestiviruses can be divided into two different biotypes, cytopathogenic (cp) and non-cytopathogenic (ncp) viruses. While cp BVDV induces apoptosis and cell death (Zhang et al., 1996) and expresses nonstructural protein 3 (NS3) as well as uncleaved NS2/3, inoculation with ncp BVDV leads to persistent infection of cell cultures and expression of NS2/3 (Donis and Dubovi, 1987). The pestivirus genome consists of a single-stranded RNA of positive orientation. The RNA has a length of approximately 12.3 kb and contains one large open reading frame (ORF), which is flanked by nontranslated regions (NTR) at both genome ends (Collett et al., 1988a; Meyers et al., 1989). The pestiviral ORF is translated into one polyprotein, which is co- and post-translationally processed into 11 (ncp BVDV) or 12 (cp
BVDV) mature proteins by viral and cellular proteases. Pestivirus virions consist of four structural proteins, a capsid (C) protein and three glycosylated envelope proteins (E\textsubscript{RNS}, E1, E2) (Collett et al., 1991; Rümenapf et al., 1993; Donis, 1995). BVDV antibodies are directed against E\textsubscript{RNS}, E2, and NS3 (Donis et al., 1991) and neutralizing activity was predominantly demonstrated for E2-specific antibodies (Donis et al., 1988; Bolin, 1993). Envelope protein E2 is reported to form a heterodimer with E1 as well as E2–E2 homodimers (Weiland et al., 1990), and it has been suggested that E2 plays a major role in virus attachment and entry (Donis, 1995). On the basis of sequence analysis and determination of the N-termini, it was shown that processing of E2 is mediated by host cell signalase. Typical hydrophobic signal sequences were identified upstream of the E2 N-terminus within E1 and a transmembrane anchor of about 40 hydrophobic amino acids was demonstrated at the C-terminus of E2 (Rümenapf et al., 1993).

Studies on the replication of BVDV have been considerably facilitated by reverse genetic systems and the discovery of autonomously replicating subgenomic RNAs (replicons) (Behrens et al., 1998; Meyers et al., 1996). The infectious BVDV clone of the cytopathogenic strain CP7 is based on the plasmid pA/BVDV. Deletion of a genomic insertion, which was shown to be responsible for cytopathogenicity, resulted in plasmid pA/BVDV/Ins\textsuperscript{−}. Using pA/BVDV/Ins\textsuperscript{−}, the ncp BVDV strain NCP7 could be recovered (Meyers et al., 1996). Furthermore, pA/BVDV and pA/BVDV/Ins\textsuperscript{−} were used to define the first BVDV self-replicating RNAs with genetic deletions similar to that of so-called defective interfering interfering particles (DI), e.g., DI9 transcribed from pA/BVDV/D9 (Behrens et al., 1998; Meyers et al., 1996; Tautz et al., 1999). Pestivirus self-replicating RNAs are important tools for an understanding of virus replication, assembly, and egress. So far, there are only a few reports concerning BVDV replicons and trans-complementation of BVDV replicons. Grassmann et al. (2001) trans-complemented defects in the nonstructural protein 5A and Harada et al. (2000) characterized the E2-p7-coding region using E2-p7-deleted replicons and complementing cell lines. Trans-complementation of deleted parts of the genome can be used for the identification of trans-acting elements of the BVDV genome, but can also allow the construction of a novel type of pestivirus vaccine using complemented virions transferring defective genomes in vivo. For CSFV, a set of self-replicating RNAs with various deletions within the genome has been characterized (Moser et al., 1999). Additionally, E\textsubscript{RNS}-deleted CSFV-replicons were generated and trans-complemented using a swine kidney cell line (SK6) constitutively expressing CSFV E\textsubscript{RNS}. The resulting virions were able to infect SK6 cells without the production of infectious virus progeny and could be passaged on E\textsubscript{RNS}-expressing SK6 cells. Pigs were protected against lethal CSFV challenge after immunization with these complemented virions (Widjiojoatmodjo et al., 2000).

The aims of this study were (i) the characterization of novel self-replicating mutated BVDV RNA genomes (replicons) and the investigation of their potency for expression of foreign genes, and (ii) the attempt of efficient trans-complementation of different replicons using a helper cell line providing all structural BVDV proteins. We used the infectious clone pA/BVDV/Ins\textsuperscript{−} to construct different BVDV deletion mutants. The produced deletion mutants were able to replicate and to express an inserted reporter gene encoding green fluorescent protein (GFP), but did not produce infectious progeny virus. By using a cell line constitutively expressing all four BVDV structural proteins, two different autonomously replicating E2 deletion mutants were efficiently trans-complemented. The resulting virions were able to infect bovine target cells and to transfer the replicons without generating replication-competent virus progeny.

Results

Construction and characterization of BVDV replicons

After establishment of an infectious clone for BVDV CP7 (plasmid pA/BVDV), a variant of the construct with a deletion of a 27-nucleotide insertion was generated that allowed recovery of ncp BVDV NCP7 (plasmid pA/BVDV/Ins\textsuperscript{−}) (Meyers et al., 1996). Based on pA/BVDV/Ins\textsuperscript{−}, several replicons were constructed by deletion of sequences encoding E2 and p7 (Fig. 1). The replicons contained a deletion of the complete E2 region (plasmid NCP7ΔE2, amino acids position 694 to 1066, Fig. 1), of the E2 gene with an intact transmembrane region (imtr) where only the ER luminal part of E2 is deleted (NCP7ΔE2lum, amino acid position 694 to 1018, Fig. 1), or of the complete E2-p7 gene region (NCP7ΔE2p7, amino acid position 694 to 1143, Fig. 1). All deletions were confirmed by nucleotide sequencing of the resulting cDNA clones. Prototype replicon DI9 transcribed from plasmid pA/BVDV/D9 (Behrens et al., 1998; Meyers et al., 1996) and replication-defective RNA transcribed from plasmid NCP7Δ3’imtrAatII (Fig. 1) were used as controls.

Subsequently, in vitro transcribed full-length replicon RNA was transfected into BVDV-negative PT cells. Transient expression of NS2/3 and E\textsubscript{RNS} could be detected from 24 h posttransfection by immunofluorescence (IF) for all replicons except NCP7Δ3’imtrAatII (NS3 and E\textsubscript{RNS}-negative) and DI9 (E\textsubscript{RNS}-negative). However, no infectious recombiant BVDV could be recovered, even after serial passages and copassages using highly susceptible KOP-R cells (Table 1). The intensity of NS2/3-specific immunostaining of NCP7ΔE2lum-, NCP7ΔE2-, or NCP7ΔE2p7-transfected PT cells was comparable to cells transfected with full-length NCP7 RNA and more than 85% of the cells were positive in IF (Table 1, Fig. 2). Transfection of full-length NCP7 RNA into PT cells resulted in virus titers of up to 10\textsuperscript{3} infectious units (IU)/ml 24 h posttransfection (p.t.). As expected, electroporation of cells with RNA of the pro-
totype replicon DI9 (Behrens et al., 1998) did not lead to recovery of infectious BVDV, but a strong immunofluorescence following NS3 staining was observed. RT-PCR (Fig. 3A) and Northern blot analysis (Fig. 3B) of PT cells transfected with NCP7 replicons and full-length NCP7 demonstrated presence of BVDV-specific RNA of the expected size containing the correct deletions. However, RNA bands of the deletion mutants were weaker in Northern blot than the bands of full-length NCP7 RNA and DI9 RNA (Fig. 3B). This observation was confirmed by repeated Northern blot experiments (data not shown). No replication was detectable after transfection of NCP7\_H9004\_3\_H11032\_ntrAatII RNA (Fig. 3B). In summary, deleted BVDV genomes, which were replication-competent but lacked the ability of generating virus progeny, were constructed and could efficiently be transfected into bovine cells.

Replicons of this kind are frequently used as expression systems in mammalian cell lines. The feasibility of the expression of foreign proteins by BVDV replicons was examined by the insertion of the gene encoding the GFP into the E2-p7-deleted replicon NCP7\_\Delta\_\_E2p7 (NCP7\_\Delta\_\_E2p7\_GFP). After transfection of in vitro transcribed NCP7\_\Delta\_\_E2p7\_GFP RNA into PT cells, GFP fluorescence could be detected in more than 80% of transfected cells (Fig. 4A). Subsequent NS2/3 IF staining was positive for a comparable number of cells (data not shown). Furthermore, RT-PCR analysis with NCP7-specific primers flanking the E2-p7 region and with GFP-specific primers demonstrated the correct insertion of the GFP gene (Fig. 4B). By using NCP7\_\Delta\_\_E2p7\_GFP, we were able to demonstrate expression of a foreign gene by an ncp BVDV replicon.

Establishment of a PT cell line expressing BVDV structural proteins

To do complementation studies with the BVDV replicons, a bovine cell line was established that constitutively expresses BVDV structural proteins. PT cells were trans-
Table 1
Replication and trans-complementation of BVDV NCP7 RNAs with in-frame deletions (replicons)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Replication(^a) after RNA transfection in PT cells (24 h)</th>
<th>1st passage(^b) (24 h, 48 h, 72 h) on KOP-R cells</th>
<th>Titer of virus progeny(^c) 24 h after transfection of complementing PT_805 cells (IU/ml)</th>
<th>4th passage(^d) of virus progeny on KOP-R cells</th>
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<tr>
<td>NCP7</td>
<td>++ (P)</td>
<td>++ (10^7 IU/ml) (P)</td>
<td>10^{7.3} (P)</td>
<td>++ (P)</td>
</tr>
<tr>
<td>NCP7ΔE2lum</td>
<td>++</td>
<td>φ</td>
<td>10^{6.3}</td>
<td>φ</td>
</tr>
<tr>
<td>NCP7ΔE2</td>
<td>++</td>
<td>φ</td>
<td>10^{6.6}</td>
<td>φ</td>
</tr>
<tr>
<td>NCP7ΔE2p7</td>
<td>++</td>
<td>φ</td>
<td>&lt;10^6</td>
<td>nd(^g)</td>
</tr>
<tr>
<td>DI9</td>
<td>++</td>
<td>φ</td>
<td>&lt;10^6</td>
<td>nd(^g)</td>
</tr>
</tbody>
</table>

\(^a\) PT cells were transfected with in vitro transcribed RNA and IF-stained for NS3 expression after 24 h.

\(^b\) KOP-R cells were inoculated with undiluted supernatants 24, 48, and 72 h after transfection of PT cells (1 ml per 10^5 cells). KOP-R cells were stained with NS3-specific mabs 5 days postinoculation.

\(^c\) PT_805 cells were transfected with in vitro transcribed RNA and the supernatants were titrated after 24 h using KOP-R cells.

\(^d\) KOP-R cells were inoculated using supernatants of transfected PT_805 cells with a titer > 10^6 IU/ml (1 ml per 10^5 cells).

\(^e\) P indicates the detection of virus plaques after NS3-staining (>10 cells).

\(^f\) φ = no BVDV-specific immunofluorescence; + = weak positive NS3-IF-signal and <50% IF-positive cells, ++ = positive NS3-IF-signal and >50% IF-positive cells, +++ = strong-positive NS3-IF-signal and >50% IF-positive cells.

\(^g\) nd = not done.

Fig. 2. IF analysis of PT cells transfected with RNAs transcribed from NCP7ΔE2lum (A), NCP7ΔE2 (B), and NCP7ΔE2p7 (C). At 24 h posttransfection, NS2/3 expression was analyzed by IF using the mab mix WB103/105. Mock-transfected control cells remained NS2/3-negative (D).
were tested for BVDV ERNS and E2 expression using monoclonal antibodies (mabs). After G418 selection and passaging, resistant cell clones were identified. The presence of the Geneticin resistance gene allowing selection with G418 and the HCMV immediate early promoter allowed the expression of E1, E2 of ncp BVDV strain PT810 under the control of the E2-p7 region. RNA was extracted from 10^6 cells transfected with plasmid pCDNA_C-E2, which contains the complete region encoding the structural proteins C, E RNS, E1, E2 of ncp BVDV strain PT810. RT-PCR analysis of selected replicons with primer pairs flanking the E2-p7 region. RNA was extracted from 10^6 cells transfected with transcribed RNA. All RT-PCR products were of the expected size between 176 bp (NCP7ΔE2p7) and 1528 bp (NCP7) and verified the different deletions introduced into the E2 or E2-p7 region. (B) Northern blot analysis of ~10 μg total RNA isolated from PT cells transfected with the replicons DI9, NCP7ΔE2lum, NCP7ΔE2, NCP7ΔE2p7, and the full-length NCP7 RNA with a digoxigenin-labeled NS3 RNA probe. RNA from cells transfected with the nonpuriﬁcation construct NCP7Δ3’ntrAatII served as a negative control. Arrows indicate the position in the gel of RNA of 12.3 kb (NCP7), 8 kb (DI9), and 2.3 kb (20 ng control RNA). For semiquantitative analyses, RNA from DI9-transfected cells was used undiluted and at dilutions of 1:10 and 1:20. NCP7 and the deletion mutant replicons demonstrated RNA in the correct size and with an amount in the range of the diluted DI9 RNA.

Fig. 3. (A) RT-PCR analysis of selected replicons with primer pairs flanking the E2-p7 region. RNA was extracted from 10^6 cells transfected with transcribed RNA. All RT-PCR products were of the expected size between 176 bp (NCP7ΔE2p7) and 1528 bp (NCP7) and verified the different deletions introduced into the E2 or E2-p7 region. (B) Northern blot analysis of ~10 μg total RNA isolated from PT cells transfected with the replicons DI9, NCP7ΔE2lum, NCP7ΔE2, NCP7ΔE2p7, and the full-length NCP7 RNA with a digoxigenin-labeled NS3 RNA probe. RNA from cells transfected with the nonpuriﬁcation construct NCP7Δ3’ntrAatII served as a negative control. Arrows indicate the position in the gel of RNA of 12.3 kb (NCP7), 8 kb (DI9), and 2.3 kb (20 ng control RNA). For semiquantitative analyses, RNA from DI9-transfected cells was used undiluted and at dilutions of 1:10 and 1:20. NCP7 and the deletion mutant replicons demonstrated RNA in the correct size and with an amount in the range of the diluted DI9 RNA.

In vitro transcribed RNA of the replicons and of full-length NCP7 was transfected into complementing PT_805 cells. At 24 h p.t., monolayers of PT_805 cells were examined for NS2/3 expression and culture supernatants were passaged and titrated using highly susceptible KOP-R cells. For all replicons and full-length NCP7, NS2/3 staining following RNA transfection led to immunofluorescence patterns similar to that obtained with PT cells. Between 30 and 95% of transfected cells were reactive with NS3-speciﬁc antibodies (data not shown). Infectious recombinant BVDV, however, was only detected in supernatants from PT_805-cells transfected with NCP7ΔE2lum, NCP7ΔE2, or NCP7 RNA. Virus titers of the resulting complemented viruses NCP7ΔE2_trans and NCP7ΔE2lum_trans varied between 10^6.3 and 10^6.6 IU/ml at 24 h p.t. (Table 1). After transfection of full-length NCP7 RNA into complementing PT_805 cells, virus titers of up to 10^7.3 IU/ml could be detected at 24 p.t. (Table 1). Therefore, titers of trans-complemented viruses from supernatants collected at 24 h p.t. were approximately 10,000-fold higher than virus titers obtained at 24 h after transfection of full-length NCP7 RNA into non-complementing PT cells (Table 1). In addition, no complemented BVDV was detected after passaging of PT_805 cell-culture supernatants transfected with NCP7ΔE2p7 or DI9 replicon RNAs at all times p.t. (Table 1).

Using IF of KOP-R cells infected with NCP7ΔE2_trans and NCP7ΔE2lum_trans, expression of E RNS (data not shown) and NS2/3 but not E2 could be detected (Table 1, Fig. 7). Expression pattern indicated that supernatants of transfected PT_805 cells contained infectious virus that can infect and replicate in KOP-R cells. Titration experiments and subsequent IF staining demonstrated that no cell-to-cell spread occurred, and that neither NCP7ΔE2_trans nor NCP7ΔE2lum_trans were able to form NS2/3-positive plaques or secondary infections in these cells (Table 1). At higher dilutions, only single cells or small foci of cells were positive by IF-staining for NS2/3. Control experiments with
BVDV NCP7 on KOP-R cells resulted in antigen-positive virus plaques as demonstrated for ERNS, NS2/3, and E2 (data not shown). The infection of KOP-R cells with trans-complemented viruses could be blocked with BVDV-neutralizing antisera (S-BVD_pos, S-BVD_E2) and no infectivity was detected after inoculation of KOP-R cells (100% neutralization). In a control reaction, incubation with a BVDV antibody-free serum (S-BVD_neg) did not result in virus neutralization (Table 2).

Surprisingly, infection of PT_805 cells with complemented BVDV NCP7ΔE2_trans or NCP7ΔE2lum_trans at a multiplicity of infection (m.o.i.) of 5 resulted in isolated infection of only a few single cells as determined by IF staining for NS2/3 at 24 h.p.i. (Table 3, Fig. 8). Further-
more, the number of infected PT_805 cells did not increase by passaging of cells or supernatants or by prolonged incubation times of up to 5 days (data not shown). Titration of the resulting supernatants on KOP-R cells yielded constant virus titers (single cells infected) of $10^1$ to $10^2$ IU/ml (Table 3). The titers of complemented viruses were independent from both the incubation time and the passage level (data not shown). As a control, PT_805 cells were infected using BVDV strain PT810 or NCP7 at an m.o.i. of 5. Here, also only single cells were positive after NS3-specific IF staining at 24 h p.i. In addition, virus plaques could be detected using IF-staining after more than 48 h postinfection of PT_805 cells (Fig. 8). Original PT cells, however, which were inoculated with the same virus preparation, could be easily infected with BVDV PT810 and NCP7 (plaque formation demonstrated using IF staining; Fig. 8) or complemented NCP7ΔE2_trans and NCP7ΔE2lum_trans viruses (no plaque formation, data not shown). The reason for this nonpermissiveness of PT_805 cells expressing BVDV structural proteins remains to be determined.

**Discussion**

Pestivirus genomes with deletions were first described as defective interfering particles for BVDV and CSFV (Kupfermann et al., 1996; Meyers and Thiel, 1995), and autonomous replication of such defective genomes was demonstrated (Behrens et al., 1998; Moser et al., 1999; Tratschin et al., 1998). From these experiments it became obvious that neither the BVDV nonstructural proteins Npro, p7, NS2, nor the structural proteins are essential for BVDV RNA replication. In addition, defective interfering particles were reported to be packaged by helperviruses (Kupfermann et al., 1996).

Here, we constructed NCP7 mutants with deletions of E2 (with or without transmembrane region) or E2-p7 without...
introducing a frameshift within the BVDV ORF. Transfection of in vitro transcribed RNA of the replicons NCP7ΔE2lum, NCP7ΔE2, and NCP7ΔE2p7 resulted in RNA replication. In all experiments, the prototype replicon DI9 (Meyers et al., 1996) and a replication-defective NCP7Δ3'ntrAatII deletion mutant served as controls. In none of the cases was virus progeny detectable after passage of the supernatants of cells transfected with the deletion mutants. However, virus progeny with a titer of approximately 10^2 to 10^3 IU/ml could be detected at 24 h after transfection of full-length BVDV NCP7 RNA in supernatants of transfected PT cells. Recently, it was shown that p7 is essential for formation of infectious BVDV (Harada et al., 2000). Although deletion of p7 alone prevented generation of infectious viruses, Harada et al. (2000) could further demonstrate that a cell line expressing both p7 and E2, but not p7 alone, could rescue the E2-p7-deleted replicon. It can be concluded from these data that E2 is essential for infectious virion production. Our experiments with NCP7ΔE2lum and NCP7ΔE2 now provide direct evidence for the essential nature of E2. In addition, our data confirm that p7 is essential, since the E2p7 deletion could not be trans-complemented with PT_805 cells. After reinsertion of the gene coding for E2, the resulting revertant NCP7revE2 BVDV was indistinguishable from original NCP7 (data not shown). Furthermore it was shown that deletion of E2-p7, E2, or E2lum did not severely impact the ability of RNA self-replication. Nevertheless, Northern blot analysis of the replicons argues for a better RNA replication with full-length NCP7 and prototype replicon DI9 than with the deletion mutants. In a series of CSFV deletion mutants (Moser et al., 1999), replicons with different levels of autonomous RNA replication were also detected, and some of the reported mutants were not replication competent.

After in-frame insertion of a GFP-coding gene into the E2 locus of the NCP7ΔE2p7 deletion mutant, the resulting construct NCP7ΔE2p7_GFP was shown to replicate after RNA transfection into PT cells and to express the inserted reporter gene. Expression of foreign genes using self-replicating RNAs is well known for members of the genus Alphavirus (e.g., Sindbis virus replicons), where expression is driven by a subgenomic promotor (Schlesinger and Dubensky, 1999), and in the family Flaviviridae in the case of the Kunjin virus replicon (Varnavski and Khromykh, 1999). The novel non-cytopathogenic BVDV replicon vector expressing GFP allows the convenient visualization of replicons, independent from fixation and IF staining. Furthermore, self-replicating BVDV RNAs may be used for long-term expression of other foreign proteins, especially in ruminant cell lines and possibly in ruminants, e.g., after trans-complementation of virions. The reported transfection efficiency and the noncytopathic characteristics could make BVDV replicons a useful expression system not only for bovine cells, but also for other mammalian cell lines.

Trans-complementation of structural proteins has been reported for CSFV (Widjojoatmodjo et al., 2000), BVDV (Harada et al., 2000), and Kunjin virus (Khromykh et al., 1998). In addition to complementation by helper viruses, the
First passage on KOP-R cells

<table>
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<tr>
<th></th>
<th>anti-NS3</th>
<th>anti-E2</th>
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<tr>
<td><strong>BVDV NCP7</strong></td>
<td>A</td>
<td>F</td>
</tr>
<tr>
<td><strong>NCP7ΔE2lum</strong></td>
<td>B</td>
<td>G</td>
</tr>
<tr>
<td><strong>trans</strong></td>
<td>C</td>
<td>H</td>
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<td><strong>NCP7ΔE2</strong></td>
<td>D</td>
<td>I</td>
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<td><strong>trans</strong></td>
<td>E</td>
<td>J</td>
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<tr>
<td><strong>DI9</strong></td>
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missing gene products were also provided by Semliki Forest virus replicons or cell lines constitutively expressing the deleted structural proteins. For the here-described trans-complementation of BVDV replicons, self-replicating RNAs were directly transfected into the bovine cell line PT_805 expressing the structural proteins C, E2<sup>RNS</sup>, E1, and E2 of BVDV. After transfection into PT_805 cells, replicons with deletions of the E2 sequence (NCP7ΔE2lum and NCP7ΔE2) were efficiently complemented in trans. The resulting virus titers of about 5 × 10<sup>6</sup>IU/ml as early as 24 h after transfection noticeably exceeded those reported for the parental NCP7 clone (Meyers et al., 1996) and other complemented BVDV (Harada et al., 2000). To our knowledge, this is the first report of a highly efficient complementation of BVDV E2 deletion mutants and the first report of a bovine cell line constitutively expressing all structural proteins of BVDV. Transfection of full-length BVDV NCP7 RNA into PT_805 cells also resulted in 10,000-fold higher virus titers compared to virus titers obtained 24 h after transfection of NCP7 RNA into parental PT cells. One possible explanation could be that the presence of additional BVDV structural proteins in PT_805 cells enhanced assembly and/or egress of infectious virions. An early and more efficient packaging of full-length RNA in the presence of “excess” structural proteins may be responsible for this phenomenon. In this context, it is noted that previous reports also describe low virus yields at 24 h after transfection of RNA transcribed from pA/BVDV/Ins<sup>–</sup> (Meyers et al., 1996). In addition, virus titers of less than 10<sup>3</sup> TCID<sub>50</sub>/ml were reported for the infectious cDNA clone of cp BVDV strain NADL during the first 48 h p.t. (Vassiliev et al., 1997). However, since NCP7 titers reach levels similar to that of other BVDV strains after replication for several days (Meyers et al., 1996), it is also possible that the structural proteins provided by the PT_805 cell line compensate for a general defect at the early phase of BVDV assembly and egress. Selection of a cell clone which is more permissive to BVDV replication is extremely unlikely due to the fact that an independently constructed second cell line (PT_803) expressing all BVDV structural proteins showed the same characteristics (data not shown). Further experiments with additional infectious cDNA clones and cell lines providing only single structural proteins (e.g., E2) are in progress.

Infection of noncomplementing bovine cells with the trans-complemented virions resulted in replication without release of infectious virus particles. Such complemented virions were indistinguishable from wild-type BVDV in virus neutralization experiments and were completely neutralized by a BVDV-specific serum as well as by a BVDV E2-specific serum. Recombinations yielding infectious wild-type BVDV were not detected in any of the complementation experiments. Even after four consecutive passages on highly susceptible KOP-R cells, no rescued BVDV was seen in transfection supernatants containing more than 10<sup>6</sup> IU/ml trans-complemented virions (Table 1). However, homologous RNA recombination is a possible risk for plus-stranded RNA viruses in general (Hill et al., 1997; Schlesinger and Weiss, 1994). RNA recombination was also described for wild-type BVDV in persistently infected cattle following application of a modified live BVDV vaccine (Becher et al., 1999) or after experimental inoculation (Fritzemeier et al., 1995), but the incidence seems to be very low, because similar complementation experiments using CSFV replicons with deletions in the E<sup>RNS</sup>-coding region did not result in replication-competent recombinants, even after 10 serial passages of the defective viruses using an E<sup>RNS</sup>-expressing cell line (Widjojoatmodjo et al., 2000). While CSFV E<sup>RNS</sup>-expressing SK6 cells allowed infection with complemented virions (Widjojoatmodjo et al., 2000), a markedly reduced susceptibility of complementing PT_805 cells to BVDV infection could be demonstrated. Only single virus foci were observed following BVDV PT810 or NCP7 inoculation using an m.o.i. of 5. The cells, however, could efficiently replicate BVDV, since transfection of full-length NCP7 RNA into the complementing cells led to high BVDV titers at 24 h p.t. Harada et al. (2000) reported a BVDV E2p7- and a p7-expressing cell line, which could not be infected with BVDV. The observed phenomenon is referred to as virus interference, but the mechanisms leading to interference are not well understood. Probably as a consequence of the interference, complemented virions NCP7ΔE2<sup>trans</sup> and NCP7ΔE2lum<sup>trans</sup> could not be passaged using PT_805 cells. In contrast, complemented CSFV

Table 2

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<th>BVDV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IF analysis 3 days postinoculation of the neutralization mix</th>
<th>S-BVD&lt;sub&gt;pos&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>S-BVD&lt;sub&gt;E2&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>S-BVD&lt;sub&gt;neg&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>NCP7</td>
<td></td>
<td>Ø</td>
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<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>NCP7ΔE2lum&lt;sup&gt;c&lt;/sup&gt;_trans</td>
<td></td>
<td>Ø</td>
<td>Ø</td>
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<td>Ø</td>
<td>Ø</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Complemented viruses were adjusted to a titer of 10<sup>4</sup> IU/ml.

<sup>b</sup> Cattle serum prepared against BVDV type 1.

<sup>c</sup> Sheep serum specifically directed against BVDV type 1 E2.

<sup>d</sup> BVDV antibody free cattle serum.

<sup>e</sup> Ø = no IF-positive cells, + = more than 100 IF-positive cells/well.

Fig. 7. IF analyses of trans-complemented viruses. PT_805 cells were transfected with replicon RNA and supernatants were harvested at 24 h p.t. for inoculation of KOP-R cells. At 24 h p.t., KOP-R cells were analyzed by IF using NS3- and E2-specific monoclonal antibodies (WB103/105; WR215). Infectious virions could be passaged from supernatants of PT_805 cells transfected with NCP7, NCP7ΔE2lum, and NCP7ΔE2 (Fig. 7A, B, and C), but not from NCP7ΔE2p7- or DI9-transfected PT_805 cultures (Fig. 7D, E, I, and J). An E2 expression (mab CA3) could only be detected in cells inoculated with supernatants from PT_805 cells transfected with full-length NCP7 RNA (Fig. 7F, G, and H).
replicons with a deletion in the E RNS -coding region could be efficiently propagated using CSFV E RNS -expressing SK6 cells and there is a reported increase of the titers of complemented CSFV during passaging (Widjojoatmodjo et al., 2000). Nevertheless, our results demonstrate that BVDV structural proteins can play a role in the blocking of superinfection, and that BVDV envelope protein E2 may be a candidate causing the described form of interference.

In summary, we were able to demonstrate trans-complementation of self-replicating E2-deleted BVDV RNAs and enhancement of BVDV replication using a bovine cell line expressing all BVDV structural proteins. The described trans-complemented BVDV as well as the helper cell line PT_805 may open new ways to study pestivirus entry, packaging, release, and interference. Furthermore, complemented BVDV may be useful for the development of safe and efficient BVDV vaccines.

Materials and methods

Cells

PT cells (RIE11 CCLV), a permanent bovine kidney cell line, and KOP-R cells (RIE244, CCLV), a diploid bovine esophageal cell line, were obtained from the collection of cell lines in veterinary medicine at the Federal Research Center for Virus Diseases of Animals, Insel Riems (CCLV). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS). PT cells were chosen due to their applicability for DNA/RNA transfection and for generation of constitutively expressing cell lines. KOP-R cells were selected due to their susceptibility to BVDV infection and their suitability for BVDV propagation.

Monoclonal antibodies, BVDV antisera, and BVDV neutralization

For the detection of BVDV proteins, mab WB210 (IgG1, anti-E RNS, CVL, Weybridge), 01-03 (anti-E RNS, kindly provided by Christian Schelp, Intervet BV) WB215 (IgG2b, anti-E2, CVL, Weybridge), CA3 (anti-E2, Institute for Virology, TiHo Hannover), mab-mix WB103/105 (anti-NS3, CVL, Weybridge), and C16 (anti-NS3, Institute for Virology, TiHo, Hannover) were used (Edwards et al., 1988, 1991; Peters et al., 1986). IF analysis using a fluorescence-activated cell sorter (FACS; Becton–Dickinson) was performed as previously described (Grummer et al., 2001). Cells were detached with a trypsin solution, fixed with 1% paraformaldehyde (PFA; Sigma) on ice for 15 min, and permeabilized for 5 min with 0.0025% digitonin (in PBS; Sigma) at room temperature. After two washing steps with phosphate-buffered saline without Ca2+/Mg2+ (PBS–), BVDV-specific mabs were added for 15 min. Binding of the mabs was detected using ALEXA488 goat anti-mouse conjugate (Molecular Probes). Stained cells were resuspended in 100 μl propidium iodide (PI; Sigma) solution and analyzed by flow cytometry (5000 cells/sample). For IF analysis using a fluorescence microscope or a confocal laser scanning microscope (CFM), cell cultures were fixed with 3% PFA and permeabilized with 0.0025% digitonin (stain-
mixed in a 24-well plate with 50 μl of 5% H9262. Subsequently, the monolayer was stained for BVDV-NS2/3 (staining of E2 or ERNS). Subsequently, cell cultures were incubated for 15 min and finally washed and analyzed using a fluorescence microscope (Olympus). For double staining, the protein-specific mabs were detected with type-specific FITC-(anti-mouse IgG2b, Medac, Germany) or Cy3-labeled (anti-mouse IgG1, Medac, Germany) conjugates. Double-stained cell cultures were analyzed using confocal laser scanning microscopy (Zeiss, LSM510).

BVDV was neutralized with a pooled anti-BVDV serum (S-BVD_pos), which was collected from cattle experimentally infected with ncp BVDV strain PT810 (Beer et al., 2000). The homologous neutralization titer of this serum was 1:4096. For E2-specific neutralization, serum from sheep, immunized with an E2-expressing modified Vaccinia Virus Ankara (MVA), was used (S-BVD-E2) (Beer et al., unpublished data). A BVDV antibody-free cattle serum (S-BVD_neg) served as a control. For neutralization of BVDV, 100 μl of virus suspension (10^4 TCID_50/ml) was mixed in a 24-well plate with 50 μl of undiluted serum and incubated at 37°C. After 2 h, KOP-R cells (2 × 10^5) were transferred to the mixture and incubated for 3 days at 37°C. Subsequently, the monolayer was stained for BVDV-NS2/3 by indirect immunofluorescence (Grummer et al., 2001).

Radioimmunoprecipitation

PT_805 cells (1.5 × 10^6) were labeled with 35S-protein cell-labeling mixture (Amersham Pharmacia Biotech). In brief, cell-culture medium was replaced with DMEM without methionine and cysteine (Gibco) and incubated for 60 min at 37°C. Subsequently, the cells were labeled with 100 μCi of 35S-protein cell-labeling mixture for 16 h at 37°C. Washed twice with PBS, and lysed with RIPA buffer [1% Triton X-100, 10 mM sodium phosphate (pH 7.1), 1% sodium deoxycholate, 0.1% SDS, 0.1 M sodium fluoride, 10 mM EDTA, and 1 μg/ml Aprotinin]. The lysate was clarified by centrifugation at 40,000 rpm for 30 min and subjected to radioimmunoprecipitation analysis. The clarified lysates were incubated with an E2-specific rabbit serum for 60 min with rocking. Pansorbin cells (Calbiochem) were added and incubated for 1 h at 4°C with rocking. Immunoprecipitates were washed three times with RIPA wash buffer [0.3% Triton X-100, 1 M NaCl, 10 mM sodium phosphate (pH 7.1), 10 mM EDTA] and then washed finally with RIPA wash buffer lacking Triton X-100. Washed immunoprecipitates were resuspended in sample buffer, heated to 95°C for 5 min, and centrifuged at 12,000 g for 1 min. Immunoprecipitated proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and exposed to X-ray film for 48 h.

Western blot

PT_805 cells (1.5 × 10^6) were lysed by adding 300 μl of lysis buffer [1% Triton X-100, 20 mM sodium phosphate (pH 7.1), 150 mM NaCl, 2 mM EDTA]. The cell lysate was cleared of cell debris by low-speed centrifugation at 10,000 g for 5 min. For separation of proteins, 20 μl of lysate was electrophoresed through a 10 or 12% SDS–PAGE and then proteins were transferred onto a nitrocellulose membrane (Schleicher & Schüll). The membrane was incubated for 1 h at room temperature with a mix of anti-E_RNS mabs WB210 (1:100 dilution) and 01-03 (1:500 dilution) or a capsid antiserum (kindly provided by Tillmann Rümenapf, University of Giessen, Germany; 1:10,000 dilution) in TBST buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl, 0.1% Tween 20). After washing with TBST buffer, the membrane was incubated at room temperature for 1 h with an anti-mouse (Dianova; 1:30,000 dilution) or an anti-rabbit (Dianova; 1:20,000 dilution) horseradish conjugate. Following washing, the membrane was incubated with SuperSignal chemiluminescent detection reagent (Pierce) and exposed to film.

In vitro transcription and electroporation

In vitro transcription of linearized full-length cDNA constructs and replicons was performed by T7 RiboMax Large-Scale RNA Production System (Promega) according to the manufacturer’s instructions. The amount of RNA was estimated by ethidium bromide staining after agarose gel electrophoresis. For transfections, 1 × 10^7 PT, PT_805, or KOP-R cells were detached using a trypsin solution, washed with PBS, mixed with 1–5 μg of in vitro synthesized RNA, and electroporated (two pulses at 850 V, 25 μF, 156 ω) using an EasyJet Plus (EquiBio) transfection unit.

Establishment of C-E_RNS-E1-E2 expressing PT cells

The genomic region encoding the structural proteins (C-E_RNS-E1-E2) of ncp BVDV strain PT810 (Wolfmeyer et al., 1997) was cloned as a chemically synthesized synthetic open reading frame (Syn-ORF) kindly provided by Tobias Schlapp, Bayer AG, Leverkusen). It consisted of 2694 nucleotides extending from nucleotide 890 to 3584 of the nucleotide sequence of BVDV strain NADL (Collett et al., 1988b) and was inserted into the pcDNA3.1 expression plasmid (Invitrogen) using restriction sites KpnI and NotI. The pcDNA3.1 plasmid allows the efficient expression of proteins under control of the HCMV immediate early promoter/enhancer. Stable cell lines can be selected with G418 (Gibco, Life Technologies) using the Neomycin/Geneticin resistance gene. The nucleotide sequence of Syn-ORF had been changed to remove splice sites (Schmitt et al., 1999), but retained the original amino acid sequence of ncp BVDV strain PT810 (Accession No. AY078406). Additionally, the first codon of Syn-ORF was changed to a metionine to
allow expression of the polyprotein under the control of the 
HCMV immediate-early promoter present in pcDNA3.1, and a stop codon was inserted behind the last codon. The resulting construct pCDNA_C-E2 (1 µg) was used to transfect PT cells with the SUPERFECT reagent (Qiagen). At 2 days after transfection, cell-culture medium was changed to DMEM supplemented with 10% bovine serum and 1 mg of G418 per milliliter. G418-resistant colonies were isolated, replated several times, and stained for ERNS and E2 expression using mabs WB210, WB215, and CA3.

Polymerase chain reaction (PCR) and sequencing

For PCR, a PTC-200 thermal cycler (MJ Research, Inc.) was used. DNA-based amplification was done by Expand High-Fidelity PCR System (Roche Molecular Biochemicals) according to the supplier’s protocol. Primers for generation of the respective constructs are summarized in Table 4. For RT-PCR, total RNA of virus-infected cells was extracted using the TRIZOL reagent (Gibco-Life Technologies). cDNA was amplified from the RNA template by using reverse transcriptase (AMV; Promega) and a sequence-specific primer. The synthesized cDNA was amplified with a thermostable Taq Polymerase (Promega) and PCR products were directly sequenced. RNA for RT-PCR experiments was treated with RNase-free DNases and the RT step was omitted to detect contamination with cDNA. Replication defective NCP7Δ3’ntrAatII served as a negative control.

Sequencing was carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences). Nucleotide sequences were read with a LI-COR automatic sequencer (MWG Biotech) and were analyzed using the Wisconsin software package version 9.1 (Genetics Computer Group, USA).

RNA analysis

Total RNA for Northern blot analysis was extracted from 5 × 10^6 electroporated cells by using TRIZOL (Gibco-Life Technologies). Five to ten micrograms of RNA were separated using a denaturing 1% agarose gel. RNA transfer, hybridization, and chemiluminescent detection were performed following standard supplier protocols (Roche Molecular Biochemicals). Using a DIG-labeled riboprobe, which is complementary to nucleotides 5153 to 5774 of the BVDV genome, the detection of positive-stranded replicon RNA or of BVDV NCP7 RNA (Accession No. U63479) was done. As a negative control, replication-defective RNA transcribed from NCP7Δ3’ntrAatII (Table 4) was transfected.

Plasmid constructs

Plasmids were amplified in Escherichia coli TOP10F’ cells (Invitrogen). Restriction enzyme digestion and cloning procedures were performed according to standard protocols. Plasmid DNA was purified by Qiagen Plasmid Mini, Midi, or Maxi kits. The primers used for PCR or sequencing (labeled with IRD 800) were custom synthesized (MWG-Biotech). Primer pairs for the construction of deletion mutants are given in Table 4. The full-length BVDV cDNA clone pA/BVDV/Ins− and the D9 cDNA construct pA/BVDV/D9 have been previously described (Meyers et al., 1996). Mutant BVDV clones shown in Fig. 1 were constructed on the basis of the full-length cDNA clone pA/BVDV/Ins−. Location of restriction sites are indicated by superscript numbers corresponding to their cleavage positions in the BVDV CP7 genome (Fig. 1, Table 4). The used KpnI restriction sites almost exactly span the NCP7 E2p7 region. However, nucleotides coding for one amino acid from E2 remained and a genomic region coding for the first six amino acids of NS2 was additionally deleted following KpnI digestion and ligation.

For construction of NCP7Δ2p7 and NCP7Δ2p7_GFP, plasmid pA/BVDV/Ins− was digested with KpnI and religated. The in-frame deletion comprised E2 and p7 sequences. To establish a GFP-expressing replicon, a PCR fragment of plasmid pEGFP-N1 (Clontech Laboratories) was amplified with appropriate primers GFP_KpnI and GFP_Pr_KpnI, digested with KpnI, and ligated in-frame into KpnI-digested plasmid NCP7Δ2p7 (Fig. 1, Table 4). The NCP7Δ2E2 and NCP7Δ2lum plasmids (lum: ER luminal region of E2, without the transmembrane region) were constructed as above, without the transmembrane domain.

Table 4

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Genomic region in BVDV CP7 (nucleotides)</th>
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<tr>
<td>GFP-KpnI</td>
<td>CAAGGTACCCTATGGAGGCAAGGCGAGGAG</td>
<td>– (+ sense)</td>
</tr>
<tr>
<td>GFPR-KpnI</td>
<td>TCTAGATTACCCTTTAGCACAGCTCGTCATGCCC</td>
<td>– (− sense)</td>
</tr>
<tr>
<td>p7</td>
<td>CAAGGTACCCTATGGAGGCAAGGCGAGGAG</td>
<td>3567–3586 (+ sense)</td>
</tr>
<tr>
<td>p7R</td>
<td>GCCTAGTTACCCTTGGGCA</td>
<td>3785–3804 (− sense)</td>
</tr>
<tr>
<td>tmr-E2</td>
<td>CAAGGTACCCTATGGAGGCAAGGCGAGGAG</td>
<td>3423–3442 (+ sense)</td>
</tr>
</tbody>
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*a Restriction enzyme sites are underlined; overlaps to facilitate restriction enzyme digestion are in italics, and additional nucleotides for in-frame ligation or generation of translation start codons are in bold.
nt 3423–3785) into the KpnI2447 site of plasmid NCP73E2p7 (Fig. 1, Table 4). For construction of NCP73Δ3ntrAatII plasmid pA/BVDV/Ins— was digested with AatII and Klenow DNA polymerase. After re-ligation, deletion of nucleotides 12,242 to 12,245 was confirmed by sequencing (Fig. 1). Further details of the plasmid construction, including single cloning steps, are available on request.

Complementation experiments

For trans-complementation experiments, in vitro transcribed BVDV replicon RNA was transfected into PT_805 cells by electroporation. Cell-culture supernatants were collected 24 to 72 h p. t., clarified by centrifugation (10,000 g, 5 min), and titrated using KOP-R cells. At the day of collection, replication of BVDV was monitored by IF staining.

Virus titers of the complemented viruses were determined as infectious units. Cell-culture supernatants were titrated in triplicate in log10 steps and 1 ml was inoculated onto KOP-R cells seeded in 24-well plates. After 12 h of incubation at 37°C, cells were washed, detached with a trypsin solution, and counted. An aliquot of the cells was stained by IF using a BVDV NS3-specific mab and analyzed by flow cytometry. Infectious units were calculated according to the formula:

\[
\text{Number of cells in the well} \times \% \text{ IF-positive cells} \\
\times \text{dilution factor} = \text{titer in IU/ml}
\]

For calculations, only wells with >5 and <30% IF-positive cells were considered.

Acknowledgments

We thank Doreen Reichelt and Gabriela Adam for excellent technical assistance and Tillmann Rümenapf (University of Giessen, Germany) for providing the capsid specific antiserum. We are also grateful to Nikolaus Osterrieder for helpful discussions and critical reading of the manuscript, and to Uwe Fischer for the technical support with the confocal microscope.

References