5-10-2009

A Replicon *Trans*-Packaging System Reveals the Requirement of Nonstructural Proteins for the Assembly of Bovine Viral Diarrhea Virus (BVDV) Virion

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Published in *Virology* **387**:2 (May 10, 2009), pp. 331–340; doi 10.1016/j.virol.2009.02.019

Submitted November 25, 2008; revised December 28, 2008; accepted February 7, 2009; published online March 26, 2009.

A replicon *trans*-packaging system reveals the requirement of nonstructural proteins for the assembly of bovine viral diarrhea virus (BVDV) virion

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Abstract

A selective *trans*-packaging system was developed to produce and isolate bovine viral diarrhea virus (BVDV) pseudo-particles with complementing reporter replicons and their packaging proteins expressed *in trans* with recombinant vaccinia virus. The encapsidation of replicon rNS3-5B was dependent not only on the *in trans* expression of structural proteins C, E^{rns}, E1 and E2, but also the nonstructural proteins, p7 and contiguous precursor NS2-3-4A. Nonstructural p7, NS4B, NS5A or NS5B could be expressed *in cis* and *in trans* with precursor NS2-3-4A without significantly affecting virion assembly efficiency. NS2-3-4A was identified as an *in trans* functional precursor in virion assembly. BVDV genomes with mutant NS5B, which did not undergo active replication, were packaged 5-fold less efficiently than the intact genomes demonstrating the importance of replication in virion packaging. These results suggest that genome replication and assembly are closely associated, consistent with a model in which these two steps are coupled for maximum efficiency.

Keywords: BVDV, pseudo-particles, *trans*-packaging, complementation, NS2-3-4A precursor, replication, virion assembly

Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of domestic livestock and wild ruminants. The virus is a member of the genus *Pestivirus*, which together with the *Flavivirus* and *Hepacivirus* genera constitute the family *Flaviviridae* (Van Regenmortel, 1999). The genome of BVDV is approximately 12.3 kb in length and consists of a single long open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (NTRs) (Collett *et al.*, 1988). Translation of the ORF is mediated by an internal ribosome entry site (IRES) (Poole *et al.*, 1995). The ORF is translated into a single large polyprotein which is co- and post-translationally processed into at least 11 mature structural and nonstructural (NS) viral proteins as follows: N^{pro}-capsid (C)-E^{rns}-E1-E2-p7-NS2-3-NS4A-NS4B-NS5A-NS5B (Collett *et al.*, 1988; Elbers *et al.*, 1996; Tautz *et al.*, 1997). N^{pro} is an autoprotease that cleaves at its own carboxyl-terminus to release the C protein (Rumenapf *et al.*, 1998). The cleavages of C, E^{rns}, E1, E2 and p7 are catalyzed by host cellular signal peptidases (Rumenapf *et al.*, 1993, 1998). The N-terminal, one-third of NS3 encodes a serine protease that functions in concert with cofactor NS4A to mediate processing of all downstream NS proteins (Collett *et al.*, 1988; Failla *et al.*, 1994). BVDV isolates are divided into noncytopathic (ncp) and cytopathic (cp) biotypes that are distinguished by their effect on cultured cells (Gillespie *et al.*, 1960). Expression of NS3 as a separate protein is a hallmark of cp-BVDV (Donis and Dubovi, 1987). NS3 is co-linear to the C-terminal portion of NS2-3 and its expression by cp-BVDV is the result of complex genetic changes (Meyers *et al.*, 1996). However, an almost complete cleavage of NS2-3 in ncp-BVDV during the early hours of infection has been reported recently, indicating that temporal modulation of NS2-3 processing is crucial for replication and pathogenicity (Lackner *et al.*, 2004).

Genetic analyses have shown that genome replication requires the five NS proteins, NS3, NS4A, NS4B, NS5A and NS5B, of which only NS5A is complementable *in trans* (Grassmann *et al.*, 2001; Moser *et al.*, 1999; Tautz *et al.*, 1999). The known relevant catalytic functions in this complex are the nucleotidyl transferase of NS5B (Lai *et al.*, 1999; Steffens *et al.*, 1999), as well as the ATPase and RNA helicase of NS3 (Grassmann *et al.*, 1999). The roles of NS4A, NS4B and NS5A in RNA replication are unknown (Qu *et al.*, 2001). Pestivirus virions are enveloped particles between 40–60 nm in diameter. They consist of viral genomic RNA, the C protein, the envelope glycoproteins E^{rns}, E1, and E2, as well as the lipid membrane envelope. Electron microscopy of infected cells has suggested that particles bud into the lumen of cytoplasmic vesicular structures (Ohm-

ann, 1990). The mechanism by which genomic RNA is incorporated into virions during budding remains unknown. The spatial relationships between the sites of RNA replication and virion assembly are not known and RNA packaging signals have not been identified in any of the *Flaviviridae* members. There is a growing body of evidence indicating that *Pestivirus* NS proteins participate in the assembly of infectious virions (Agapov *et al.*, 2004; Ansari *et al.*, 2004). However, it is not known how the NS proteins contribute to the assembly process. One possibility is that certain NS proteins participate in coupling the genome replication process to that of virion assembly. The results described in this study demonstrate a significant involvement of NS proteins in genome packaging and also suggest that genome packaging is more efficient when coupled to an active replicase complex.

Results

Development of an efficient BVDV virion packaging assay system

We recently reported the development and use of a bicistronic replicon derived from the cp-BVDV strain NADL that expresses firefly luciferase to facilitate detection of its biological activity within electroporated cells (Ansari *et al.*, 2004; Horscroft *et al.*, 2005; Vassilev and Donis, 2000). This replicon, termed rNS3-5B, expresses the minimal complement of NS proteins required for genome replication (Ansari *et al.*, 2004; Behrens *et al.*, 1998). To study the role of NS proteins in BVDV genome packaging into virions, we constructed an additional reporter replicon, termed rp7-NS5B (Figure 1A). The upstream cistron of this replicon expresses firefly luciferase and neomycin phosphotransferase, whereas the second cistron expresses the polyprotein encoding the complete set of NS proteins starting from p7 to NS5B, hence rp7-NS5B is approximately 1.5 kb longer than rNS3-5B. The expression of the second cistron is mediated by the encephalomyocarditis virus (ECMV) IRES. The RNA transcripts derived from both replicons resulted in the expression of increased levels of firefly luciferase following RNA electroporation into bovine cells. Luciferase activity was detected as early as 24 h post-electroporation and was maximal at 48 h (Figure 1B). Both replicons, rNS3-5B and rp7-NS5B, expressed equivalent levels of luciferase. In contrast, cells electroporated with rNS3-5Bgdd, showed only background levels of luciferase activity, indicating that the significant levels of luciferase activity observed after rNS3-5B or rp7-NS5B were dependent on the polymerase activity of NS5B to mediate RNA amplification (Figure 1B). More importantly, luciferase levels were found to correlate with the intracellular amounts of rep-

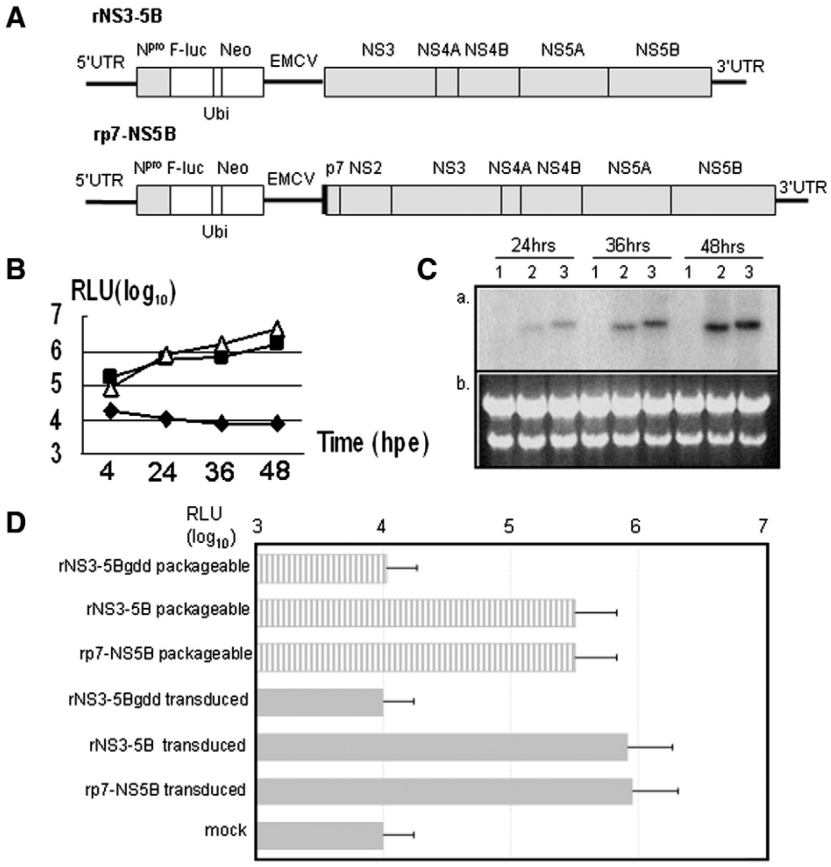

Figure 1. Replication and packaging of subgenomic BVDV replicons by helper virus. **(A)** Schematic diagram of bicistronic reporter BVDV replicons rNS3-5B and rp7-NS5B as indicated: F-luc = firefly luciferase, Ubi = ubiquitin, Neo = neomycin phosphotransferase and EMCV = internal ribosome entry site of EMCV. **(B)** Firefly luciferase activity from total cell lysates measured at the indicated hours post-electroporation (hpe) with RNA transcripts of rNS3-5B (filled square), rp7-NS5B (open triangle) and rNS3-5Bgdd (diamond), respectively. All values were normalized for electroporation efficiency by measuring the *Renilla* luciferase from a monocistronic RNA co-electroporated with the replicon RNA transcripts. Data represents one of the repeated experiments. **(C)** Northern blotting analysis to determine replication efficiency of rNS3-5B (lane 2), rp7-NS5B (lane 3) in NCL1 cells at the indicated hours (hrs) after electroporation with RNA transcripts. RNA derived from rNS3-5Bgdd served as a control (lane 1). Panel **a** shows replicon RNA in total cellular RNA extracted from 1×10^6 cells. Panel **b** is an image of the ethidium bromide stained agarose gel of total cellular RNA that was blotted and probed in panel a. **(D)** Transduction of luciferase expression to naïve (indicator) cells using supernatant collected from cells persistently infected by ncp-BVDV-2 helper virus for 36 h before electroporation with reporter BVDV replicon RNAs. Luciferase activities were tested 48 h post-RNA electroporation or infection with packaged/pseudotyped replicon RNAs. Packageable replicons refer to replicons recovered from source cells transfected with RNA transcripts and used for the transduction of indicator cells. Transduced replicons refer to replicons recovered from indicator cells following inoculation with packaged replicons.

licon RNA as indicated by northern blot hybridization (Figure 1C). The luciferase levels from the BVDV replicons were normalized for electroporation efficiency by quantification of a co-electroporated RNA encoding *Renilla* luciferase. To this end, a fraction of the electroporated cells was cultured separately in which *Renilla* luciferase activity was monitored at 4 h post-electroporation (data not shown). An excellent correlation was observed between normalized RNA levels as detected by northern blot analysis and normalized firefly luciferase expression at all time points analyzed (Figs. 1B, C). However, it appeared that the kinetics of RNA accumulation was slightly different between the two reporter replicons, with rNS3-5B RNA accumulation delayed relative to rp7-NS5B.

Previous studies have demonstrated that BVDV replicons can be packaged by infecting with helper BVDV virus, like the naturally-occurring pestivirus defective interfering (DI) particles that they emulate (Behrens *et al*., 1998; Meyers and Thiel, 1995). To ascertain if the introduction of foreign RNA elements into the BVDV subgenomic RNA might interfere with its encapsidation, we electroporated the reporter replicon RNAs into helper BVDV genotype 2 (BVDV-2) infected cells and analyzed the cell culture medium harvested 2 days after electroporation for its ability to transduce luciferase expression in bovine cells. The results revealed that both versions of reporter replicons were able to transduce luciferase expression in the indicator cell monolayer (Figure 1D) and there were no significant differences between the rNS3-5B and rp7-NS5B replicons in the release of virions into the culture fluids (Figure 1D). The levels of luciferase expression in the transduced cells were higher than in the electroporated cells. These results indicated that replicons rNS3-5B and rp7-NS5B retain the necessary *cis*-elements for packaging, although it is not known if BVDV structural proteins can exclude foreign RNA molecules.

A recent report indicated that encapsidation of the BVDV genome was strictly dependent on the presence of the NS2-3 protein (Agapov *et al*., 2004). The finding that the two reporter replicons dif-

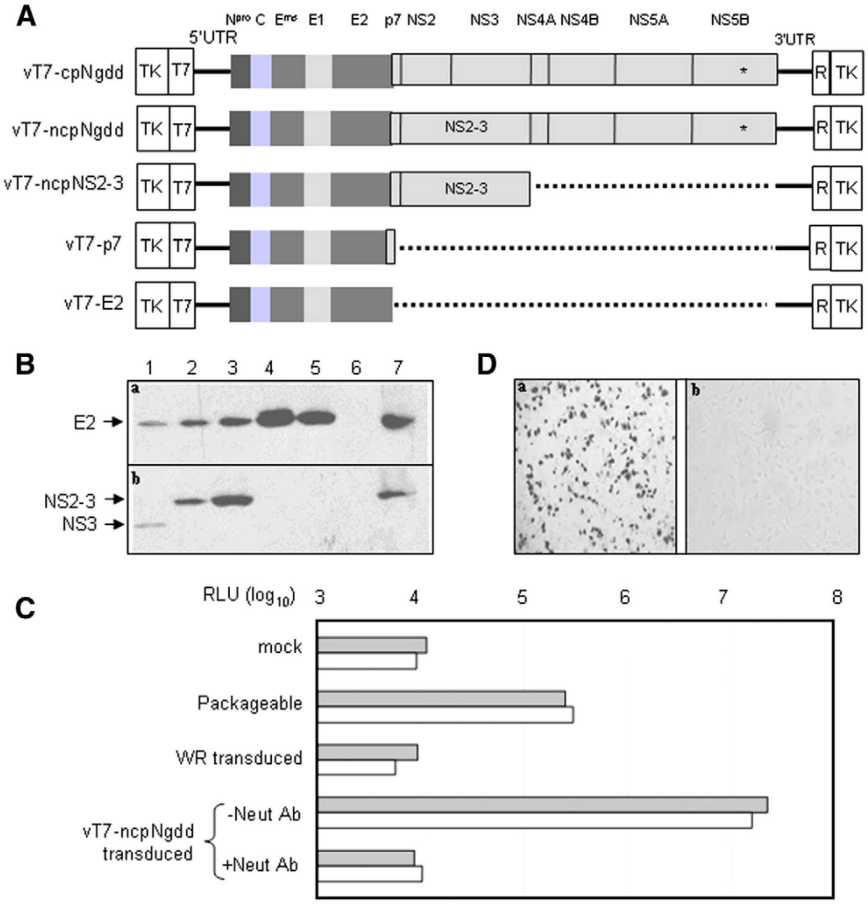

Figure 2. Packaging of BVDV replicons using recombinant vaccinia viruses expressing different lengths of BVDV proteins *in trans*. **(A)** Schematic representation of recombinant vaccinia virus genome expressing different lengths of BVDV proteins. The thymidine kinase (TK) gene of vaccinia virus WR was replaced with BVDV coding regions having truncations from the C-terminal end of the BVDV genome: T7 = T7 promoter, R = Ribozyme of hepatitis D virus. Asterisk (*) with NS5B indicates the mutation of GDD motif to ATR. **(B)** Western blot analysis of NCL1 cells infected with recombinant vaccinia virus expressing different length of BVDV proteins. Total cell lysate corresponding to 2×10^4 NCL1 cells was loaded into each lane of an 8% SDS-PAGE gel, electrophoresed, transferred to a membrane and protein detected using monoclonal antibodies against E2 (panel a) or NS3 (panel b). Lanes 1 to 5 represent NCL1 cells infected with recombinant vaccinia virus vT7-cpNgdd, vT7-ncpNgdd, vT7-ncpNS2-3, vT7-p7 and vT7-E2, respectively. Lane 6 is normal control cells while lane 7 represents NCL1 cells persistently-infected with ncp-BVDV. **(C)** *Trans*-packaging of BVDV replicons using recombinant vaccinia virus vT7-ncpNgdd. NCL1 cells were infected with wild type (WR) or vT7-ncpNgdd vaccinia viruses after 36 h post-RNA electroporation of the two replicons. The *trans*-packaged replicon pseudo-particles from the cells were filtered and applied onto naive cells. Luciferase activities were tested 48 h after electroporation or infection of replicons. White bars represent replicon rNS3-5B, while gray bars represent replicon rp7-NS5B. A fraction of the supernatant was incubated with 100 serum neutralizing units of BVDV neutralizing antibody for 30 min at room temperature prior to infection of naive NCL1 cells. Data represents one of the repeated experiments. **(D)** Semliki Forest virus replicons fail to be packaged by vaccinia virus vT7-ncpNADLgdd. NCL1 cells were electroporated with transcripts derived from SFV3-lacZ. Four hours post-RNA electroporation, cells were infected with vaccinia virus vT7-ncpNADLgdd and stained for expression of β-galactosidase after 8 h (panel a). The supernatant from these cells was collected 24 h post-RNA electroporation, passed through a 0.1 mm filter and applied to fresh NCL1 naive cells (indicator cells) and further analyzed for expression of β-galactosidase after 12 h (panel b).

fering in the expression of NS2-3 were efficiently incorporated into particles in the presence of helper ncp-BVDV infection prompted us to determine the minimum complement of viral proteins required for the incorporation of the subgenomic reporter replicon into virus particles. To this end, we engineered a helper vaccinia virus expression system that could generate either the entire wt BVDV genomic RNA and efficiently encode the BVDV polyprotein in infected cells or any selected mutants of interest (e.g. deletion mutants or mutants with BVDV polyprotein having a catalytically inactive NS5B) (Figure 2A). The engineered vaccinia viruses, vT7-NADL and vT7-cpNgdd, expressed the entire polyprotein of BVDV in infected cells (Figure 2B) and nearly 100% of the cells were positive by indirect immunofluorescence staining using an anti-E2 monoclonal antibody (Mab) (data not shown). Western blot analyses of vT7-cpNgdd infected cells revealed expression and correct processing of E2 and NS3 (Figure 2B). The vT7-NADL released infectious BVDV into the culture medium (data not shown).

To assess the efficiency of the complete set of BVDV proteins expressed by the vaccinia system in encapsidating BVDV reporter replicons into functional viral particles, we analyzed the expression of luciferase in indicator cells inoculated with cell culture medium from the packaging cells, the rNS3-5B or rp7-NS5B RNA electroporated cells following infection with the helper vT7-ncpNgdd recombinant vaccinia virus. The culture fluid from the packaging cells was passed through a 0.1 μm filter to remove the vaccinia virus and the filtered fluid inoculated onto a fresh monolayer of indicator cells to assess transduction of luciferase expression. Luciferase activity measured from the indicator cells revealed the delivery of the replicon rNS3-5B and rp7-NS5B to the new monolayer (Figure 2C). Delivery of the two packaged replicons have comparable efficiency. In contrast, wild type vaccinia virus WR could not *trans*-package the BVDV replicon, hence only background levels of luciferase activity were detected (Fig 2C). Furthermore the transduction of luciferase activity to the indicator cells was blocked by pre-incubation of the cell culture fluids with BVDV neutralizing Mabs against E2 (Figure 2C) indicating that the packaged replicon contained authentic BVDV E2 protein on the surface. Taken together, these results demonstrate that the replicon RNAs were encapsidated and transduced luciferase expression in the target cells. These encapsidated virions were also able to pass through a 0.1 μm filter with which vaccinia virus virions were effectively excluded.

BVDV virion packaging machinery is highly selective

Although the packaging of the reporter replicon by *trans*-complementing with recombinant BVDV-vaccinia appears to recapitulate the natural process of DI propagation, the mechanism responsible for packaging the RNA is still unknown. The efficiency of virus assembly would be enhanced by the selectivity of the genome packaging machinery. Such selectivity should be correlated with the failure to encapsidate a non-cognate viral RNA genome that is present in abundant quantities within the cytoplasm of the packaging cells.

To demonstrate the selectivity of *Pestivirus* genome encapsidation we chose to exploit an alphavirus replicon system due to the similarities between Togaviruses and Flaviviruses in terms of RNA replication and assembly (Strauss and Strauss, 1994). We used a Semliki Forest virus (SFV) alphavirus replicon (SFV3-lacZ, Invitrogen, Carlsbad, CA) that accumulates to high levels in electroporated cells (Liljestrom and Garoff, 1991). This replicon encodes the SFV NS polyprotein from the genomic promoter, as well as the *Escherichia coli* β-galactosidase (β-gal) reporter protein from a subgenomic promoter (Liljestrom and Garoff, 1991). Preliminary studies showed that a SFV replicon replicated efficiently in several bovine cells after electroporation (data not shown). The SFV3-lacZ RNA transcripts

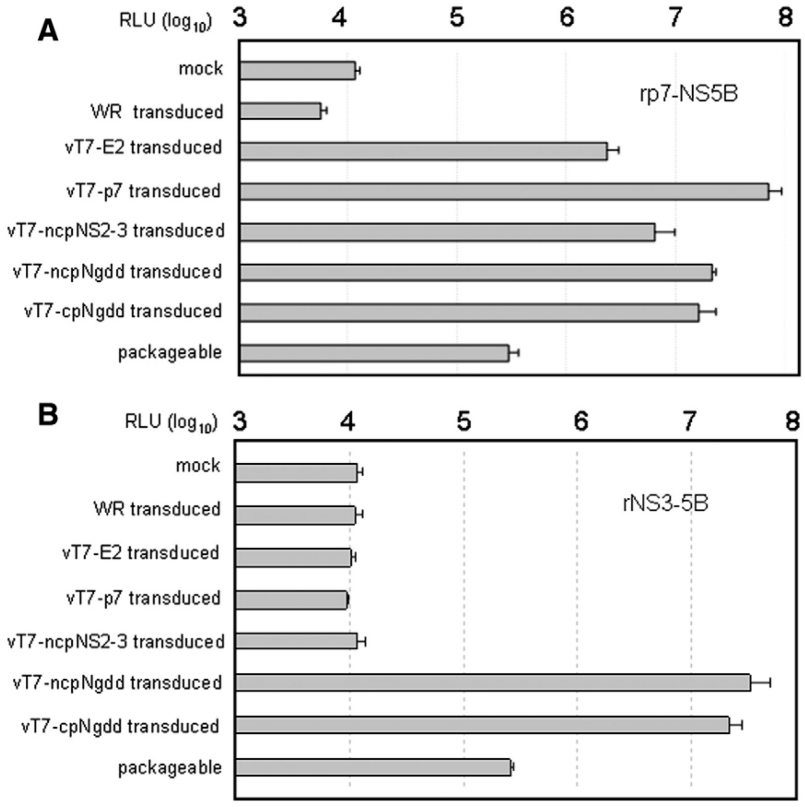

Figure 3. Requirement of different sets of BVDV NS proteins to package rp7-NS5B and rNS3-5B replicons. NCL1 cells were infected with different recombinant vaccinia virus 36 h after being electroporated with replicons **(A)** rp7-NS5B or **(B)** rNS3-5B. The *trans*-packaged replicon pseudoparticles from RNA electroporated cells were filtered and applied to naive cells. Luciferase activities were measured 48 h after electroporation or infection of replicons.

electroporated into bovine cells and infected with helper vT7-ncp-NADLgdd or BVDV-2 directed the expression of high levels of β-galactosidase in the packaging cells (Figure 2D, panel a). However, no β-galactosidase could be detected in the indicator cells inoculated with the cell culture filtrates from RNA electroporated cells (Figure 2D, panel b). These results indicated that the pestivirus packaging machinery has a strong selection for the RNA genome, although the packaging signals at the RNA level have not yet been defined.

Segregation of BVDV polyprotein expression between NS3 and NS4A leads to the failure of virion packaging

The results presented above validated the use of the recombinant vaccinia helper system to encapsidate and transduce BVDV replicons. Previous studies had indicated that NS2, NS3, NS4A and NS5B played a role in the assembly of infectious virions (Agapov *et al*., 2004; Ansari *et al*., 2004). To determine the minimum complement of viral proteins required *in trans* to package the rp7-NS5B RNA (expressing all the NS proteins) into infectious particles, we engineered a vaccinia virus vT7-E2 which expresses the BVDV structural proteins, C, E^{rns}, E1 and E2, with the autoprotease, N^{pro}, at the N-terminus of C to conserve the normal biogenesis of its N-terminus (Figure 2A). The N^{pro} is neither a component of virions, nor is it required for pestivirus virion assembly (Lai *et al*., 1999). Preliminary studies showed that vaccinia recombinants expressed and processed both E2 and NS2-3/NS3 as expected, due to the presence of signal sequences and the Jiv90 element, respectively (Lackner *et al*., 2006).

Our results indicate that vT7-E2 helper transduced replicon rp7-NS5B (luciferase) to target cells (Figure 3A). However, the efficiency of this process was significantly lower compared to that of the vT7-ncpNADLgdd or vT7-cpNADLgdd helper packaged, suggesting that some of the BVDV NS proteins contributed to efficient virion assembly. Therefore, we constructed and evaluated another vaccinia BVDV recombinant that expressed the structural proteins and p7 (vT7-p7). This helper vaccinia-BVDV recombinant yielded equivalent *trans*-packaging efficiency as the complete polyprotein from vT7-ncpNADLgdd or vT7-cpNADLgdd (Figure 3A). These results indicated that only the structural proteins are required *in trans* to package cognate replicons expressing all the NS proteins. The efficiency of the process is greatly enhanced by the addition of p7 to the polyprotein that gives rise to the structural proteins, suggesting a possible interaction between E2 and p7 (role for the precursor E2-p7) in replicon packaging, and by extension, in the assembly of progeny virions during BVDV infections.

In contrast with the rp7-NS5B replicon, the rNS3-5B failed to be packaged by the structural proteins expressed under identical conditions (Figure 3B). Furthermore, rNS3-5B failed to be packaged by helper expression of additional NS proteins, p7 and NS2-NS3 (Figure 3B). The functionality of the complementing vT7-ncpNS2-3 recombinant vaccinia was demonstrated by packaging of the rp7-NS5B, suggesting that failure to package is not due to undetected mutations within structural genes. Furthermore, a different version of recombinant vaccinia virus, vT7-cpNS3, expressing polyprotein N^{pro}-NS3 derived from cp-BVDV strain NADL, also failed to package

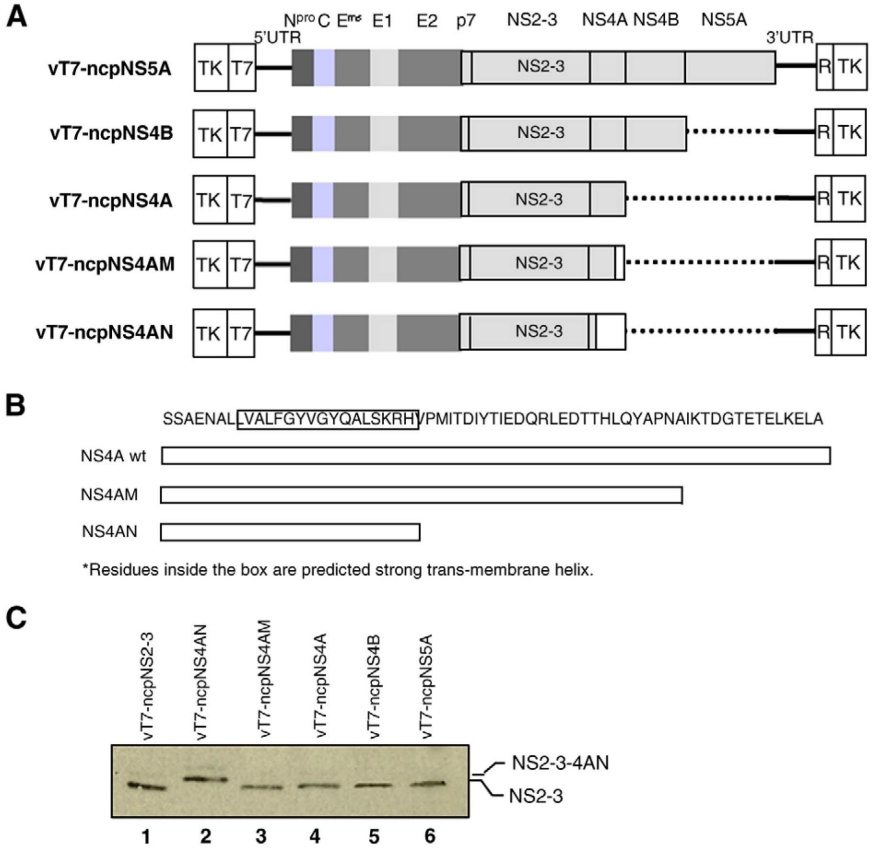

Figure 4. Characterization of recombinant vaccinia viruses expressing truncated ncp-BVDV polyproteins and its utilization for packaging of rNS3-5B and rp7-NS5B replicons. **(A)** Schematic representation of recombinant vaccinia virus genome expressing ncp-BVDV polyproteins. The thymidine kinase (TK) gene of vaccinia virus WR was replaced with different sizes of truncated BVDV polyprotein, respectively. T7 = T7 promoter, R = Ribozyme of hepatitis D virus. **(B)** NS4A amino acid sequences included in vaccinia virus vT7-NS4A, vT7-NS4AM (retained middle domain but deleted C-terminal) and vT7-NS4AN (retained only N-terminal). **(C)** The expression and processing of NS3 in cells infected with these recombinant vaccinia viruses. Cell lysate corresponding to 2×10^4 NCL1 cells were loaded in each lane of 8% SDS-PAGE and detection of NS3 was performed by western blotting using a primary monoclonal antibody against NS3. Note NS4AN was not processed from NS2-3 in the vT7-ncpNS4AN vaccinia construct.

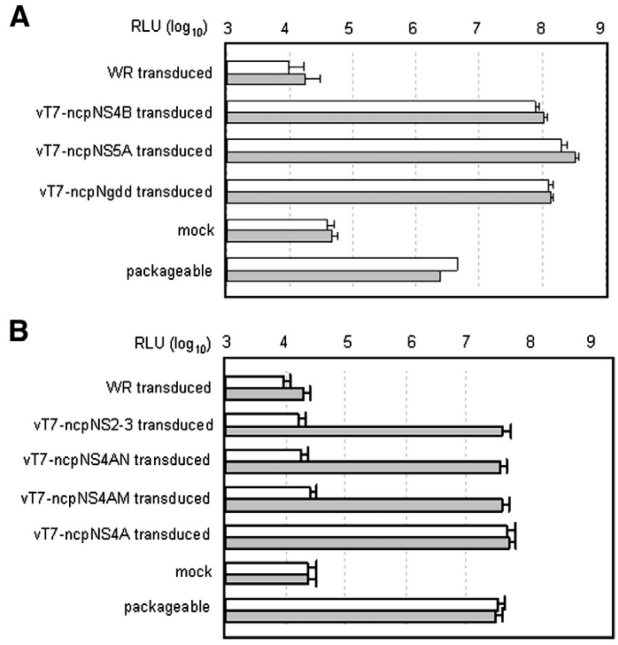

Figure 5. Mapping of minimum sets of nonstructural polyproteins required for *trans*-packaging of rNS3-5B. NCL1 cells were infected with different recombinant vaccinia viruses 36 h after being electroporated with replicon rNS3-5B or rp7-NS5B. The *trans*-packaged replicon pseudo-particles from the packaging cells were filtered and passed to naive cells. Luciferase activities in packaging cells and indicator cells were measured 48 h after electroporation or infection of replicons. White bars represent replicon rNS3-5B, while gray bars represent replicon rp7-NS5B. **(A)** Mapping of the nonstructural polyproteins required for *trans*-packaging of rNS3-5B and rp7-NS5B replicon using vT7-ncpNS4B, vT7-ncpNS5A and vT7-ncpNS5B *in trans*. **(B)** Mapping of the nonstructural polyproteins required for *trans*-packaging of rNS3-5B and rp7-NS5B replicon using vT7-ncpNS2-3, vT7-ncpNS4AN, vT7-ncpNS4AM and vT7-ncpNS4A *in trans*.

rNS3-5B (data not shown). Taken together, these results suggest that the polyprotein downstream of NS3 is involved in efficient packaging of rNS3-5B.

It was surprising that vT7-cpNS3 could not *trans*-complement rNS3-5B for packaging. It is worth noting that the cells harboring the rNS3-5B replicon, expressed precursor NS3-NS4A-NS4B-NS5A-NS5B that could be processed into functionally mature proteins. Similarly, recombinant vaccinia virus, vT7-cpNS3, expressed precursor N^{pro}-E^{rns}-E1-E2-p7-NS2-3 that could be processed by host cellular signal peptidase. The two precursors overlap in the NS3 region, therefore, the cells harboring both rNS3-5B and vT7-cpNS3 expressed two truncated polyproteins that covered the entire BVDV genome. We demonstrated that efficient *trans*-packaging of rNS3-5B could be achieved by co-infection with recombinant vaccinia virus vT7NADLgdd, which expresses the entire BVDV polyprotein with the GDD motif in NS5B mutated to null the replication activity (Figure 3B). Thus, the failure of rNS3-5B to be packaged by vT7-cpNS3 indicated that contiguous expression of nonstructural proteins NS2-3 with its downstream polyproteins is essential for the efficient incorporation of rNS3-5B into functional virus particles.

Contiguous expression of NS2-3-4A is essential for efficient virion packaging

To identify the critical NS proteins responsible for the failure to incorporate rNS3-5B genomes into virion particles, we analyzed their packaging using a recombinant vaccinia virus that expressed the structural proteins with the NS protein segment, p7-NS4A or p7-NS5A, as a single polyprotein derived from ncp-BVDV (Figure 4A). Western blotting showed that all the vaccinia recombinants expressed and processed E2 and NS2-3 proteins correctly (Figure 4C, lanes 4, 5, 6). Recombinants vT7-NS4B, vT7-NS5A and vT7-NS4A packaged the rNS3-5B replicon with equivalent efficiency to that of rp7-NS5B, respectively (Figs. 5A, B). These results indicate that all of the proteins upstream of, and including NS4A could be provided *in trans* for efficient virion packaging. However, we have demonstrated that rNS3-5B could not be efficiently *trans*-packaged by recombinant vaccinia virus expressing polyprotein N^{pro}-NS3. The packaging efficiency of the rNS3-5B replicon by complementation with vT7-NS4A was comparable with vT7-NS4B, vT7-NS5A and vT7-Ngdd (Figs. 5A, B). These results suggest that the sequence context downstream of NS4A is not essential for NS2-3-4A processing or complex formation. However, we could not exclude the possibility of involvement of the polyprotein, NS4B-5B region, in virion packaging (Ansari *et al.*, 2004).

The C-tail of NS4A is indispensable for efficient virion packaging

The most significant role of NS4A is to function as a cofactor in the NS3-mediated cleavage of the viral polyproteins (Tautz *et al.*, 2000; Xu *et al.*, 1997). Because the central domain of NS4A is critical for this function, we engineered a C-terminal deletion NS4A mutant, vT7-NS4AM, and another mutant lacking both the C-terminal domain and the middle domain of NS4A, vT7-NS4AN. The hydrophobic N-terminus of NS4A is predicted to adopt a helix conformation, which is likely to insert itself into membranes (Figure 4B). Western blotting results showed that NS2-3 was processed efficiently from its NS4AM precursor (Figure 4C, lane 3). It is important to note that the NS2-3-4AN precursor expressed from vT7-NS4AN was larger than the normal NS2-3 molecule by ~ 2.2 kDa, indicating that the N-terminal domain of NS4A molecule was retained with NS2-3 precursor (Figure 4C, lane 2). Both replicons, rNS3-5B and rp7-NS5B, were efficiently packaged by the recombinant vaccinia virus, vT7-ncpNS4A (Figure 5B). However, none of the vaccinia virus recombinant NS4A deletion mutants, vT7-NS4AN and vT7-NS4AM, were able to complement packaging of the rNS3-5B replicon (Fig 5B). A summary of the packaging of the two replicons with different recombinant vaccinia virus mutants is given in Table 1. These findings suggest that the C-tail of NS4A is indispensable for efficient virion packaging.

The close link between BVDV virion packaging and replication machinery

Our results correlate with recent findings on the involvement of several NS proteins in the packaging of genomes generated by an active viral replicase complex (Agapov *et al.*, 2004; Ansari *et al.*, 2004). Furthermore, Agapov *et al.*, 2004 demonstrated the importance of the NS2-3 precursor in the production of infectious virions, although NS2 was dispensable for genome replication (Agapov *et al.*, 2004). Taken together with the results presented above, the role of NS2 and NS2-3 precursors may involve the engagement of the replicase

Table 1. Summary of BVDV replicon *trans*-packaging results with different recombinant vaccinia viruses expressing different lengths of BVDV polyproteins.

	rp7-NS5B	rNS3-5B
vT7-E2	+	−
vT7-p7	+	−
vT7-ncpNS2-3	+	−
vT7-ncpNS4AN	+	−
vT7-ncpNS4AM	+	−
vT7-ncpNS4A	+	+
vT7-ncpNS4B	+	+
vT7-ncpNS5A	+	+
vT7-ncpNgdd	+	+
vT7-cpNgdd	+	+

Replicon rp7-NS5B contains BVDV polyprotein from p7 to NS5B, while rNS3-5B contains polyprotein from NS3 to NS5B. Recombinant vaccinia viruses express BVDV polyproteins from N^{pro} to E2, p7, NS3, NS4A N-terminus, NS4A middle domain, NS4B, NS5A, NS5B, respectively. +: packageable; −: unpackageable.

complex by the structural proteins. To examine the recognition of the replication machinery by the structural proteins, we analyzed the production and release of virions by recombinant vaccinia virus carrying the WT BVDV genome and the BVDV genome having an inactive NS5B. Cells were infected with these recombinant vaccinia viruses and culture supernatants collected at 16 h post-infection. Total cell lysates and culture supernatants were analyzed by western blot analysis to assess the levels of BVDV E2 protein expression in infected cells and supernatant which will indicate the virion released into the media. Fractions that co-sedimented with standard BVDV infectious virions (density 1.12 to 1.14) were found to contain detectable E2 (Figure 6, lane 6) and to be infectious to bovine cells (data not shown).

The relative amounts of E2 present in the culture supernatant of cells infected with vaccinia virus recombinants expressing wt NS5B or the catalytically inactive NS5Bgdd were determined by densitometry and normalized by the relative amounts of total E2 expressed in the infected cells. Vaccinia-derived BVDV genomes with inactive NS5B but otherwise normal polyprotein, released only 20% of the virions compared to the cells infected with vT7-cpNADL (Fig 6, lanes 5 and 6). Furthermore, the virion produced by vT7-cpNgdd were noninfectious in bovine cells (data not shown). On the other hand the amount of expressed E2 in vT7-Ngdd infected cells (lane 2) was slightly greater than the vT7-cpNADL (lane 3). This difference may be due to the fact that in vT7-cpNADL infected cells, part of the E2 is used for authentic virion production, while in vT7-cpNgdd infected cells most of the E2 is retained inside the cells. It was also evident that viral genome replication in vT7-cpNADL infection did not lead to increased production of E2 inside the cells (lane 3) as compared to vT7-cpNgdd (lane 2) due to the production of high levels of transcripts generated by vaccinia infection. Hence, the expressed protein from viral replication is masked by expression products derived from vaccinia transcripts. These results strongly suggest that active genome replication

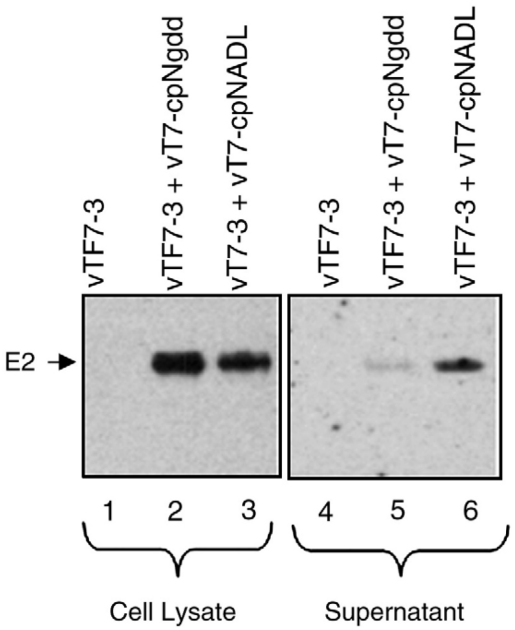

Figure 6. Lack of genome replication is correlated with reduced efficiency of virion assembly and release. RK-13 cells were infected with both T7 helper vaccinia virus vTF7-3 and vT7-cpNADL or vT7-cpNgdd, respectively. The supernatant which contains both rVV and BVDV virions was filtered through 0.1 mm filter and centrifuged to pellet the virions. The infected cell lysates and virion pellets were resolved on 8% SDS-PAGE and western blot analysis was performed using a monoclonal antibody against BVDV E2. Lanes 1–3 represent cell lysates from the infected cells with vTF7-3, vTF7-3 + vT7-cpNgdd and vTF7-3 + vT7-cpNADL, respectively, while lanes 4–6 indicate the supernatant from their corresponding infected cells.

enhances the production and/or release of virions and are also consistent with the previous report where involvement of the NS5B in genome packaging is demonstrated (Ansari *et al.*, 2004).

Discussion

We have developed an efficient BVDV reporter replicon packaging system, in which both the minimum replicon, rNS3-5B, and the long replicon, rp7-NS5B, replicated and assembled with comparable efficiency. We employed a recombinant vaccinia virus system to express the BVDV packaging proteins rather than a BVDV helper virus or dual replicon approach (Agapov *et al.*, 2004). The pure progeny BVDV virions can be easily separated from recombinant vaccinia virions by filtration. The efficiency of the *trans*-packaging system using recombinant vaccinia virus expressing polyprotein from N^{pro} to NS4A is comparable to that using a helper BVDV virus, which occurs in nature. Furthermore, the introduction of recombinant vaccinia virus during the late stages of BVDV replicon replication minimizes viral recombination opportunity. Our unpublished data has shown that a chimeric BVDV genome up to 15 kb in length could be efficiently assembled into infectious virions. Therefore, the relatively large cloning capacity of the replicons accommodates different purposes. The desired heterologous genes could be effectively cloned into the firefly luciferase or neomycin phosphotransferase gene position. Thus, this replicon *trans*-packaging system can serve as a gene delivery system at the cellular or animal level.

Consistent with our results, Agapov *et al.* (2004) reported that uncleaved NS2-3 was required for the production of infectious BVDV. Both, the system used by Agapov *et al.* (2004) and our system demonstrated the importance of the expression of NS4A *in cis* with NS2-3 protein. In contrast to our results, Agapov *et al.* (2004) determined that the expression of NS4A *in cis* with NS2-3 was not essential for, but markedly increased the packaging efficiency. This difference may be due to the different packaging systems used in the studies. Agapov *et al.* (2004) used a second BVDV replicon to express the functional NS2-3-4A *in trans*, while we used a recombinant vaccinia virus to express the functional NS2-3-4A *in trans*. This may result in different protein localization and different packaging efficiencies.

High levels of NS3 are expressed by cp-BVDV which is well correlated with an enhanced accumulation of intracellular viral RNA as compared to ncp-BVDV strains (Lackner *et al.*, 2004). However, virion production from cp-BVDV is not significantly different than that from ncp-BVDV in tissue culture. This indicated that a greater level of viral genome and packaging proteins were not enough for increased virion production. It has been demonstrated that NS2-3 was involved in virion assembly. However, it was surprising that expression of NS2-3-4A, but not NS2-3, could complement replicon assembly in our packaging system, suggesting that the NS2-3-4A precursor was involved in virion assembly. We also observed that extending NS2-3-4A to NS2-3-4A-NS4B or NS2-3-4A-NS4B-NS5A did not increase the packaging efficiency of rNS3-5B. This demonstrated that the region downstream of NS4A could be separated from NS2-3-4A for assembly function.

Thus, the contiguous expression of N^{pro}-E^{rns}-E1-E2-p7-NS2-3 with NS4A contributes to the successful *trans*-packaging of rNS3-5B. A previous report suggested that p7 could be separated from E2 and NS2 with efficient virion packaging (Harada *et al.*, 2000). Therefore, our results indicate that the contiguous expression of NS2-3-4A is essential for efficient *trans*-packaging of rNS3-5B. Based on the information that NS3 cannot be functionally replaced by uncleaved NS2-3, as well as, the strict correlation between NS3 level and efficiency of RNA replication (Lackner *et al.*, 2004), this strongly suggested that NS3, but not NS2-3, is an essential component of the viral replication complex. Thus, we propose that precursor NS2-3-4A, provided by the recombinant vaccinia viruses, was involved in efficient virion *trans*-packaging but not virus replication.

Having shown the importance of the NS2-3-4A precursor protein, we suggest that cp-BVDV also employs this precursor for the virion assembly function. Thus, pestiviruses exploit polyprotein precursors

for different viral functions. The temporal regulation of NS2-3-4A autoprocessing is likely necessary for the switch between the phases of highly active viral RNA replication and virus morphogenesis. The processing speed, order and ratio of the polyprotein precursor may regulate different stages of the BVDV life cycle, i.e. translation, replication and assembly. Although we did not provide evidence for a direct role of the NS proteins in the recruitment of actively replicating genomic RNA to assembly sites, the involvement of the NS proteins in virion assembly provides an obvious candidate.

Materials and methods

Viruses, cells and reagents

Rabbit kidney (RK13, CCL-37), monkey kidney (CV1, CCL-70), and thymidine kinase-deficient human osteosarcoma (TK-143B, CRL-8303) cells were obtained from the American Type Culture Collection (Manassas, VA). Bovine uterine cells (NCL1) and monoclonal antibodies against BVDV proteins were gifts from Dr. Edward Dubovi (Cornell University, Ithaca, NY). BVDV genotype 1 (BVDV-1) isolate NADL (cytopathogenic) and vaccinia virus strain WR were obtained from the American Type Culture Collection. BVDV genotype 2 (BVDV-2) isolate 890 was a gift from Dr. Julia Ridpath (USDA, Ames, IA). Recombinant vaccinia virus producing the T7 RNA polymerase (vTF7-3) was a gift from Dr. Bernard Moss (NIH, Bethesda, MD). DNA restriction/modification enzymes and luciferase assay systems were purchased from Promega (Madison, WI). Fetal bovine serum (FBS), γ-irradiated FBS (γ-FBS), equine serum, antibiotics for tissue culture (penicillin and streptomycin premix), minimum essential medium (MEM), Dulbecco's MEM (DMEM) and oligonucleotides were purchased from Invitrogen (Carlsbad, CA). The NCL1 cells were grown in MEM supplemented with penicillin–streptomycin and 10% equine serum, while CV1 and TK-143B cells were grown in DMEM supplemented with penicillin and 10% FBS. All cells were maintained at 37 °C with 5% CO_2. Plasmids encoding the full-length BVDV NADL genome and its derivatives lacking the Jiv cellular insert that mediates cytopathogenicity (Rinck *et al.*, 2001) were described previously (Vassilev *et al.*, 1997). A plasmid that allows transcription of RNA molecules encoding *Renilla* luciferase with expression driven by the encephalomyocarditis virus (EMCV) IRES was described previously (Ansari *et al.*, 2004).

Construction of bicistronic subgenomic BVDV reporter replicons

Bicistronic reporter replicons, rNS3-5B and rNS3-5Bgdd, were derived from the pNADLp15 full-length infectious genome of the BVDV strain NADL described previously (Ansari *et al.*, 2004; Vassilev *et al.*, 1997). Compared to rNS3-5B, rNS3-5Bgdd has a disabled NS5B by virtue of replacing the conserved GDD motif (amino acid residues 447 to 449, using NS5B numbering) with ATR (Ansari *et al.*, 2004). Another replicon, rp7-NS5B, was constructed as described for rNS3-5B, with the EMCV IRES inserted upstream of p7 rather than NS3. The junction between N^{pro} and p7 consisted of a stretch of nucleotides encoding for short amino acid segments of the N- and C-termini of capsid (C) protein, to mediate the correct membrane targeting of p7 and its subsequent processing. To this end, the central domain of the capsid (nucleotides 909–1079, encoding amino acids 175–232 of the wt virus polyprotein) (Genbank accession AJ133738) was deleted by overlap extension PCR.

Construction of recombinant vaccinia viruses expressing BVDV viral proteins

Construction of recombinant vaccinia viruses by homologous recombination between a transfer plasmid carrying BVDV sequences and the vaccinia virus WR genome was performed as previously described (Ausubel *et al.*, 1989). The cloning sites in the vaccinia transfer plasmid were flanked by the vaccinia virus DNA sequences, TK_L (left) and TK_R (right). Different sizes of the BVDV truncated polyproteins were first cloned into the vaccinia transfer vector, pSC59 (Ausubel *et al.*, 1989). The transfer vectors were engineered to contain a T7 promoter with a transcription start site at nucleotide 1 of the BVDV genome that contained either full-length BVDV genome cDNA or deletions in the 3′ end of the open reading frame to generate C-terminal truncated BVDV polyproteins. All constructs contained an intact BVDV 3′ UTR immediately followed by the hepatitis D virus ribozyme to yield a genuine BVDV 3′ end.

Vaccinia recombinants were generated in CV1 cells by transfecting with the transfer plasmid DNA followed by infection with vaccinia virus WR. Selection of recombinant vaccinia viruses lacking functional TK was performed by passage in TK^-143B cells in the presence of 5-bromodeoxyuridine (BrdU). Candidate recombinant viruses were co-infected with vTF7-3 helper virus followed by the detection of BVDV E2 protein expression in infected CV1 cells using indirect immunofluorescence microscopy and a specific mouse monoclonal antibody against E2. The plaques expressing BVDV E2 were purified three times and the expression and proper processing of all the structural and NS proteins was confirmed by western blotting. The resulting recombinant vaccinia viruses (rVV) were termed vT7-cpNADL, which expresses the full-length BVDV genomic RNA (from the cpNADL strain) and vT7-cpNgdd, which expresses the full-length BVDV genomic RNA of cpNADL with the GDD motif of NS5B mutated to ATR. Analogous vaccinia virus recombinants, termed vT7-ncpNADL and vT7-ncpNgdd encode BVDV genomes that differ from the previous ones in their NS2 protein lacking the insert of bovine origin. Additional rVV were generated to express truncated versions of the BVDV polyprotein namely: vT7-E2, vT7-p7, vT7-ncpNS2-3, vT7-ncpNS4AN, vT7-ncpNS4AM, vT7-ncpNS4A, vT7-ncpNS4B, and vT7-ncpNS5A. These viruses express the ncp-BVDV NADL strain polyprotein spanning from N^{pro} to E2, p7, NS3, NS4A N-terminus, NS4A middle domain, NS4A, NS4B and NS5A, respectively.

In vitro RNA transcription and electroporation

DNA templates for *in vitro* transcription were produced by PCR amplification of the region containing the T7 promoter and the entire replicon of the relevant plasmids using Klentaq polymerase (Clontech BD Biosciences, Palo Alto, CA). *In vitro* RNA transcription was performed using the T7-MEGAscript kit (Ambion, Austin, TX) as described previously (Ansari *et al.*, 2004). The pIRES-*Renilla* plasmid was linearized with XbaI enzyme and used for RNA transcription as described previously (Ansari *et al.*, 2004). At all times the electroporation efficiency of the replicon RNA was monitored by co-electroporation of a reporter RNA encoding *Renilla* luciferase in which the expression was driven by the EMCV IRES. Corrections for electroporation efficiency were not necessary due to the repeatedly consistent levels of *Renilla* luciferase expression (10^5 RLU) in each experiment.

RNA transcripts from a Semliki Forest Virus replicon, encoding β-galactosidase (pSFV3-LacZ) (Invitrogen, Carlsbad, CA), were prepared using plasmid DNA linearized with SpeI. Capped RNA transcripts were synthesized *in vitro* with SP6-polymerase in the presence of $m^7G(5')ppp(5')G$ cap analog by using the mMessage mMachine kit (Ambion, Austin, TX). RNA concentration following transcription reactions was determined fluorometrically using Ribogreen (Molecular Probes, Eugene, OR). An aliquot of the transcription reaction was analyzed by formaldehyde agarose gel electrophoresis to determine the integrity of the transcripts. Transcription reactions were stored at − 80 °C in small aliquots for electroporation purposes.

NCL1 cells (70 to 80% confluent) were trypsinized, washed two times with ice-cold DMEM and resuspended at a concentration of 2×10^7 cells/ml in cytomix as described previously (Ansari *et al.*, 2004). Briefly, transcripts (2 μg) were electroporated into 2×10^6

NCL1 cells in a 100 µl volume using a 0.2 cm gap cuvette with a single electrical pulse discharge of 235 V, 100 µF and 48 Ohms. Replication of the electroporated RNA was quantified by northern blot analysis of cells harvested at various times post-electroporation as described previously (Ansari *et al.*, 2004). Electroporation experiments were repeated a minimum of three times, starting from independent *in vitro* RNA synthesis reactions.

Reporter assays

Firefly and *Renilla* Luciferase assays were performed using the Dual Luciferase Assay system (Promega, Madison, WI) following the manufacturer's protocol. Briefly, the NCL1 cells were harvested and lysed in lysis buffer. An equivalent of 1×10^5 cells was used for the measurement of luciferase enzyme activity. Light emission was measured using a TopCount Luminescence Counter (Packard Instruments, Meriden, CT). To detect expression of β-galactosidase in transfected cells, monolayers were fixed with 0.2% paraformaldehyde in PBS for 5 min at room temperature. After 3 washes in PBS, the cells were incubated in a chromogenic substrate solution containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-13-d-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS. The appearance of blue color in the cell monolayers was monitored following incubation at 37 °C. Images were captured using wide-field digital photomicroscopy at 6-hour intervals for a period of 24 h.

Infections with recombinant vaccinia viruses, BVDV or packaged replicons

Bovine cell monolayers were rinsed with PBS and inoculated with cell culture fluids containing vaccinia recombinants, BVDV or packaged replicon virions in a volume sufficient to cover the entire cell monolayer. After adsorption for 1 h at 37 °C the supernatant was removed, monolayer rinsed and supplemented with prewarmed complete media and incubated in a 37 °C incubator until further analysis.

Western blotting

NCL1 cells (~ 80% confluency) were infected with each recombinant vaccinia virus (multiplicity of infection, MOI = 5) together with T7 helper vaccinia virus (MOI = 3) to achieve a greater level of expression. Total cell lysates were collected 12 h after vaccinia infection while ncp-BVDV or cp-BVDV-infected cells were harvested at 24 h post-infection. Cell lysate, the equivalent of ~ 2×10^4 cells, was loaded into each well of an 8% SDS-PAGE gel. Proteins were separated and electrotransferred to nitrocellulose membranes (Hybond-C, Amersham, Arlington Heights, IL). Proteins were detected using monoclonal antibodies against E2 (Antibody #4b6) or NS3. Antibody binding was detected using a chemiluminescent assay as described before (Ansari *et al.*, 2004).

Trans-packaging of BVDV replicons following infection with either recombinant vaccinia viruses expressing BVDV proteins or with BVDV-2

Approximately 2 µg of replicon RNA transcript and 0.4 µg of *Renilla* reporter RNA were mixed and electroporated into 2×10^6 NCL1 cells. Aliquots of the transfected cells, termed the packaging cells, were evenly distributed into ten fractions and plated in a 12-well-plate. One well was harvested for luciferase activity at 48 h post-electroporation to quantify the levels of packageable replicon RNA in the electroporated cells. The remaining nine wells were used for infection with recombinant vaccinia viruses expressing BVDV proteins or with BVDV-2 at 36 h post-electroporation. A system that employs a dual vaccinia T7 promoter–T7 RNA polymerase co-infection was used to express BVDV RNA (Fuerst *et al.*, 1987). To this end, cells previously electroporated with replicon RNA were co-infected with the recombinant vaccinia viruses expressing BVDV under T7 promoter control (MOI = 5) and vTF7-3 recombinant vaccinia virus constitutively expressing T7 RNA polymerase (MOI = 1). The cells were incubated for an additional 24 h after which the medium containing packaged replicons was harvested, passed through a 0.1 µm filter, reducing the helper vaccinia virus titer to undetectable levels and stored at 4 °C for further use. To assess the presence of packaged replicons, the culture filtrates (0.5 ml) were used to inoculate fresh NCL1 cells in duplicate (termed indicator cells) in 12-well plates. The indicator cells were cultured for 48 h and harvested to measure luciferase activity as a reporter of the number of packaged replicons delivered to the indicator cells by functional virions.

Virion release from cells infected with recombinant vaccinia virus expressing wt or replication-incompetent BVDV genome

The RK-13 rabbit kidney cells (~ 5×10^5) were co-infected with vTF7-3 (MOI = 3) and vT7-cpNADL or vT7-cpNgdd (MOI = 5), for 1 h at 37 °C. The infected cells were rinsed with PBS and cultured in DMEM containing 2.5% γ-irradiated FBS for an additional 22 h. To investigate the presence of released BVDV virions, culture fluids were collected and centrifuged at 13,000 *g* for 15 min at 4 °C to remove cell debris and the supernatant filtered through a 0.1 mm filter to remove the vaccinia virions. The filtrate was then centrifuged using a 10% sucrose cushion and the fraction containing BVDV virions (density of 1.12 to 1.14) was collected and analyzed by western blotting as described above. The infected cell monolayer was harvested in SDS-PAGE sample buffer for subsequent analysis by western blotting to determine the relative structural protein expression levels in the different samples.

Acknowledgments

A contribution of the University of Nebraska Agricultural Research Division, supported by funds provided through USDA/NRI grant 2002-35204-11619 to R.O.D. is acknowledged. We thank Dr. E. J. Dubovi for antibodies and bovine cell lines. We also thank the UNL genome research facility for all the DNA sequencing. The members of the Donis lab are acknowledged for their help and valuable suggestions.

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