Autonomously Replicating RNAs of Bungowannah Pestivirus: E^{RNS} Is Not Essential for the Generation of Infectious Particles

Anja Dalmann,^a Ilona Reimann,^a Kerstin Wernike,^a Martin Beer^a

^aInstitute of Diagnostic Virology, Friedrich Loeffler Institute, Greifswald-Insel Riems, Germany

ABSTRACT Autonomously replicating subgenomic Bungowannah virus (BuPV) RNAs (BuPV replicons) with deletions of the genome regions encoding the structural proteins C, E^{RNS}, E1, and E2 were constructed on the basis of an infectious cDNA clone of BuPV. Nanoluciferase (Nluc) insertion was used to compare the replication efficiencies of all constructs after electroporation of *in vitro*-transcribed RNA from the different clones. Deletion of C, E1, E2, or the complete structural protein genome region (C-E^{RNS}-E1-E2) prevented the production of infectious progeny virus, whereas deletion of E^{RNS} still allowed the generation of infectious particles. However, those Δ E^{RNS} viral particles were defective in virus assembly and/or egress and could not be further propagated for more than three additional passages in porcine SK-6 cells. These “defective-in-third-cycle” BuPV Δ E^{RNS} mutants were subsequently used to express the classical swine fever virus envelope protein E2, the N-terminal domain of the Schmallenberg virus Gc protein, and the receptor binding domain of the Middle East respiratory syndrome coronavirus spike protein. The constructs could be efficiently complemented and further passaged in SK-6 cells constitutively expressing the BuPV E^{RNS} protein. Importantly, BuPVs are able to infect a wide variety of target cell lines, allowing expression in a very wide host spectrum. Therefore, we suggest that packaged BuPV Δ E^{RNS} replicon particles have potential as broad-spectrum viral vectors.

IMPORTANCE The proteins N^{PRO} and E^{RNS} are unique for the genus *Pestivirus*, but only N^{PRO} has been demonstrated to be nonessential for *in vitro* growth. While this was also speculated for E^{RNS}, it has always been previously shown that pestivirus replicons with deletions of the structural proteins E^{RNS}, E1, or E2 did not produce any infectious progeny virus in susceptible host cells. Here, we demonstrated for the first time that BuPV E^{RNS} is dispensable for the generation of infectious virus particles but still important for efficient passaging. The E^{RNS}-defective BuPV particles showed clearly limited growth in cell culture but were capable of several rounds of infection, expression of foreign genes, and highly efficient *trans*-complementation to rescue virus replicon particles (VRPs). The noncytopathic characteristics and the absence of preexisting immunity to BuPV in human populations and livestock also provide a significant benefit for a possible use, e.g., as a vector vaccine platform.

KEYWORDS Bungowannah virus, E^{RNS}, pestivirus, foreign gene expression, replicon, viral vector, virus replicon particles

Bungowannah virus (BuPV), a member of the species *Pestivirus F* of the genus *Pestivirus* within the family *Flaviviridae* (1), is associated with the porcine myocarditis syndrome (PMC) in young pigs (2, 3). The first outbreak of BuPV was detected in Australia in New South Wales in 2003, but until now, the virus had not spread to any other region in Australia or to other countries worldwide (4, 5). Bungowannah virus is genetically and antigenically very divergent from the four classical pestivirus species bovine viral diarrhea virus 1 (BVDV-1; only recently reclassified as *Pestivirus A*), bovine

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Address correspondence to Martin Beer, martin.beer@fli.de.

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viral diarrhea virus 2 (BVDV-2; *Pestivirus B*), border disease virus (BDV; *Pestivirus D*), and classical swine fever virus (CSFV; *Pestivirus C*). The atypical pestivirus BuPV is mostly related to the porcine lateral shaking inducing neurodegenerative agent (LINDA) virus, which was identified in Austria in 2015 (6), and the most recently in toothed whales detected *Phocoena pestivirus* (PhoPeV) (7). Moreover, the *in vitro* cell tropism of BuPV differs markedly from that of other pestiviruses. With the exception of CSFV, which can only infect cells of swine origin, pestiviruses are able to infect a variety of cell cultures derived from cloven-hoofed animals (8). However, the broadest *in vitro* cell tropism was described for BuPV. Besides bovine, ovine, and porcine cells, cell lines of human, mouse, bat, and monkey origin are also susceptible to BuPV (9).

Pestivirus particles are small, enveloped virions. The genome is a positive-sense single-stranded RNA (ssRNA) of about 11.5 to 13 kb that consists of a single open reading frame (ORF) flanked by nontranslated regions (NTRs) at both genome ends (10–15). The 5′ and 3′ NTRs are highly structured and required for RNA replication and translation (16–18). The 5′ NTR contains an internal ribosomal entry site (IRES) for cap-independent translation initiation (19, 20). The 3′ NTR exhibits highly variable as well as highly conserved regions (17).

The ORF encodes a single polyprotein which is co- and posttranslationally cleaved by both cellular and viral proteases into the mature structural proteins capsid (C), E^{RNS}, E1, and E2 and the nonstructural proteins N^{PRO}, p7, NS2-NS3 (NS2, NS3), NS4A, NS4B, NS5A, and NS5B (10, 12, 21, 22). The nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B are essential components of the pestiviral replication machinery (23–25). The positively charged capsid protein forms the viral capsid and is responsible for the packaging of the viral RNA, probably by a histone-like protein-RNA interaction (26). The viral envelope is composed of the three glycoproteins E^{RNS}, E1, and E2 embedded in a lipid membrane of host cellular origin. In virus particles and pestivirus-infected cells, disulfide-linked E2 and E^{RNS} homodimers and E1-E2 heterodimers can be detected (21, 23, 27–29). The envelope protein E^{RNS} is like the nonstructural protein N^{PRO} unique to pestiviruses. N^{PRO} is described as an interferon antagonist in host cells and is not essential for virus replication in cell culture (30, 31). However, while the function of E^{RNS} in the pestiviral life cycle is not fully understood, it has been shown that E^{RNS} is involved in virus entry by binding to glucosaminoglycans (32), that the uptake of E^{RNS} bound to cells is powered by clathrin-dependent endocytosis, and that E^{RNS} also acts as interferon antagonist (33). Unlike the other pestiviral glycoproteins E1 and E2, E^{RNS} lacks a typical membrane anchor but exhibits a long amphipathic α -helix (34, 35). A very special feature of E^{RNS} is its intrinsic RNase activity, which is an important virulence factor (36–39).

Autonomously replicating subgenomic pestivirus RNAs (replicons) were originally identified as defective interfering RNAs that could be packaged by a helper virus. Reverse genetics systems were used to generate first self-replicating RNAs analogous to the defective interfering RNAs *in vitro* (25, 40, 41). Pestivirus replicons became important tools to study molecular mechanisms of pestivirus replication, assembly, and egress (42, 43). So, it became clear that neither the structural proteins C, E^{RNS}, E1, and E2 nor the nonstructural proteins p7 and NS2 are required for RNA replication but are indispensable for the generation of infectious virus particles (25, 42, 44). Interestingly, viable CSFV mutants with deletions within the capsid protein encoding the genomic region and a second-site mutation in the C-terminal region of NS3 could be rescued (45). Replicons expressing heterologous genes, e.g., the green fluorescent protein (GFP) or a luciferase reporter gene, have also been used for antiviral drug screening and small interfering RNA (siRNA) testing (46–50). Packaging of replicons, generated by *trans*-complementation in cell lines providing the deleted genes, has been shown to have a high potential for the development of safe and efficacious marker vaccines (43, 51, 52). Furthermore, replicons expressing homologous genes have been successfully used as nontransmissible vaccine vectors (53–55).

In the present study, we constructed BuPV replicons with deletions within the regions encoding the structural proteins C, E^{RNS}, E1, and E2 and compared their

replication efficiencies. All generated mutants were able to replicate autonomously and, with exception of the mutants with E^{RNS} deletions, did not produce any detectable infectious virus progeny. Here, we could for the first time show that the deletion of BuPV E^{RNS} does not prevent the generation of infectious virus particles. However, the virus particles could not be propagated in porcine SK-6 cells efficiently, and after several passages, infectious virus was no longer detectable. Replicons with E^{RNS} deletions were able to express foreign genes, as shown exemplarily for CSFV envelope protein E2, an immunogenic domain of Schmallenberg virus (SBV) Gc, and the immunogenic receptor binding domain (RBD) of the Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein, and could be *trans*-complemented in a newly established cell line constitutively expressing the BuPV E^{RNS} protein.

RESULTS

Construction and characterization of BuPV replicons. The BuPV replicons were constructed analogously to efficiently replicating BVDV and CSFV replicons (43, 51, 56) by using the BuPV plasmid pA/BV as the template. The different replicons exhibited either a deletion of 123 bases within the capsid protein (BVΔC, amino acids [aa] 208 to 248), a deletion of 456 bases within E1 (BVΔE1, aa 506 to 657), a deletion of 468 bases within E^{RNS} (BVΔE^{RNS}_156, aa 328 to 483), a complete deletion of E2 (BVΔE2, aa 695 to 1071), or a complete deletion of the structural protein-encoding region (BVΔC-E2; aa 208 to 1071) (Fig. 1A). At 72 h posttransfection (hpt) of *in vitro*-transcribed RNA of the full-length BuPV plasmid pA/BV or the replicon constructs, the expression of nonstructural protein NS3 could be detected by immunofluorescence (IF) staining, indicating that all *in vitro*-synthesized RNAs were able to replicate autonomously after electroporation into cells. No apparent differences in fluorescence intensity were observed between the different replicon constructs after electroporation. Expression of the deleted structural proteins was not detected (BVΔE2, negative in E2 staining; BVΔE^{RNS}, negative in E^{RNS} staining; BVΔC-E2, negative in E2 and E^{RNS} staining) (Fig. 1B). Because BuPV-C- and BuPV-E1-specific antibodies are not available, the absence of the capsid protein C in BVΔC and of the E1 protein in BVΔE1 RNA-electroporated cells could not be confirmed by staining or Western blotting but could be shown by reverse transcription PCR (RT-PCR) analysis of the RNAs. In order to examine whether recombinant virus was generated, fresh SK-6 cells were inoculated with filtered supernatants of the electroporated cells sampled at 72 hpt. To exclude a carryover event from electroporated cells or cell fragments, the supernatants were centrifuged and filtered after harvesting to remove residual cells from the supernatants. As expected, infectious virus progeny could not be recovered from supernatants of cells electroporated with RNA of the constructs BVΔC, BVΔE1, BVΔE2, and BVΔC-E2.

Surprisingly, in cells inoculated with supernatants of BVΔE^{RNS}_156 RNA-electroporated cells, a large number of BuPV protein-expressing foci could be observed, indicating that infectious recombinant virions (BVΔE^{RNS}_156) could be rescued, and this could not be observed for any other constructed replicons. To further exclude any carryover event, the passaging of BVΔE^{RNS}_156 RNA-electroporated cells onto fresh SK-6 cells was repeated, but with an extensive washing step after 2 h. Again, BuPV protein-expressing foci could be observed (Fig. 2). However, the rescued virions showed a clear growth defect and could not efficiently be further propagated.

Comparison of the replication efficiencies of the BuPV replicons. In order to compare the replication efficiencies of the BuPV replicons, corresponding constructs expressing the nanoluciferase reporter gene between N^{PRO} and C (pA/BV_Nluc, BVΔC_Nluc, BVΔE^{RNS}_156_Nluc, BVΔE2_Nluc, BVΔE1_Nluc, BVΔC-E2_Nluc) were generated. An insertion of the first 21 nucleotides (nt) of the capsid gene upstream and a short GSG linker (9 nt) as well as the teschovirus 2A^{PRO} sequence (59 nt) downstream of the Nluc coding region facilitates the correct cleavage of Nluc from the viral polyprotein by the autoproteolytic activity of N^{PRO} and teschovirus 2A^{PRO} (Fig. 3A). A replication-incompetent construct (pA/BV_GAA_Nluc) was generated by mutation of the GDD RNA

FIG 1 (A) Schematic representation of the infectious BuPV full-length cDNA backbone clone pA/BV and the replicon constructs generated in this study. Light gray boxes represent the nonstructural proteins, and dark gray boxes represent the structural proteins. Deletions are represented by horizontal dotted lines. Lines at the left and right ends represent NTRs. (B) IF analysis of SK-6 cells electroporated with *in vitro*-transcribed RNA of the full-length clone pA/BV and replicon constructs BVΔC, BVΔE^{NS}₁₅₆, BVΔE2, BVΔE1, and BVΔC-E2 or inoculated with transfection supernatants (first passage) using a pan-pestivirus NS3-specific antibody (anti-NS3), BuPV-specific monoclonal E2 antibody (anti-E2), and BuPV^{E^{NS}} monoclonal antibody (anti-E^{NS}) at 72 hpt. Mock- and pA/BV-electroporated cells were used as controls. Bar, 100 μm.

polymerase motif in NS5B to GAA (Fig. 3A) and served as a negative control in the Nluc assays.

Electroporation of SK-6 cells with *in vitro*-transcribed RNA from the full-length cDNA clone pA/BV_Nluc and the replicons with Nluc insertions (BVΔC_Nluc, BVΔE^{NS}₁₅₆_Nluc, BVΔE2_Nluc, BVΔE1_Nluc, BVΔC-E2_Nluc) resulted in RNA replication and production of viral proteins comparable to that of the corresponding constructs without the Nluc gene. To determine whether the Nluc insertion had an effect on the virus replication, growth kinetic studies were performed with the wild-type BuPV (BV_wt) and the recombinant viruses pA/BV and pA/BV_Nluc in SK-6 cells. Growth kinetics studies demonstrated comparable replication efficiencies of BV_wt and the recombinant viruses pA/BV and pA/BV_Nluc, and virus titers of about 10^{6.75} to 10^{7.525} 50% tissue culture infective dose (TCID₅₀)/ml could be measured at 72 h postinfection

FIG 2 BVΔE^{RNS}_156 RNA-electroporated cells were centrifuged, filtered, and passaged onto fresh SK-6 cells; the cells were then incubated for 72 h (A) or the medium was removed after 2 h, the cells were washed with phosphate-buffered saline, fresh DMEM was added, and then the cells were incubated for 72 h (B). After the incubation period, the cells were fixed and stained using an anti-NS3-specific MAb.

(hpi) (Fig. 3B). Furthermore, Nano-Glo luciferase assays demonstrated the expression of biologically active Nluc from all replication-competent constructs with Nluc insertions.

In order to quantify the Nluc expression as a measure of replication efficiency of the different replicon constructs, supernatants of electroporated cells were removed at 4,

FIG 3 (A) Schematic representation of cDNA constructs with an Nluc reporter gene insertion, which is indicated by hatched boxes. 7 aa capsid, 7 N-terminal amino acids of BuPV capsid protein; Teschovirus 2A^{pro}, teschovirus 2A protease. The mutated nucleotides introduced into the NS5B gene of the RNA-dependent RNA polymerase-defective construct BV_GAA are shown in capital letters. (B) Growth characteristics of pA/BV, BV_wt, and pA/BV_Nluc in SK-6 cells. All represented viruses replicate efficiently, and comparable virus titers could be determined at the depicted hours postinfection. (C) Nluc activity in SK-6 cells electroporated with *in vitro*-transcribed RNA of the replicon constructs BVΔC_Nluc, BVΔE^{RNS}_156_Nluc, BVΔE1_Nluc, BVΔE2_Nluc, and BVΔC-E2_Nluc or the full-length clone BV_Nluc and the replication-incompetent cDNA clone BV_GAA_Nluc at 4, 24, 48, and 72 hpt, normalized to BV_GAA_Nluc. Three technical and biological replicates were measured for each construct; error bars represent standard deviations, and a horizontal dotted line represents the dynamic range of the luminometer.

24, 48, and 72 hpt, and cells were assayed for Nluc activity. Figure 2C shows the values normalized to the replication-incompetent GAA mutant. In parallel, we monitored the consistent expression of NS3 in all samples by IF staining (data not shown).

Nluc activity was initially detected at 4 hpt, and at this time point, no significant differences could be shown for the electroporated replicons and the control.

At 24 hpt, maximum relative light unit (RLU) values of around 6×10^6 could be estimated for the replicons BVΔC_Nluc, BVΔE^{RNS}_156_Nluc, BVΔE2_Nluc, and BVΔC-E2_Nluc. BVΔE1_Nluc replicated on a slightly reduced level, with maximal values of 3.6×10^6 RLU (Fig. 3C). Virus production could already be detected at 24 h after electroporation of pA/BV_Nluc, and the maximum Nluc values of pA/BV_Nluc were higher at this and the following time points than for the replicons. With the exception of construct BVΔE1_Nluc, there were no significant differences in RNA replication of the deletion replicons over the investigated time (Table 1).

Characterization of BuPV replicons with E^{RNS} deletions. We next investigated whether deletions within functional regions of BuPV E^{RNS} or the size of the E^{RNS} deletion had an impact on the generation of infectious progeny virus. BuPV replicons with deletions of 55 to 112 amino acids within E^{RNS} (BVΔE^{RNS}_55, BVΔE^{RNS}_88, and BVΔE^{RNS}_112) or a complete E^{RNS} deletion of 222 amino acids (BVΔE^{RNS}) were constructed (Fig. 4A). In the construct BVΔE^{RNS}_55, the deletion of 55 aa between aa 84 and aa 139 in the middle of E^{RNS} does not affect any of the functional domains. In the construct BVΔE^{RNS}_88, the deletion from aa 51 to aa 139 covers the second RNase domain with its catalytic center H⁸⁰ (BuPV-E^{RNS} aa numbering [57–59]). In deletion mutant BVΔE^{RNS}_112, in which aa 51 to aa 163 have been deleted, the first cluster of basic amino acids (¹⁴⁰KKKGH¹⁴³), which has been shown to be important for double-stranded RNA (dsRNA) binding and *regulation* of interferon (IFN) induction in pestivirus-infected cells (32, 58), is also deleted. In replicon BVΔE^{RNS}_156, amino acids between positions 51 and 207 were deleted, while 51 N-terminal amino acids, including the first RNase domain with its active center H³¹ and the 15 C-terminal amino acids harboring a part of the amphipathic helix with a second cluster of basic amino acids (²⁰⁹KIENTKKK²¹⁶) responsible for membrane binding (34, 35) were retained.

To investigate the influence of the deletions on RNA replication, the constructs were transfected into SK-6 cells by electroporation, and expression of NS3, a marker for autonomous pestivirus RNA replication, could be detected. Furthermore, E^{RNS} staining of the cells was negative. The intensity of NS3 immunostaining and the number of NS3-positive cells differed only marginally among the replicons (Fig. 4B).

Transfection of all different replicons with an E^{RNS} deletion resulted in the generation of infectious progeny virus, and a large number of small foci of infected cells could be observed. However, all E^{RNS}-deleted recombinant viruses showed a significant growth defect and could only be passaged up to two further rounds in SK-6 cells (Fig. 4B).

The BVDV-1 plasmid NCP7ΔE^{RNS} (51) and a BVDV-2 plasmid, pA/BVDV-2ΔE^{RNS} (I. Reimann, unpublished data), were used as controls; however, infectious virus could never be rescued (data not shown).

For further characterization of the generated particles, a neutralization test was performed using serum of an animal inoculated experimentally with the wild-type virus. All BVΔE^{RNS} virus particles could be neutralized, indicating that these are actually infectious and not artifacts of the electroporation process (Fig. 4C). Transfection supernatants incubated with a negative serum, which was used as a control, were not neutralized.

Establishment of a BuPV E^{RNS}-expressing SK-6 cell line and trans-complementation studies. For a further characterization of the different E^{RNS} deletion mutants, we established an SK-6 cell line (SK-6_BV-E^{RNS}) that constitutively expresses BuPV E^{RNS} (Fig. 4D). SK-6 cells were electroporated with plasmid pCAGGS_BuPV-E^{RNS}, which contains the complete region encoding the BuPV structural protein E^{RNS} under the control of the chicken beta-actin (CAG) promoter, and a puromycin resistance gene. IF staining of

TABLE 1 Significance analysis of replication efficiencies of the BuPV replicons expressing nanoluciferase and pA/BV_Nluc^a

Time point and comparison	Significant?	Summary ^b	Adjusted <i>P</i> value
4 h			
BVΔC_Nluc vs BVΔE ^{RNS} _156__Nluc	No	NS	>0.9999
BVΔC_Nluc vs BVΔE1_Nluc	No	NS	>0.9999
BVΔC_Nluc vs BVΔE2_Nluc	No	NS	>0.9999
BVΔC_Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔC_Nluc vs pA/BV_Nluc	No	NS	>0.9999
BVΔE ^{RNS} _156__Nluc vs BVΔE1_Nluc	No	NS	>0.9999
BVΔE ^{RNS} _156__Nluc vs BVΔE2_Nluc	No	NS	>0.9999
BVΔE ^{RNS} _156__Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔE ^{RNS} _156__Nluc vs pA/BV_Nluc	No	NS	>0.9999
BVΔE1_Nluc vs BVΔE2_Nluc	No	NS	>0.9999
BVΔE1_Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔE1_Nluc vs pA/BV_Nluc	No	NS	>0.9999
BVΔE2_Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔE2_Nluc vs pA/BV_Nluc	No	NS	>0.9999
BVΔC-E2_Nluc vs pA/BV_Nluc	No	NS	>0.9999
24 h			
BVΔC_Nluc vs BVΔE ^{RNS} _156__Nluc	No	NS	0.833
BVΔC_Nluc vs BVΔE1_Nluc	Yes	****	<0.0001
BVΔC_Nluc vs BVΔE2_Nluc	No	NS	0.0904
BVΔC_Nluc vs BVΔC-E2_Nluc	No	NS	0.1087
BVΔC_Nluc vs pA/BV_Nluc	No	NS	0.1383
BVΔE ^{RNS} _156__Nluc vs BVΔE1_Nluc	Yes	**	0.0077
BVΔE ^{RNS} _156__Nluc vs BVΔE2_Nluc	No	NS	0.7003
BVΔE ^{RNS} _156__Nluc vs BVΔC-E2_Nluc	No	NS	0.7465
BVΔE ^{RNS} _156__Nluc vs pA/BV_Nluc	Yes	**	0.0041
BVΔE1_Nluc vs BVΔE2_Nluc	No	NS	0.3186
BVΔE1_Nluc vs BVΔC-E2_Nluc	No	NS	0.2785
BVΔE1_Nluc vs BV_Nluc	Yes	****	<0.0001
BVΔE2_Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔE2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔC-E2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
48 h			
BVΔC_Nluc vs BVΔE ^{RNS} _156__Nluc	No	NS	0.9853
BVΔC_Nluc vs BVΔE1_Nluc	Yes	***	0.0004
BVΔC_Nluc vs BVΔE2_Nluc	No	NS	0.1805
BVΔC_Nluc vs BVΔC-E2_Nluc	No	NS	0.2317
BVΔC_Nluc vs pA/BV_Nluc	Yes	***	0.0003
BVΔE ^{RNS} _156__Nluc vs BVΔE1_Nluc	Yes	**	0.0054
BVΔE ^{RNS} _156__Nluc vs BVΔE2_Nluc	No	NS	0.5467
BVΔE ^{RNS} _156__Nluc vs BVΔC-E2_Nluc	No	NS	0.6283
BVΔE ^{RNS} _156__Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔE1_Nluc vs BVΔE2_Nluc	No	NS	0.3958
BVΔE1_Nluc vs BVΔC-E2_Nluc	No	NS	0.3242
BVΔE1_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔE2_Nluc vs BVΔC-E2_Nluc	No	NS	>0.9999
BVΔE2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔC-E2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
72 h			
BVΔC_Nluc vs BVΔE ^{RNS} _156__Nluc	No	NS	0.7169
BVΔC_Nluc vs BVΔE1_Nluc	Yes	*	0.0186
BVΔC_Nluc vs BVΔE2_Nluc	No	NS	0.6407
BVΔC_Nluc vs BVΔC-E2_Nluc	No	NS	0.7918
BVΔC_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔE ^{RNS} _156__Nluc vs BVΔE1_Nluc	Yes	****	<0.0001
BVΔE ^{RNS} _156__Nluc vs BVΔE2_Nluc	Yes	*	0.0406
BVΔE ^{RNS} _156__Nluc vs BVΔC-E2_Nluc	No	NS	0.0792
BVΔE ^{RNS} _156__Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔE1_Nluc vs BVΔE2_Nluc	No	NS	0.5450
BVΔE1_Nluc vs BVΔC-E2_Nluc	No	NS	0.3856
BVΔE1_Nluc vs pA/BV_Nluc	Yes	****	<0.0001

(Continued on next page)

TABLE 1 (Continued)

Time point and comparison	Significant?	Summary ^b	Adjusted <i>P</i> value
BVΔE2_Nluc vs BVΔC-E2_Nluc	No	NS	0.9999
BVΔE2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001
BVΔC-E2_Nluc vs pA/BV_Nluc	Yes	****	<0.0001

^aOne-way ANOVA followed by Tukey's multiple-comparison test was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA).

^b*, <0.05; **, <0.01; ***, <0.001; ****, <0.0001; NS, not significant.

puromycin-resistant cell clones using the BuPV E^{RNS}-specific monoclonal antibody (MAb) revealed BuPV E^{RNS} expression in more than 90% of the cells. IF staining of the cells using pan-pestivirus NS3-specific and BuPV E2-specific MAbs served as negative controls (Fig. 4D). After continuous passaging of the SK-6_BV-E^{RNS} cell lines for 6 months in the presence of puromycin, the portion of E^{RNS}-expressing cells remained stable (data not shown).

To investigate whether BuPV E^{RNS} can be complemented in *trans*, *in vitro*-transcribed RNA of the constructs BVΔE^{RNS}_55, BVΔE^{RNS}_88, BVΔE^{RNS}_112, BVΔE^{RNS}_156, and BVΔE^{RNS} was electroporated into the SK-6_BV-E^{RNS} cell line. At 72 hpt, supernatants of the electroporated cells were collected, and secreted *trans*-complemented virion replicon particles (VRPs) (BVΔE^{RNS}_55_comp, BVΔE^{RNS}_88_comp, BVΔE^{RNS}_112_comp, BVΔE^{RNS}_156_comp, and BVΔE^{RNS}_comp) with virus titers of 10^{3.25} to 10^{4.0} TCID₅₀/ml could be detected in all transfection supernatants (Fig. 4E). Subsequent serial passages in the SK-6_BV-E^{RNS} helper cell line resulted in virus titers of about 10⁷ TCID₅₀/ml after nine passages for all mutants, independent of the size of the E^{RNS} deletion.

In contrast, in alternating passaging experiments using the parental SK-6 cells and SK-6_BV-E^{RNS} cells, the titers of the *trans*-complemented viruses decreased clearly in all cases after two passages in SK-6 cells but increased immediately after one passage in complementing SK-6_BV-E^{RNS} cells, before dropping down again to virus titers just below 10¹ TCID₅₀/ml (Fig. 4F). Finally, after three consecutive passages in SK-6 cells, again no progeny virus could be detected.

These results could be confirmed by electroporation of BVΔE^{RNS}_156 in SK-6 cells and passaging the supernatants of electroporated cells in both SK-6 and SK-6_BV-E^{RNS} cells. Supernatants of the first to fifth passages in SK-6 cells were again passaged once in SK-6_BV-E^{RNS} cells, and virus titers as well as quantification cycle (*C_q*) values of BuPV RNA were determined (Fig. 4G and H). In passage one, virus titers of 10^{0.75} TCID₅₀/ml in SK-6 cells and 10^{6.5} TCID₅₀/ml in SK-6_BV-E^{RNS} cells could be determined (Fig. 4H). In this experiment, in passage two, no virus could be detected in SK-6 cells by IF staining; however, in SK-6_BV-E^{RNS} cells, *trans*-complemented virus was detectable and high virus titers of 10^{5.75} TCID₅₀/ml could be obtained. Only after three passages in SK-6 cells, no infectious virus could be rescued after passaging these supernatants in SK-6_BV-E^{RNS} cells (= passage P4) (Fig. 4H). The real-time RT-PCR results confirmed these results, as the viral genome of BVΔE^{RNS}_156 was detectable in SK-6 cells up to passage two, with *C_q* values of 29.8 in SK-6 cells and 19.93 in SK-6_BV-E^{RNS} cells, and in passage four, no viral RNA was detectable in either cell line (Fig. 4G).

From these results and from sequence analyses of the generated VRPs (passage 9 in SK-6_BV-E^{RNS} cells, alternating passages 7 and 8), we conclude that neither genetic changes such as adaptive mutations nor recombination with the E^{RNS} gene of the cell line occurred and that the VRPs are stable in SK-6_BV-E^{RNS} cells.

The growth defect of BVΔE^{RNS} is not related to replication efficiency but is a consequence of virion assembly and/or egress. In order to investigate whether the growth defect of the E^{RNS}-defective VRPs is caused by reduced RNA replication efficiency, we analyzed the replication capacity of BVΔE^{RNS}_156 replicon RNA and the full-length BuPV RNA after electroporation into SK-6 cells earlier. The results show that the full-length construct replicates only at a slightly higher level at different time points (Fig. 3C).

FIG 4 (A) Schematic representation of the BuPV genome and the generated replicons with deletion of various functional domains within the E^{RNS}. Horizontal dotted lines represent the deletions; RNAD_I, RNase domain I; RNAD_{II}, RNase domain II; dsRNAD, double-stranded RNA binding domain; horizontal black lines represent conserved cysteines. Numbers above vertical lines on the bar indicate amino acid positions. (B) IF analysis of SK-6 cells electroporated with the respective replicons (BVΔE^{RNS}₁₅₆, BVΔE^{RNS}₁₁₂, BVΔE^{RNS}₈₈, BVΔE^{RNS}₅₅, BVΔE^{RNS}). Supernatants were harvested, and the cells were immunostained at 72 hpt using the pan-pestivirus NS3-specific MAb and the BuPV E^{RNS}-specific MAb. Supernatants contained infectious recombinant viruses that could be passaged for up to two further rounds, as demonstrated by IF staining with NS3 (1st passage, 2nd passage, 3rd passage). Bar, 100 μm. (C) Neutralization test of BVΔE^{RNS} replicons with BuPV-positive serum. Supernatants from electroporated SK-6 cells were neutralized, and IF staining was used for analysis with BuPV E2-specific MAb. (D) IF analysis of the generated SK-6 cell line expressing the BuPV E^{RNS} protein (SK-6_BV-E^{RNS}). IF staining with the BuPV E^{RNS}-specific MAb led to positive IF signals, while IF staining using the NS3-specific MAb and the BuPV E2-specific MAb remained negative. (E and F) Passage history of *trans*-complemented VRPs with E^{RNS} deletions in SK-6_BV-E^{RNS} cells (E) and alternating in SK-6 cells and SK-6_BV-E^{RNS} cells (F). SK-6_BV-E^{RNS} cells were electroporated with the respective replicons. At 72 hpi, supernatants were harvested (TF, transfection supernatant) and the generated complemented viruses (BVΔE^{RNS}_{156_comp}, BVΔE^{RNS}_{112_comp}, BVΔE^{RNS}_{88_comp}, BVΔE^{RNS}_{55_comp}, BVΔE^{RNS}_{comp}) were serially passaged using complementing SK-6_BV-E^{RNS} cells with increasing virus titers. In contrast, alternating passages in complementing (boxed passage numbers) and noncomplementing cells always resulted in a dramatic drop in virus titers, demonstrating the limited replication of the packaged replicons in noncomplementing cells and the absence of recombination events. Endpoint titers are given in log₁₀ TCID₅₀/ml. After electroporation of SK-6 cells with BVΔE^{RNS}₁₅₆, supernatants were harvested and passaged using SK-6 or SK-6-BV-E^{RNS} cells. Viral titers (H) and C_q values (G) were determined after every passage.

FIG 5 Quantitative RT-PCR analysis of SK-6 cells and SK-6_BV-E^{RNS} cells infected with BVΔE^{RNS}_156_comp and full-length BuPV. RNA copy numbers in SK-6 and SK-6_BV-E^{RNS} cells infected with the packaged replicon BVΔE^{RNS}_156_comp and rBuPV at an MOI of 0.1 were estimated per well at 2 and 48 hpi. SK-6 and SK-6_BV-E^{RNS} cells inoculated with supernatants of SK-6_BV-E^{RNS} cells electroporated with BVΔE1 were used as a negative control. Experiments were done in biological duplicates. Significance analysis was performed by ANOVA using GraphPad Prism software. Asterisks indicate significant differences between groups.

In order to have a closer look at these processes, SK-6 cells and the helper cell line SK-6_BV-E^{RNS} were infected with recombinant BuPV (rBuPV) or packaged BVΔE^{RNS}_156_comp VRPs, as an example for the E^{RNS}-deleted mutants, at a multiplicity of infection (MOI) of 0.1 TCID₅₀/ml. After virus adsorption and several washing steps, to ensure that the starting material did not affect subsequent investigations, supernatants and cells of the same well were separated and RNA was isolated from both fractions. BVΔE1 was used as a negative control. The RNA amounts were quantified 2 and 48 hpi by real-time RT-PCR using BuPV-specific primers and an external standard. At 2 hpi, very low copy numbers and no apparent differences were estimated between the replicon and full-length RNA levels in SK-6 and SK-6_BV-E^{RNS} cells and supernatants. This indicates for both cell lines that the infection with VRPs as well as rBuPV were under equal conditions and the same amounts of input RNA (Fig. 5).

As expected, no BuPV-specific RNA could be detected in cells inoculated with supernatants of BVΔE1 electroporated SK-6_BV-E^{RNS} cells. However, compared to the 2-h RNA values, at 48 hpi, a significant increase in full-length BuPV RNA could be detected for both cell lines in the cells as well as in the supernatants (SK-6_BV-E^{RNS} cells, $P = 0.0001$, supernatant, $P = 0.0202$; SK-6 cells, full-length BuPV, $P = 0.043$, supernatant, $P = 0.0028$). For BVΔE^{RNS}_156, significantly increased RNA copy numbers could be likewise detected in the complementing cell line after 48 hpi compared to the 2-h values (cells, $P = 0.0155$; supernatant, $P = 0.0187$). In the SK-6 cells, however, no statistically significant difference was observed (Table 2). Comparable increases in RNA copy numbers by about 3 log steps were measured in the cell fraction for BVΔE^{RNS}_156 in both cell lines, whereas the copy number in the supernatant did not change significantly 48 hpi in the noncomplementing cell line. In contrast, RNA copy numbers of around 10⁷ in cells and 10⁹ in the supernatant could be detected in the complementing cell line infected with the BVΔE^{RNS}_156 replicon particles or rBuPV at 48 hpi. Significantly higher RNA copy numbers of the full-length BuPV RNA than of BVΔE^{RNS}_156 replicon RNA could be observed in SK-6 cells, with P values of 0.0468 in cells and 0.0028 in the supernatant at that time point. This indicates a defect of BVΔE^{RNS} in the assembly or release of RNA-carrying virions.

BuPV replicons with E^{RNS} deletions are suitable for the stable expression of heterologous viral genes and selected immunogenic domains. To investigate whether the generated BVΔE^{RNS} replicons are a suitable system for expressing foreign

TABLE 2 Significance analysis of RNA copy numbers from pA/BV-, BVΔE^{RNS}-comp-, and BVΔE1-infected SK-6 or SK-6-BV-E^{RNS} cells^a

Cell type or supernatant and comparison	Significant?	Summary ^b	Adjusted <i>P</i> value
SK-6-BV-E ^{RNS} supernatant			
2h BVΔE ^{RNS} vs 2h pA/BV	No	NS	>0.9999
2h BVΔE ^{RNS} vs 2h BVΔE1	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h BVΔE ^{RNS}	Yes	*	0.0187
2h BVΔE ^{RNS} vs 48h pA/BV	Yes	*	0.0202
2h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
2h pA/BV vs 2h BVΔE1	No	NS	>0.9999
2h pA/BV vs 48h BVΔE ^{RNS}	Yes	*	0.0187
2h pA/BV vs 48h pA/BV	Yes	*	0.0202
2h pA/BV vs 48h BVΔE1	No	NS	>0.9999
2h BVΔE1 vs 48h BVΔE ^{RNS}	Yes	*	0.0187
2h BVΔE1 vs 48h pA/BV	Yes	*	0.0202
2h BVΔE1 vs 48h BVΔE1	No	NS	>0.9999
48h BVΔE ^{RNS} vs 48h pA/BV	No	NS	0.9611
48h BVΔE ^{RNS} vs 48h BVΔE1	Yes	*	0.0187
48h pA/BV vs 48h BVΔE1	Yes	*	0.0202
SK-6-BV-E ^{RNS} cells			
2h BVΔE ^{RNS} vs 2h pA/BV	No	NS	>0.9999
2h BVΔE ^{RNS} vs 2h BVΔE1	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h BVΔE ^{RNS}	Yes	*	0.0155
2h BVΔE ^{RNS} vs 48h pA/BV	Yes	***	0.0001
2h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
2h pA/BV vs 2h BVΔE1	No	NS	>0.9999
2h pA/BV vs 48h BVΔE ^{RNS}	Yes	*	0.0155
2h pA/BV vs 48h pA/BV	Yes	***	0.0001
2h pA/BV vs 48h BVΔE1	No	NS	>0.9999
2h BVΔE1 vs 48h BVΔE ^{RNS}	Yes	*	0.0155
2h BVΔE1 vs 48h pA/BV	Yes	***	0.0001
2h BVΔE1 vs 48h BVΔE1	No	NS	>0.9999
48h BVΔE ^{RNS} vs 48h pA/BV	No	NS	0.0594
48h BVΔE ^{RNS} vs 48h BVΔE1	Yes	*	0.0155
48h pA/BV vs 48h BVΔE1	Yes	***	0.0001
SK-6 supernatant			
2h BVΔE ^{RNS} vs 2h pA/BV	No	NS	>0.9999
2h BVΔE ^{RNS} vs 2h BVΔE1	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h pA/BV	Yes	**	0.0028
2h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
2h pA/BV vs 2h BVΔE1	No	NS	>0.9999
2h pA/BV vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h pA/BV vs 48h pA/BV	Yes	**	0.0028
2h pA/BV vs 48h BVΔE1	No	NS	>0.9999
2h BVΔE1 vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h BVΔE1 vs 48h pA/BV	Yes	**	0.0065
2h BVΔE1 vs 48h BVΔE1	No	NS	>0.9999
48h BVΔE ^{RNS} vs 48h pA/BV	Yes	**	0.0028
48h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
48h pA/BV vs 48h BVΔE1	Yes	**	0.0028
SK-6 cells			
2h BVΔE ^{RNS} vs 2h pA/BV	No	NS	>0.9999
2h BVΔE ^{RNS} vs 2h BVΔE1	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h BVΔE ^{RNS} vs 48h pA/BV	Yes	*	0.043
2h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
2h pA/BV vs 2h BVΔE1	No	NS	>0.9999
2h pA/BV vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h pA/BV vs 48h pA/BV	Yes	*	0.043
2h pA/BV vs 48h BVΔE1	No	NS	>0.9999
2h BVΔE1 vs 48h BVΔE ^{RNS}	No	NS	>0.9999
2h BVΔE1 vs 48h pA/BV	Yes	*	0.043
2h BVΔE1 vs 48h BVΔE1	No	NS	>0.9999

(Continued on next page)

TABLE 2 (Continued)

Cell type or supernatant and comparison	Significant?	Summary ^b	Adjusted <i>P</i> value
48h BVΔE ^{RNS} vs 48h pA/BV	Yes	*	0.0468
48h BVΔE ^{RNS} vs 48h BVΔE1	No	NS	>0.9999
48h pA/BV vs 48h BVΔE1	Yes	*	0.043

^aOne-way ANOVA followed by Tukey's multiple-comparison test was performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA).

^b*, <0.05; **, <0.01; ***, <0.001; NS, not significant.

genes, the CSFV E2 protein, the N-terminal domain of the SBV glycoprotein Gc (GcN), and the receptor-binding domain (RBD) of the MERS-CoV spike protein served as model immunogens (60–62). Based on BVΔE^{RNS}₁₅₆, we generated replicons expressing GcN by insertion of two GcN domains connected by a 16-aa-long linker sequence (BVΔE^{RNS}_{2GcN}) or the RBD of MERS-CoV spike protein (BVΔE^{RNS}_{RBD}). For the correct processing of both the BuPV C and E1 proteins by cellular signalases, in both replicons the foreign genes were flanked by 51 N-terminal amino acids and 15 C-terminal amino acids of BuPV E^{RNS}. The CSFV E2-expressing replicon BVΔE^{RNS}_{CSFV_E2} was constructed on the basis of BVΔE^{RNS} with a complete E^{RNS} deletion and was inserted between C and E1 of the replicon (Fig. 6A). Since both BuPV E^{RNS} and CSFV E2 are pestiviral glycoproteins with a C-terminal region, which allows cleavage from the polyprotein by cellular signalases, the complete substitution of CSFV E2 for BuPV E^{RNS} should not negatively affect correct processing of BVΔE^{RNS}_{CSFV-E2}.

The successful expression of the different foreign genes could be demonstrated by immunostaining of SK-6 cells electroporated with *in vitro*-transcribed RNA of the replicon constructs BVΔE^{RNS}_{2GcN}, BVΔE^{RNS}_{RBD}, and BVΔE^{RNS}_{CSFV_E2}, respectively, at 72 hpt, using SBV GcN-specific, MERS-CoV RBD-specific, or CSFV E2-specific MAbs. In contrast, full-length BuPV electroporated cells, which have been used as a control, reacted negatively in IF staining with those MAbs.

After electroporation, all replicon RNA-transfected cells were positive by NS3 staining, while the mock-transfected cells reacted negatively and pA/BV, which was used as positive control, reacted correctly positive. Transfection supernatants were used for inoculation of SK-6 cells, and at 72 hpi, small foci of IF-positive cells could be detected. However, the number of IF-positive foci was lower than that observed after the first passage of BVΔE^{RNS} and BVΔE^{RNS}₁₅₆ virions without insertions of foreign genes, and no progeny virus could be further passaged in SK-6 cells (Fig. 6B).

However, the expression of foreign genes had no negative effect on the packaging of the replicon RNA in SK-6_{BV-E^{RNS}} helper cells. After electroporation of the constructs BVΔE^{RNS}_{2GcN}, BVΔE^{RNS}_{RBD}, and BVΔE^{RNS}_{CSFV_E2} into complementing SK-6_{BV-E^{RNS}} cells, RNA replication could be detected by NS3-specific immunostaining and expression of the SBV GcN domain, MERS-CoV RBD, and CSFV E2 was confirmed at 72 hpt (Fig. 6C). Virus titers between 10^{3.875} and 10^{5.625} TCID₅₀/ml could be determined at 72 hpt (Fig. 6C). The complemented viruses could be further passaged in SK-6_{BV-E^{RNS}} cells; the titers are given in Fig. 5.

Stable expression of foreign genes. In order to investigate the stability of protein expression after six passages of the complemented viruses in SK-6_{BV-E^{RNS}} cells, virus titers were also determined by IF staining using the respective foreign-gene-specific MAbs. For all complemented viruses, the titers determined by NS3 immunostaining as well as by SBV GcN, MERS-CoV RBD, and CSFV E2 immunostaining did not differ significantly and indicated a stable expression of the foreign genes (Fig. 6C). Recombination events could be excluded, since the complemented viruses could not be continuously passaged in noncomplementing SK-6 cells.

The expression of the SBV GcN domain, the receptor-binding domain of MERS-CoV S1 spike protein, and CSFV E2 protein could also be shown by Western blotting. SK-6 cells were inoculated with the complemented viruses BVΔE^{RNS}_{RBD_comp}, BVΔE^{RNS}_{2GcN_comp}, BVΔE^{RNS}_{CSFV_E2_comp}, and BVΔE^{RNS}_{156_comp} or remained noninfected (negative control). In lysates of cells infected with BVΔE^{RNS}_{2GcN}, the

FIG 6 Analysis of chimeric replicons and chimeric VRPs. (A) Schematic depiction of the constructed chimeric replicons with deletions within the E^{RNS} coding region. Structural proteins are shown in dark gray, nonstructural proteins are shown in light gray, and foreign genes are (Continued on next page)

double GcN domain could be detected as a fusion protein with an estimated size of approximately 62 kDa, similar to the calculated size. In lysates of cells infected with BVΔ^{ERN5}_RBD_comp, MERS-CoV RBD, which was also expressed as a fusion protein, could be detected at 38 kDa, slightly higher than the calculated molecular weight of 34 kDa. In lysates of cells infected with BVΔ^{ERN5}_CSFV_E2_comp, expression of CSFV E2 with a calculated size of about 55 kDa could be demonstrated by Western blotting as well (Fig. 6D).

Expression of foreign genes does not alter the cell tropism of VRPs. In order to investigate whether the chimeric BuPV VRPs were able to infect cells originating from the most relevant target species of SBV (sheep and cattle) and MERS-CoV (Bactrian camel, human), cells of these species were inoculated with the chimeric VRPs or VRPs without insertions (negative control). At 72 hpi, RNA replication and expression of GcN, RBD, and CSFV-E2 could be detected in sheep (SFT-R), human (HEK-293), Bactrian camel (TT-R), and cattle (KOP-R) cells. In addition, the RBD domain was successfully expressed in llama (LGL-2-R) and alpaca (LPK-1-R, LPL-2-R) cells (data not shown). IF analysis of the infected cells demonstrated that the broad cell tropism of BuPV also applies to the chimeric VRPs (Fig. 7A and B). In order to determine the infection rate of the cell lines with the respective VRPs and to compare the chimeric VRPs with the BuPV VRPs without foreign genes, fluorescence-activated cell sorting (FACS) analysis was performed. For this comparison, the measured values of the construct BVΔ^{ERN5} were set as 100% for each of the tested cell lines, and it could be demonstrated that all replicons showed a high infection rate in SK-6 cells (>86%) and a slightly lower infectivity in SFT cells (45% to 73%), HEK cells (52% to 75%), and KOP-R cells (49% to 73%) than that of the parental clone BVΔ^{ERN5}. An improved infection rate due to the insertion could therefore not be observed (Fig. 7C).

DISCUSSION

Autonomously replicating RNAs are important tools to study the role of single proteins, e.g., concerning their role in the viral replication cycle. Pestivirus replicons with deletions within the structural proteins C, E^{ERN5}, E1, and E2 have therefore been constructed previously and have been used for the development of safe and efficient vaccine candidates that are defective in the second cycle against BVDV and CSFV infections.

In all cases, those pestiviral replicons with deletions in the structural protein-encoding genes were not able to form infectious virus progeny, with the exception of a CSFV construct with a nearly complete deletion of the C protein. A mutation within the NS3 protein seems to compensate for the functionality of the C protein. This construct has the ability to produce infectious viruses *in vitro* (45). Furthermore, replicons could be passaged on *trans*-complementing cell lines and packaged into viral replicon particles (VRPs). For BVDV replicons, passaging and packaging were often very inefficient or even not possible due to severe interference effects (43, 51, 63). In contrast, CSFV replicons lacking the E^{ERN5} protein could be efficiently passaged on complementing helper cells (56). However, the host range of those packaged pestivirus replicons was very restricted, mainly to the natural hosts, i.e., pigs and ruminants.

FIG 6 Legend (Continued)

shown in hatched boxes. The blowups show the insertion and the surrounding genomic region. For construction of BVΔ^{ERN5}_2GcN, a double construct of the N-terminal SBV Gc region connected by a linker was inserted into the E^{ERN5}_156 deletion. The same deletion mutant was used for the insertion of the receptor binding domain of MERS-CoV (BVΔ^{ERN5}_RBD). CSFV E2 was inserted into the complete E^{ERN5} deletion mutant BVΔ^{ERN5}, resulting in BVΔ^{ERN5}-CSFV-E2. (B) IF analysis after electroporation of *in vitro*-transcribed RNA into SK-6 cells at 72 hpt by using the NS3-specific MAb WB112, a MERS-CoV RBD-specific MAb, the SBV GcN-specific MAb 5E1_A3, and the CSFV E2-specific MAb Bio275. Nontransfected cells were used as a negative control. SK-6 cells were inoculated with collected supernatants and investigated by IF staining at 72 hpi using pan-pestivirus NS3-specific MAb WB112. (C) *Trans*-complementation of the indicated constructs by RNA transfection of SK-6_BV-E^{ERN5} cells. The chimeric VRPs were passaged 6 times in SK-6_BV-E^{ERN5} cells 72 hpi and analyzed by IF staining of the cells using the pan-pestivirus NS3-specific MAb (WB112) and the insert-specific MAbs 5E1_A3 (SBV-GcN), MERS-CoV-RBD, and Bio275 (CSFV-E2). Nontransfected cells were used as a negative control. (D) SK-6 cells were infected with complemented replicons (BVΔ^{ERN5}_2GcN_comp, BVΔ^{ERN5}_RBD_comp, BVΔ^{ERN5}_CSFV_E2_comp, BVΔ^{ERN5}_156_comp), and expression of the foreign genes was analyzed at 48 hpi by Western blotting using MAbs 5E1_A3 (SBV GcN), Bio275 (CSFV E2), and PAb (MERS-CoV S1) (spike protein S1). Bar, 100 μm.

FIG 7 Infection of cells originating from the target species of SBV, CSFV, and MERS-CoV with packaged BVΔE^{RNS} replicons. (A) Sheep cells (SFT-R), cattle cells (KOP-R), cells originating from Bactrian camel (TT-R), and (B) cells of human origin (HEK-293) were infected with packaged BVΔE^{RNS}_2GcN_comp, BVΔE^{RNS}_RBD_comp, and/or BVΔE^{RNS}_CSFV_E2_comp. Infected cells were analyzed by IF staining 72 hpi by using the NS3-specific MAb WB112, a MERS-CoV RBD-specific MAb, a CSFV E2-specific MAb, and the SBV GcN-specific MAb 5E1_A3, nontransfected cells were used as a negative control, and full-length pA/BV was used as a positive control. Bar, 100 μm. (C) To determine whether the insertion influences the infection rate of the replicons, flow cytometry analysis (FACS) was performed for BVΔE^{RNS}_CSFV_E2, BVΔE^{RNS}_2GcN, and BVΔE^{RNS}_RBD. The replicon BVΔE^{RNS} and uninfected cells were used as controls. The infection rate of BVΔE^{RNS} was defined as 100%.

Here, for first time, we studied replicons of the atypical Bungowannah pestivirus. Since BuPV has only been found on one farm until now, a vaccine against this virus itself seems to be unnecessary, but since BuPV can infect a very broad range of cells of, e.g., mouse, monkey, bat, camelid, porcine, ruminant, and human origin—in sharp contrast to all other known pestiviruses (9)—and replicate efficiently in these cells, BuPV replicons might have the advantage as a universal expression vector. Therefore,

a first BuPV replicon set with the deletion of genes encoding the structural proteins was constructed and characterized. We also tested their suitability for use as expression vectors for immunogens of important human and veterinary viruses and designed a novel packaging cell line expressing E^{RNS} of BuPV.

Electroporation of the *in vitro*-transcribed RNAs of all replicon constructs resulted in efficient RNA replication, and expression of BuPV NS3 was readily detected. By also using Nluc-expressing replicon constructs, the replication efficiency of the replicon RNAs could be further investigated. Interestingly, all replicon RNAs (BVΔC_Nluc, BVΔE^{RNS}_156_Nluc, BVΔE1, BVΔE2_Nluc, BVΔC-E2_Nluc) replicated on comparable levels, indicating that the deletions did not result in altered RNA secondary structures or any other circumstances which could influence RNA replication (49, 64, 65). In accordance with previous investigations using BVDV or the majority of CSFV replicons, no infectious virus could be recovered from cells electroporated with the replicons BVΔC, BVΔE1, BVΔE2, or BVΔC-E2 (42–44, 51). Unexpectedly and in clear contrast to all previous studies, BVΔE^{RNS} RNA electroporation resulted in the production of E^{RNS} replicon-based virus particles, which could be passaged for at least two additional rounds in susceptible SK-6 cells. Although these particles were not capable of continued propagation, this is to our knowledge the first description of a viable pestivirus E^{RNS} deletion mutant. However, Wang et al. (66) demonstrated by using pseudovirions that in addition to those containing the three viral glycoproteins E1, E1, and E^{RNS}, pseudovirions were also infectious when E^{RNS} was missing. Thus, it could be shown that E^{RNS} is obviously not essential for CSFV to enter the cell. This observation is in accordance to our data, and these pseudovirions showed a lower infectivity than those containing E^{RNS} (66).

Our study therefore proves that BuPV glycoprotein E^{RNS} is not essential for the rescue of infectious virions. The resulting virions could infect and replicate in SK-6 cells. Neutralization tests demonstrated that the particles did not differ from wild-type BuPV and could be completely neutralized by BuPV-specific sera; therefore, we assume that they were not artifacts from the electroporation procedure. Nevertheless, the produced particles showed a distinct growth defect, and only small foci of infected cells could be detected. This could not be observed for any other analyzed pestivirus used as controls. Previous studies with both CSFV and BVDV replicons also clearly demonstrated that E^{RNS} is strictly essential for virion production, and supernatants of transfected cells did not contain any infectious virus. In all experiments, IF staining of susceptible cells inoculated with transfection supernatants was always negative (51, 56, 67).

Our further experiments also demonstrated that virus rescue from BuPV replicons was independent of the size or the functional domains of the E^{RNS} deletion (BVΔE^{RNS}_55, BVΔE^{RNS}_88, BVΔE^{RNS}_112, BVΔE^{RNS}_156) and could also be observed for a mutant with a complete E^{RNS} deletion (BVΔE^{RNS}). In contrast, for CSFV, it was shown that deletions within the amphipathic helix strongly influenced the functionality and properties of the E^{RNS} protein and virus production. For instance, mutations in the C-terminal region resulted in reduced activity in blocking dsRNA-induced interferon synthesis (34, 68). We consider this not an irregular nature of the virus particles, since the BuPV particles could be neutralized efficiently by an anti-BuPV serum. However, it remains unclear why Bungowannah virus particles without E^{RNS} protein can be generated. BuPV E^{RNS} has a distinctly lower molecular weight, about 38 kDa, than that of other pestiviruses (48 kDa) and exhibits a lower N-glycosylation. It can be therefore speculated that BuPV E^{RNS} is connected to the viral envelope in a different manner than the E^{RNS} of classical pestiviruses. Although it had been demonstrated that the integrity of the E^{RNS} membrane anchor is important for the recovery of infectious BVDV and CSFV (34, 35), BuPV E^{RNS} shows a deletion of 7 amino acids within this essential membrane anchor region. Furthermore, the formation of an E^{RNS}-E1 protein, which is important in the life cycle of classical pestiviruses, seems to be not essential for the BuPV life cycle (29). Most likely, these different features, alone or in combination, are the basis of the different behavior of BVΔE^{RNS} replicons.

Unfortunately, most likely because of the low number of generated E^{RNS}-deleted

BuPV particles, all attempts to visualize the defective viruses by electron microscopy were unsuccessful (data not shown).

We also investigated whether the observed growth defect of the E^{RNS} -deleted BuPV replicons is caused by reduced RNA replication compared to that of full-length BuPV RNA, disturbed virus assembly, or reduced virus egress. Our results demonstrated that the replicon BV ΔE^{RNS} _156 replicated on nearly the same level as full-length BuPV RNA. By comparing infection of SK-6 cells and SK-6_BV- E^{RNS} helper cells with that of packaged BV ΔE^{RNS} _156_comp VRPs, we could show that the newly generated E^{RNS} -defective virus particles were assembled and could be released from infected SK-6 cells. Nevertheless, virus assembly and/or egress seems to be inefficient in noncomplementing cells but could be fully compensated by the helper cell line providing BuPV- E^{RNS} in *trans*.

The novel characteristics of BV ΔE^{RNS} replicons, like the release of infectious particles with the restriction to a few further replication cycles, the highly efficient *trans*-complementation and passaging on helper cell lines, and the broad range of susceptible cell lines, render these replicons an ideal system for the long-term expression of foreign genes and the development of efficient and safe packaged replicon vaccines. We therefore investigated the *trans*-complementation of BuPV replicons with the different E^{RNS} deletions and demonstrated that all of these replicons could be efficiently complemented. For BVDV VRPs with E2 or E2 and p7 deletions, it has been reported that they could not be further propagated in cells constitutively expressing the missing proteins, probably due to virus interference (42, 43). In contrast, the packaged BuPV VRPs could be efficiently passaged in the complementing cell line, and virus titers increased to about $10^{7.0}$ TCID₅₀/ml. Slightly lower maximum virus titers of $10^{5.0}$ to $10^{5.8}$ TCID₅₀/ml have been described for packaged CSFV replicons with E^{RNS} deletions (67). In order to point out a pronounced difference in packaging efficiency, it would be interesting to compare the CSFV and BuPV VRP systems directly.

After serial passages of the VRPs in SK-6_BV- E^{RNS} cells, neither mutations within the replicon genome nor recombination of the replicon genome and the E^{RNS} gene of the complementing cells could be seen. Since the BuPV replicons with E^{RNS} deletions are viable for at least two rounds in SK-6 cells, the VRPs could also be detected after two passages in SK-6 cells. However, the virus titer was clearly reduced, which has been also demonstrated by real-time RT-PCR, as no viral RNA could be detected after three to four passages. The generation of an autonomously growing variant after passaging could also be excluded after alternating passages of the BuPV VRPs in complementing SK-6_BV- E^{RNS} cells and SK-6 cells. Neither recombination nor reversion to the wild-type BuPV occurred; however, this cannot be ruled out at higher passage numbers.

Replicons are useful tools for the expression of heterologous genes. In this context, the advantage of noncytopathogenic replicons may be that they continuously replicate at a low level in a broad range of cell lines. In contrast, cytopathogenic replicons seem to be more suitable for high-level expression in the cytoplasm (69–71). Replicon RNA of ssRNA viruses like alphaviruses (e.g., Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus), flaviviruses (e.g., Kunjin virus, West Nile virus, and yellow fever virus), measles virus, and rhabdoviruses (rabies virus, vesicular stomatitis virus) have been used as self-replicating expression systems and for vaccine development (72). These viruses are known for their high-level RNA replication in the cytoplasm, which is a prerequisite for a transient expression of heterologous genes. For the production of recombinant particles, replicons can be packaged by cotransfection of a helper vector (73) or packaging cell lines (74), of which the latter approach has been selected in the present study.

Replicon RNA vectors allow the induction of a strong protective immune response in various laboratory animals and have also been tested in clinical trials (72). In previous studies, mainly surface antigens capable of inducing neutralizing antibodies against pathogenic infectious diseases have been selected as heterologous genes to be inserted into the replicon vectors (75–77). Accordingly, the major immunogens of CSFV,

SBV, and MERS-CoV were selected to be expressed in the context of the BuPV replicon system.

We constructed chimeric BuPV replicons with insertions of three model antigens, CSFV E2 (BVΔE^{RNS}_CSFV_E2), the N-terminal domain GcN of the SBV glycoprotein Gc (BVΔE^{RNS}_2GcN), and the receptor-binding domain (RBD) of the MERS-CoV spike protein (BVΔE^{RNS}_RBD). All proteins could be expressed, and *trans*-complementation and propagation of the expression replicons using the SK-6_BV-E^{RNS} cell line was highly efficient. Titers of the expressing VRPs were comparable with those of the VRPs without insertions, and neither recombination nor loss of expression could be observed. Furthermore, the broad cell tropism of BuPV allowed, e.g., the infection of alpaca, llama, Bactrian camel, and human cells as well as cells of ovine and bovine origin by the chimeric BuPV replicons. Future studies will now focus on immunization/challenge experiments with the different constructs in the respective target animals.

In summary, the BuPV E^{RNS} deletion mutants and VRPs described in this study represent the first pestivirus replicons that could be passaged in noncomplementing cells despite the deletion of a complete envelope protein. However, the E^{RNS}-deleted BuPV replicons and VRPs are still growth defective and abortive in further growth. Nevertheless, their unique properties make BuPV ΔE^{RNS} replicons and VRPs a promising novel replicon-based expression and vaccine platform.

MATERIALS AND METHODS

Cells. SK-6 (swine kidney cells; RIE262), HEK-293 (human kidney cells; RIE197), TT-R (Bactrian camel umbilical cord cells; RIE1006), SFT-R (sheep thymus cells; RIE43), KOP-R (cattle esophagus/pharynx cells; RIE244) (Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Insel Riems, Germany [CCLV]) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂.

Plasmid constructs. Plasmids were amplified in *Escherichia coli* DH10B cells (Invitrogen, USA). Plasmid DNA was purified by use of Qiagen plasmid mini or midi kits (Qiagen, Germany). All cDNA constructs were generated by fusion PCR, a modified restriction-free cloning method (78, 79). Pairs of forward and reverse PCR primers used for the respective constructs are given in Table 3. The identity of the constructs was confirmed by DNA sequencing.

The infectious full-length cDNA clone pA/BV (9) was generated on the basis of published BuPV sequences (NCBI GenBank accession numbers NC_023176.1 and DQ901402.1). The completely synthetic construct was constituted from six plasmids harboring synthetic peptides (Geneart AG, Regensburg, Germany), which were assembled into plasmid vector pA (40) by fusion PCR cloning. In this plasmid, the full-length BuPV cDNA is flanked by the bacteriophage T7 promoter sequence at the 5' end and a SmaI restriction site at the 3' end. The construct pA/BV_Nluc was generated on the basis of plasmid pA/BV_GFP (I. Reimann, unpublished data) by replacing the eGFP gene with that of the Nluc gene (Fig. 1C). The eGFP gene has been located between the genes encoding N^{PRO} and the capsid protein C and is flanked upstream by 21 nt of the capsid gene and downstream by a linker and the teschovirus 2A^{PRO} gene.

In brief, the Nluc gene was amplified from plasmid DNA pNL1.1 (Promega, Germany) using primers Ph_CNlucF and Ph_NlucR (biomers.net GmbH, Germany) by PCR using Phusion high-fidelity PCR master mix (New England Biolabs, USA). Subsequently, the purified amplicon was used as a megaprimer in a fusion PCR with plasmid pA/BV_GFP as the template to generate full-length pA/BV_Nluc.

The subgenomic replicons (BVΔC, BVΔE^{RNS}, BVΔE^{RNS}_156, BVΔE^{RNS}_55, BVΔE^{RNS}_88, BVΔE^{RNS}_112, BVΔE2, BVΔE1, BVΔC-E2) were generated on the basis of pA/BV by complete or partial deletion of the genomic regions encoding the respective structural proteins (Fig. 1A) by fusion PCR using the appropriate primers. Nluc-expressing replicons (BVΔC_Nluc, BVΔE^{RNS}_156_Nluc, BVΔE2_Nluc, BVΔE1_Nluc, BVΔC-E2_Nluc, BV_GAA_Nluc) were constructed in the same way, using plasmid pA/BV_Nluc as the template.

Bungowannah virus replicons containing foreign genes were constructed based on replicons with deletions within the E^{RNS}-encoding region. For the construction of the replicon expressing the antigenic domain GcN of SBV, a megaprimer was amplified by using primers 2Gc_F and Erns_Gc_amino_R and plasmid syn_2xGc_amino_linker_pMK_RQ (Geneart AG, Germany) as the template DNA. Subsequently, the megaprimer was used for fusion PCR with the DNA template BVΔE^{RNS}_156 to generate the construct BVΔE^{RNS}_2GcN. The construct BVΔE^{RNS}_RBD, expressing the receptor binding domain (RBD) of the MERS-CoV spike protein, was also generated on the basis of plasmid BVΔE^{RNS}_156 but using a megaprimer, which was generated by PCR with primers BV_dErns_RBD_F and BV_dErns_RBD_R and plasmid S1_RBD_pMA-T (Geneart AG, Germany) as the DNA template. The CSFV E2-expressing plasmid BVΔE^{RNS}_CSFV_E2 was based on BVΔE^{RNS} with a complete E^{RNS} deletion. A DNA fragment was amplified by PCR by using primers BV_CSFV_E2_F and BV_CSFV_E2_R and plasmid pA/CP7_E2alf (80) as the template DNA, which was used as a megaprimer in a fusion PCR with the DNA template BVΔE^{RNS}.

For construction of the expression plasmid pCAGGS_BuPV-E^{RNS}, the genomic region encoding the C-terminal parts of the BuPV C and BuPV E^{RNS} proteins was amplified from the chemically synthesized

TABLE 3 Constructs and PCR primers used for construction

Construct	Primer	Sequence (5' → 3') ^a
BVΔC	Bungo_dC_1012F Bungo_1499R	GACAAAGCCTAAAGAGACTGAACCAGAAGCGTCCAGGAAGAAG GATTGATCCACGGCTCTATATTG
BVΔE2	Bungo_2060F Bungo_dE2_R	CTGAGCTTAATTGCAATGTC GAGCCACTATGTTCTCGTCGGCTTGCTCCCCCTATG
BVΔE ^{RNS} _156	Bungo_dErns_F Bungo_2164R	CATCTAGCAGCAGACTATGAAAGTAAGATTGAAAACACCAAGA CATCACGAAGTCCCTGTTGTC
BVΔC-E2	Ph_dCE2_Bungo_F Bungo_4021R	GACAAAGCCTAAAGAGACTGAATGGTTTGATCTCACAGCAAAG GAGACTTATTACAGCCACAAG
BVΔE ^{RNS} _55	Ph_Bungo_dErns_55F Bungo_2164R	CAAGCATGGGTGGTGAACAAGAAAGGGCACAATTCTC CATCACGAAGTCCCTGTTGTC
BVΔE ^{RNS} _88	Ph_Bungo_dErns_88F Bungo_2164R	CATCTAGCAGCAGACTATGAAAGAAAGGGCACAATTCTC CATCACGAAGTCCCTGTTGTC
BVΔE ^{RNS} _112	Ph_Bungo_dErns_112F Bungo_2164R	CATCTAGCAGCAGACTATGAAGACTTGCTCAGGATCATGG CATCACGAAGTCCCTGTTGTC
BVΔE ^{RNS}	BV_dErns_kompF Bungo_2164R	GCCTATTGGTCGTGCCGGTAGGGTCCAGTCTTACTGCCAGTGGC CATCACGAAGTCCCTGTTGTC
BVΔE1	BVdE1A_F Bungo_2874R	CAGTCCTTACTGCCAGTGGCTTTGTGGACCGAAGCAACAGC CTAGGGCATTCAAGTTCAAAGC
BV_GAA_Nluc	Bungo_GAA_F Bungo_11835R	GATACATGTGTGGAGCTGCTGGTTTCTGATAACTGAG CTGAGCCAATTAGAGAAC
pA/BV_Nluc	Ph_CNlucF Ph_NlucR	CTGACGACAAAGGAGCAAAAATGGTCTTCACACTCGAAGATTTC GAAGTTCGTGGCTCCGGAACCCGCCAGAATGCGTTCCGACAG
pCAGGS_sigE ^{RNS} _BV	CAGGS_sigErns_F CAGGS_Erns_R	GGCAAAGAATTGCGCCACCATGGAGGCCAGCCGGAAGAAGCTG GAAAATAACATATGCGGCCGCTAGCTCAGGCCTCGGCGCCGAAG
BVdE ^{RNS} _2GcN	2Gc_F Erns_GC_amino_R	CATCTAGCAGCAGACTATGAAAGTATTAAGTGCAAAAACATT CTTGGTGTTCATCTTACTGATCAGAGATAAGGTAGTGAG
BVdE ^{RNS} _RBD	BV_dErns_RBD_F BV_dErns_RBD_R	CTAGCAGCAGACTATGAATCTTTCGAAGCAAAACCTTCTGG GGTGTTCATCTTACTATATTCACGCAATTGCCTAATTGAG
BVdE ^{RNS} _CSFV_E2	BV_CSFV_E2_F BV_CSFV_E2_R	GGTCGTGCCGGTAGGGTCCAGCTAGCCTGCAAGGAAG GCCACTGGGCAGTAAGGACTACCAGCGGCAGTTGTTCT

^aRestriction enzyme sites are underlined.

and sequence-optimized ORF syn_sigERNS_BV_opt_pMA-T (Geneart AG, Germany). The amplicon was inserted into the plasmid vector pCAGGS_T7POLiresPuro (kindly provided by Stefan Finke, Friedrich-Loeffler-Institut) by fusion PCR using primers CAGGS_sigErns_F and CAGGS_Erns_R.

Further details for the generation of the plasmid constructs are available upon request.

Establishment of BuPV E^{RNS}-expressing SK6 cells (SK-6_BV-E^{RNS}). Subconfluent monolayers of SK-6 cells were transfected with plasmid pCAGGS_BuPV-E^{RNS} using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol with a 1.5-to-1 (vol/wt) Lipofectamine 3000-to-DNA ratio. In short, plasmid DNA and transfection reagent were added to Opti-MEM I (1×) plus GlutaMAX-I reduced serum medium (Gibco; Life Technologies, USA) separately and incubated for 5 min at room temperature. After 20 min of incubation at room temperature, the transfection mixture was added directly to the cell monolayer. After 48 h, the cell culture medium was replaced with fresh DMEM with 10% FCS supplemented with 2 μg/ml puromycin (Gibco, Life Technologies, USA). Single colonies were picked, replaced, and propagated under selection with 2 μg/ml puromycin and stained for E^{RNS} expression by using the BuPV E^{RNS}-specific MAb 43C3.

In vitro transcription, electroporation, and virus rescue. For transfection experiments, cDNA plasmids were linearized with SmaI (New England Biolabs, USA) and *in vitro* transcribed using a T7 RiboMax large-scale RNA production system (Promega, Germany) according to the manufacturer's instructions. The *in vitro*-synthesized RNA was transfected (1 to 5 μg) into SK-6 cells by electroporation using a Gene Pulser Xcell electroporation system (Bio-Rad, USA). After two pulses (850 V, 25 μF, 50 Ω), the cells were seeded in cell culture plates and incubated for 48 to 72 h at 37°C and 5% CO₂. For recovery of infectious virus, cell culture supernatants of the electroporated cells were harvested at 72 h post-

transfection (hpt), and after centrifugation (3,000 rpm, 5 min) and filtration with a 0.22- μ m Millex filter unit (Merck, Germany), the supernatants were passaged on fresh cells. To further exclude any carryover events, the supernatant of BV Δ E^{RNS}₁₅₆ RNA-electroporated cells was additionally passaged onto fresh SK-6 cells, the medium was removed after 2 h, the cells were washed several times with phosphate-buffered saline (PBS), and, thereafter, fresh DMEM was added.

Virus stocks were prepared by passaging the viruses on SK-6 cells. Virus titers were calculated and expressed as 50% tissue culture infective dose (TCID₅₀) per ml. The identity of the recombinant viruses was confirmed by RT-PCR and sequencing using the appropriate primers.

IF assays. For immunofluorescence (IF) analysis, electroporated or infected cells were fixed and permeabilized with 80% acetone. Subsequently, the cells were incubated with the working dilution of the monoclonal antibody of interest for 30 to 45 min. These included the pan-pestivirus NS3-specific MAb WB112 (1:500; CVL, UK), the BuPV E2-specific MAb 682/45F12 or the BuPV E^{RNS}-specific MAb 682/43C3 (1:20; M. Dauber, Friedrich-Loeffler-Institut, Insel Riems, Germany) as well as the SBV GcN-specific MAb 5E1_A3 (1:50; A. Aebischer, Friedrich-Loeffler-Institut, Insel Riems, Germany), CSFV E2-specific MAb Bio275 (1:100; Bio-X Diagnostics, Belgium), and a MERS-CoV RBD-specific MAb (1:10,000; kindly provided by B. J. Bosch, Leiden University Medical Center, The Netherlands; unpublished). After two washing steps with PBS, cell cultures were incubated with a goat anti-mouse Ig Alexa⁴⁸⁸ conjugate or, for MERS-CoV RBD, a goat anti-human Ig Alexa⁴⁸⁸ (1:1,000; Thermo Fisher Scientific, Inc., USA) for 30 min and finally washed with PBS. Images were captured with a fluorescence microscope (Nikon Eclipse) with 4 $\times$, 10 $\times$, 20 $\times$, and 40 $\times$ lens objectives.

Virus neutralization assay. One hundred microliters of supernatant from SK-6 cells electroporated with *in vitro*-transcribed RNA of BV Δ E^{RNS} deletion mutants was incubated in a 24-well cell culture plate for 2 h at 37°C with 50 μ l undiluted BuPV antibody-positive serum generated by experimental infection with the first Australian cell culture isolate (permission number 7221.3-1.1-064/11). Thereafter, an SK-6 cell suspension (2×10^4 cells/ml) was added and the plates were incubated for 72 h at 37°C. Neutralizations were detected by IF staining using the BuPV E2-specific MAb 682/45F12. As a negative control, SK-6 cells electroporated with *in vitro*-transcribed RNA of BV Δ E^{RNS} deletion mutants were incubated as described above with a BuPV-negative serum.

Nluc activity detection. For nanoluciferase (Nluc) assays, 250 ng of Smal-digested DNA of the Nluc constructs (BV Δ C_Nluc, BV Δ E^{RNS}₁₅₆_Nluc, BV Δ E2_Nluc, BV Δ C-E2_Nluc, BV Δ E1_Nluc, and BV_GAA_Nluc) was *in vitro* transcribed. A total of 1×10^7 SK-6 cells were transfected with 1 μ g synthesized RNA by electroporation. The cells were diluted with 15 ml medium, and 100 μ l cell suspension per cavity was added to a 96-well plate. After 4, 24, 48, and 72 h of incubation at 37°C and 5% CO₂, the expression of the Nluc reporter gene was detected by using a Nano-Glo luciferase assay system (Promega, USA) according to the manufacturer's protocol. Briefly, at the different time points, the supernatant was removed and the electroporated SK-6 cells were detached with fresh medium. Nano-Glo luciferase assay buffer and Nano-Glo luciferase assay substrate (Furimazine) were mixed (50:1) and added to the cells (1:1). Luciferase activity was measured as relative light units (RLU) by using a luminometer (Tecan Infinite F200 PRO; Switzerland). Three independent experiments were performed, and samples were measured in triplicate. The measured values were normalized to the values of the replication-deficient GAA mutant at the different time points. Significance analyses were performed using one-way analysis of variance (ANOVA), whereby the Tukey test was used for pairwise comparison as implemented in GraphPad Prism software 8.0.1 for Windows (GraphPad Software, USA).

Complementation studies. For *trans*-complementation experiments, *in vitro*-transcribed RNA of the BuPV replicons (BV Δ E^{RNS}₁₅₆, BV Δ E^{RNS}₁₁₂, BV Δ E^{RNS}₈₈, BV Δ E^{RNS}₅₅, BV Δ E^{RNS}, BV Δ E^{RNS}_{2GcN}, BV Δ E^{RNS}_{RBD}, BV Δ E^{RNS}_{CSFV_E2}) was electroporated into SK-6_BV-E^{RNS} cells. Cell culture supernatants were collected 72 hpt and filtered using a 0.22- μ m Millex filter unit (Merck, Germany). For detection of *trans*-complemented VRPs, SK-6 cells grown in six-well plates were inoculated with 1 ml of collected supernatant and further incubated at 37°C and 5% CO₂. At 72 h postinfection (hpi), cells were investigated by IF staining using anti-NS3-specific MAb WB 112. VRPs were serially passaged either in SK-6 or SK-6_BV-E^{RNS} cells or alternating in SK-6 and SK-6_BV-E^{RNS} cells and titrated using SK-6 cells. VRPs expressing SBV GcN (BV Δ E^{RNS}_{2GcN_comp}) were also passaged in KOP-R and SFT-R cells, and VRPs expressing MERS-CoV RBD (BV Δ E^{RNS}_{RBD_comp}) were passaged also in HEK-293T and TT-R cells. Virus titers were determined as TCID₅₀/ml by IF staining using the pan-pestivirus NS3-specific MAb WB112 or one of the insert-specific MAbs, 5E1_A3 (anti-GcN), Bio275 (anti-CSFV E2), and MERS-CoV RBD-specific MAb (anti-RBD).

PCR and sequencing. PCR was carried out using a C1000 thermal cycler (Bio-Rad, USA). DNA-based amplification was done using GoTaq polymerase (Promega, Germany) according to the supplier's protocols. For RT-PCR, total RNA of virus-infected cells was extracted using a QIAamp viral RNA minikit (Qiagen, Germany) according to the manufacturer's instruction and cDNA was amplified using a OneStep RT-PCR kit (Qiagen, Germany). Sequencing was carried out using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, USA). Nucleotide sequences were read with an automatic sequencer (3130 Genetic Analyzer; Applied Biosystems, USA) and analyzed using Geneious software (version 10.2.3; Biomatters Ltd., New Zealand).

Real-time RT-PCR. For quantification of viral RNA, SK-6 cells and SK-6_BV-E^{RNS} cells were infected with full-length BuPV or BV Δ E^{RNS}_{156_comp} at a multiplicity of infection (81) of 0.1 TCID₅₀/ml. SK-6 cells and SK-6_BV-E^{RNS} cells were also inoculated with supernatants of SK-6_BV-E^{RNS} cells electroporated with BV Δ E1 RNA (negative control). At 2 and 48 hpi, cells and supernatant of the respective wells were separated. Total RNA was extracted using TRIzol reagent (cells) or TRIzol/LS reagent (supernatant; Thermo Fisher Scientific, Inc., USA) according to the manufacturer's instruction. The real-time PCR (RT-qPCR) was

performed using an AgPath-ID one-step RT-PCR kit (Thermo Fisher Scientific, Inc., USA), the BuPV capsid protein targeting primers BV-1100-F (5'-CGAAACCAAGACTCA AGACG-3') and BV-1203-R (5'-GCAGGCT AATATTGCCCATGC-3'), and a BuPV-specific FAM-labeled probe (5'-FAM-CACAACAAGAACAAACCAGAA GCGTCC-3'). Real-time RT-PCR was carried out using a CFX 96 real-time detection system (Bio-Rad, USA) with the following thermal profile: a reverse transcription step at 45°C for 10 min, a PCR initial activation step at 95°C for 10 min, and then 42 cycles of three-step cycling consisting of denaturation at 95°C for 15 s, annealing at 57°C for 20 s, and extension at 72°C for 30 s. All samples were tested in triplicate, and to determine the genome load in the samples, an external standard was included in every real-time RT-PCR run. Two biological and three technical replicates were performed. Significance analysis was performed using one-way analysis of variance (ANOVA), whereby the Tukey test was used for pairwise comparison as implemented in GraphPad Prism software 8.4.0 for Windows (GraphPad Software, USA).

Western blot analysis. For Western blot analysis, SK-6 cells were infected with BVΔ^{ERN5}_CSFV_E2_comp, BVΔ^{ERN5}_RBD_comp, BVΔ^{ERN5}_2GcN_comp, and BVΔ^{ERN5}_156_comp, respectively, at an MOI of 0.1 TCID₅₀/ml. Total cell lysates were prepared at 48 hpi. The proteins were separated by 10% SDS-PAGE under reducing (BVΔ^{ERN5}_CSFV_E2_comp, BVΔ^{ERN5}_RBD_comp) or nonreducing (BVΔ^{ERN5}_2GcN_comp) conditions and transferred onto a nitrocellulose membrane (Bio-Rad, USA). After overnight blocking at 4°C with 1× ROTIBlock solution (Carl Roth, Germany), the membrane was incubated with the anti-GcN MAb (5E1_A3, 1:20), anti-E2 CSFV MAb (1:300; Bio-X Diagnostics, Belgium), or MERS-CoV spike protein S1 rabbit polyclonal antibody (PAb) (1:2,000; Sino Biological Inc., USA) for 2 h. As a secondary antibody, a horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:20,000; Dianova, Germany) was used. After washing, the membrane was incubated with SuperSignal-West Pico chemiluminescent detection reagent (Thermo Fisher Scientific, Inc., USA) and visualized using a ChemoCam Western blot imaging system (Intas Science Imaging Instruments GmbH, Germany). BVΔ^{ERN5}_156_comp was used as a negative control. The protein sizes were calculated by Geneious software (version 10.2.3; Biomatters Ltd., Auckland, New Zealand).

FACS analysis. For the detection of VRP infection rates in different cell lines and to investigate a possible influence of the foreign genes, FACS analysis was performed. The cells were infected with the packaged replicons BVΔ^{ERN5}_CSFV_E2_comp, BVΔ^{ERN5}_RBD_comp, BVΔ^{ERN5}_2GcN_comp, and BVΔ^{ERN5}_156_comp (MOI of 1). At 24 hpi, cells were fixed (4% paraformaldehyde), permeabilized (digitonin; Fluka Chemie GmbH, Switzerland), and incubated for 45 to 60 min with the pan-pestivirus NS3-specific MAb WB112 on ice. After a washing step with PBS, the cells were incubated with a goat anti-mouse Ig Alexa⁴⁸⁸ conjugate (Thermo Fisher Scientific, Inc., USA) for 45 to 60 min on ice, washed, and analyzed by using a FACSCalibur flow cytometer (Becton, Dickinson, USA). Two biological and technical replicates were performed. The FACS data obtained were analyzed with CellQuest Pro software (Becton, Dickinson, USA).

Data availability. All raw data are available upon request.

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