

Balance of RNA sequence requirement and NS3/NS3a expression of segment 10 of orbiviruses

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Orbiviruses are insect-transmitted, non-enveloped viruses with a ten-segmented dsRNA genome of which the bluetongue virus (BTV) is the prototype. Viral non-structural protein NS3/NS3a is encoded by genome segment 10 (Seg-10), and is involved in different virus release mechanisms. This protein induces specific release via membrane disruptions and budding in both insect and mammalian cells, but also the cytopathogenic release that is only seen in mammalian cells. NS3/NS3a is not essential for virus replication *in vitro* with BTV Seg-10 containing RNA elements essential for virus replication, even if protein is not expressed. Recently, new BTV serotypes with distinct NS3/NS3a sequence and cell tropism have been identified. Multiple studies have hinted at the importance of Seg-10 in orbivirus replication, but the exact prerequisites are still unknown. Here, more insight is obtained with regard to the needs for orbivirus Seg-10 and the balance between protein expression and RNA elements. Multiple silent mutations in the BTV NS3a ORF destabilized Seg-10, resulting in deletions and sequences originating from other viral segments being inserted, indicating strong selection at the level of RNA during replication in mammalian cells *in vitro*. The NS3a ORFs of other orbiviruses were successfully exchanged in BTV1 Seg-10, resulting in viable chimeric viruses. NS3/NS3a proteins in these chimeric viruses were generally functional in mammalian cells, but not in insect cells. NS3/NS3a of the novel BTV serotypes 25 and 26 affected virus release from *Culicoides* cells, which might be one of the reasons for their distinct cell tropism.

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INTRODUCTION

Orbiviruses are ten-segmented dsRNA viruses that belong to the family *Reoviridae*. The genus *Orbivirus* contains 22 virus species with at least 10 unclassified members (Attoui *et al.*, 2011). Well-known species are the bluetongue virus (BTV), African horsesickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV) and equine encephalosis virus (EEV), which are all transmitted by *Culicoides* biting midges and cause disease in ruminants (BTV and EHDV) or equids (AHSV and EEV). Orbiviruses are non-enveloped, architecturally complex viruses ~80 nm in diameter, which are composed of structural proteins VP1–7 organized in three capsid shells (Hewat *et al.*, 1992). In addition, at least four non-structural (NS)

viral proteins are expressed after infection (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011; Roy, 2005; Stewart *et al.*, 2015; Zwart *et al.*, 2015).

NS3 (encoded by Seg-10, ~26 kDa) and its N-terminal-truncated form NS3a (~24 kDa), expressed from a downstream in-frame start codon, function in virus release from infected cells (Celma & Roy, 2009; Wu *et al.*, 1992). These glycosylated membrane proteins are conserved among orbiviruses (Huismans *et al.*, 2004; van Niekerk *et al.*, 2003; van Staden & Huismans, 1991). A long N-terminal and a shorter C-terminal cytoplasmic domain with a centrally located small extracellular domain flanked by two transmembrane (TM) regions can be recognized (Fig. 1c) (Bansal *et al.*, 1998; French *et al.*, 1989; Hyatt *et al.*, 1991; Wu *et al.*, 1992). NS3 is a viroporin-like protein, facilitating virus release by membrane permeabilization (Han & Harty, 2004). It interacts with the calpactin light chain p11 of the cellular

One supplementary figure is available with the online Supplementary Material.

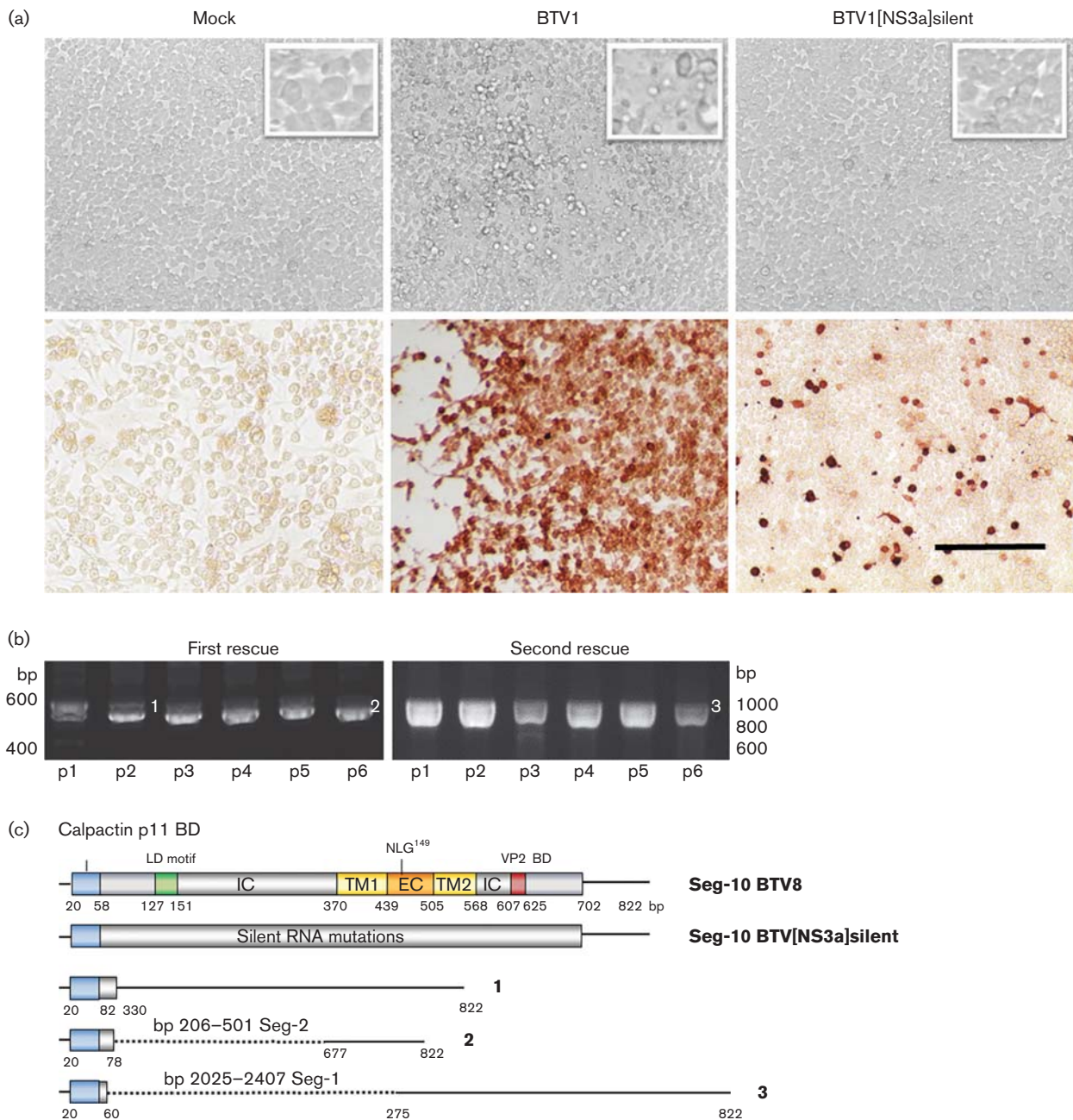


Fig. 1. (a) BSR monolayers were infected with BTV1, BTV1[NS3a]silent, or were mock infected. Upper panel: CPE at 48 h post-infection. Enlarged figures show apoptotic bodies characteristic for BTV CPE. Lower panel: VP7 immunostaining of infected monolayers. Bar, 200 μ m. (b) PCR amplification of Seg-10 of six subsequent passages of BTV1[NS3a]silent of two independent virus rescues (left and right panel). Bands that were sequenced are indicated by numbers corresponding to numbers in (c). (c) Schematic representation of Seg-10 with recognized motifs and domains in NS3/NS3a indicated. Seg-10 of BTV[NS3a]silent with synonymous mutations in the NS3a ORF is shown in grey. Sequenced Seg-10 mutants that contain deletions and that were numbered in (b) are also presented schematically, with nucleotide positions of the deleted RNA with respect to full-length Seg-10. Insertions are indicated by dashed lines.

annexin II complex and with cellular Tsg101 at its N terminus (Beaton *et al.*, 2002; Celma & Roy, 2011), which are involved in membrane-related events, secretion and intracellular trafficking (Raynal & Pollard, 1994; Wirblich *et al.*, 2006). BTV

NS3 also contains highly conserved PSAP and PPXY late domain motifs common in Tsg101-recruiting proteins (Wirblich *et al.*, 2006). The C-terminal cytoplasmic domain interacts with the outer capsid protein VP2 (Beaton *et al.*, 2002),

suggesting that NS3 supports virus release by connecting virus to cellular transport mechanisms and by disruption of the cell membrane. NS3/NS3a expression is higher in insect cells than in mammalian cells, suggesting that the NS3 function in invertebrates is more prominent (French *et al.*, 1989; Guirakhoo *et al.*, 1995; Noad & Roy, 2009; Wechsler & McHolland, 1988; Wechsler *et al.*, 1989). Release of BTV occurs by different mechanisms. In insect cells, release is non-lytic, whereas release from mammalian cells can also be lytic (Hyatt *et al.*, 1989). Indeed, virus release from mammalian cells is already observed before onset of cytopathogenic effect (CPE). Virus release early after infection occurs via budding through the cell membrane, requiring a temporary envelope, whereas later after infection virus release occurs by disruptions of the cell membrane (Hyatt *et al.*, 1989). Finally, BTV NS3/NS3a is also an inhibitor of the induction of IFN-I (Chauveau *et al.*, 2013; Doceul *et al.*, 2014).

Previously, reverse genetics has been used for rescue of BTV mutants to study the role of NS3/NS3a in virus replication (Boyce *et al.*, 2008; van Gennip *et al.*, 2012). NS3/NS3a is not essential for virus replication *in vitro*, although NS3/NS3a knockout BTV showed a disturbed release in mainly insect cells (van Gennip *et al.*, 2014). However, NS3/NS3a is essential for viraemia in the mammalian host as well as for virus propagation in *Culicoides* vectors *in vivo* (Feenstra *et al.*, 2014a, 2015).

Seg-10 also plays an important role in packaging of all ten genome segments in a cell-free virus assembly system (Lourenco & Roy, 2011). Mutations in the UTRs of Seg-10 abort virus replication, possibly by blocking RNA packaging (Boyce & McCrae, 2015). Likely, Seg-10 is the first recruited segment, followed by the other small segments, which then probably form a complex or complexes with the larger segments that are then packaged as a whole into the subcore particle (Sung & Roy, 2014). Recently, it has been shown that RNA elements in the ORF of Seg-10 are essential for virus rescue, irrespective of NS3-related expression (Feenstra *et al.*, 2014b). However, the mechanism and exact RNA sequences required are still unknown.

The BTV serogroup consists of at least 27 different serotypes of which serotypes 25–27 were identified recently (Hofmann *et al.*, 2008; Howell *et al.*, 1970; Jenckel *et al.*, 2015; Maan *et al.*, 2011). BTV25 or Toggenburg orbivirus was identified in asymptomatic infections in goats, but not in other wild or domestic ruminants (Casaubon *et al.*, 2013; Chaignat *et al.*, 2009). In goats, RNA was detected for a strikingly long period, whereas sheep showed minor symptoms and a very low viraemia in experimental infections (Vöglin *et al.*, 2013). Despite different attempts, culturing of BTV25 in cells and embryonated chicken eggs was unsuccessful (Chaignat *et al.*, 2009). Thereby, field-collected *Culicoides* species did not test positive for BTV25, and feeding of *Culicoides nubeculosus* with BTV25-positive blood did not lead to virus replication; however, this might have been due to a very low virus concentration (Planzer *et al.*, 2011). BTV26 or BTV Kuwait (Maan *et al.*, 2011) was detected in sheep, causing

mild clinical signs associated with a low and short viraemia. Interestingly, BTV26 could be isolated on mammalian cells (BHK and Vero) but without causing CPE in Vero cells, and the virus could not be isolated on *Culicoides* cells (KC) (Batten *et al.*, 2012). This difference in tropism compared with other BTV strains, which are easily isolated on KC, BSR and Vero cells, suggests that BTV25 and BTV26 might be spread by an alternative vector species or are transmitted via other mechanisms (Batten *et al.*, 2014). NS3/NS3a of BTV25–27 are more variable in some conserved regions, including a remarkable arginine to glycine mutation in the highly conserved late domain motif (PPRYAPSAP to PPGYAPSAP), which is not found in any other known BTV sequences. BTV27 was found in asymptomatic infections in goats on Corsica, France and does grow on BSR cells (Zientara *et al.*, 2014). Further research is needed to be able to conclude more about cell tropism, species infectivity and transmission of this new serotype.

Summarizing, multiple studies have hinted at the importance of Seg-10 RNA and its encoded NS3/NS3a protein in BTV replication. Here, the replication of BTV with different Seg-10 sequences in insect and mammalian cells has been studied. Further, Seg-10 of the new BTV serotypes 25 and 26 was investigated using Seg-10 mono-reassortants. A delicate balance was found between the requirement for protein expression and particular RNA sequences in Seg-10 for BTV replication *in vitro*.

RESULTS

Selection on protein expression or RNA sequence after NS3 codon exchange

Transient expression of NS3/NS3a by Seg-10(silent), which contains 180 silent mutations, was confirmed by transfection of BSR cells with Seg-10(silent) RNA and subsequent staining with NS3 mAbs (data not shown). Rescue of BTV1 with Seg-10(silent) was successful, although no CPE was observed, while VP7 staining indicated virus replication (Fig. 1a). In the first passage after virus rescue, two subpopulations of Seg-10(silent) were identified. One contained a 248 bp deletion (82–330) and the other contained a replacement of 599 bp (78–677 of Seg-10) by a 295 bp sequence originating from Seg-2 (206–501). The original sequence of Seg-10(silent) was not found, suggesting that the intended virus BTV1[NS3]silent is not viable. Independently rescued virus with Seg-10(silent) followed by six subsequent passages confirmed the instability of BTV1[NS3a]silent (Fig. 1b). Again, the original Seg-10(silent) sequence was not found. Instead, 215 bp (60–275) was deleted and replaced by 382 bp originating from Seg-1 (2025–2407) (Fig. 1c).

Exchange of the NS3a ORF of AHSV, EHDV and EEV in Seg-10 of BTV

As expected, rescue of BTV reassortants with complete Seg-10 of AHSV or EHDV failed after repeated attempts

(data not shown). BTV1-based mutants with chimeric Seg-10 containing the NS3a ORF of AHSV, EHDV or EEV fused with BTV8 UTRs were generated successfully. BTV1[NS3a]ehdv and BTV1[NS3a]eev induced CPE similar to ancestor BTV1, and replication was confirmed by immunostaining with α -VP7 mAbs. BTV1[NS3a]ahsv, however, did not induce CPE, although VP7 immunostaining indicated virus replication (Fig. 2a).

Seg-10s of each of the six passages were amplified to study stability of the chimeric segment. Seg-10 of BTV1[NS3a]ehdv and BTV1[NS3a]eev was stable (Fig. 3a), which was further confirmed by sequencing (data not

shown). Clearly, Seg-10 of BTV1[NS3a]ahsv was not stable, as smaller amplicons indicated deletions in this chimeric segment. Sequencing revealed an in-frame deletion from position 63 to 288 (Fig. 3b). Analysis of dsRNA confirmed a smaller Seg-10 for BTV1[NS3a]ahsv than for BTV1, BTV1[NS3a]ehdv and BTV1[NS3a]eev (Fig. 3c).

To investigate coincidental modification of chimeric sequences, rescue of BTV1[NS3a]ahsv, BTV1[NS3a]ehdv and BTV1[NS3a]eev was repeated. Again, BTV1 with NS3a of EHDV and EEV showed CPE in BSR cells, whereas BTV1[NS3a]ahsv did not (data not shown). BTV1[NS3a]eev was again stable (Fig. 3a). Seg-10 of the

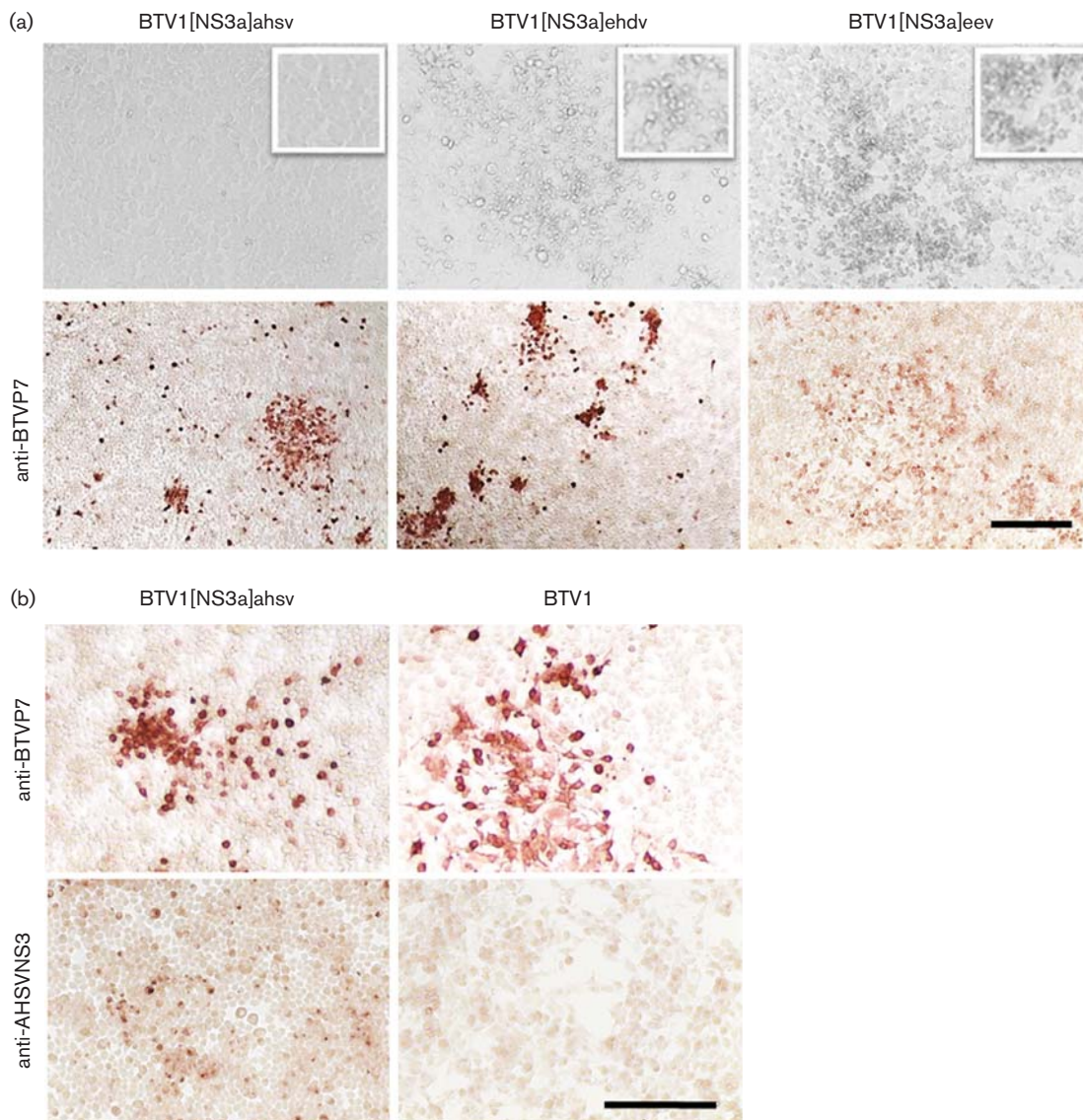


Fig. 2. (a) BSR monolayers were infected with BTV1[NS3a]ahsv, BTV1[NS3a]ehdv or BTV1[NS3a]eev. Upper panel: CPE at 48 h post-infection. Enlarged pictures show apoptotic bodies characteristic for BTV CPE. Lower panel: VP7 immunostaining of infected monolayers. (b) BSR cells infected with BTV1[NS3a]ahsv or BTV1 were immunostained with BTV VP7 (upper panel) or with AHSV NS3 (lower panel) specific mAbs. Bar, 200 μ m.

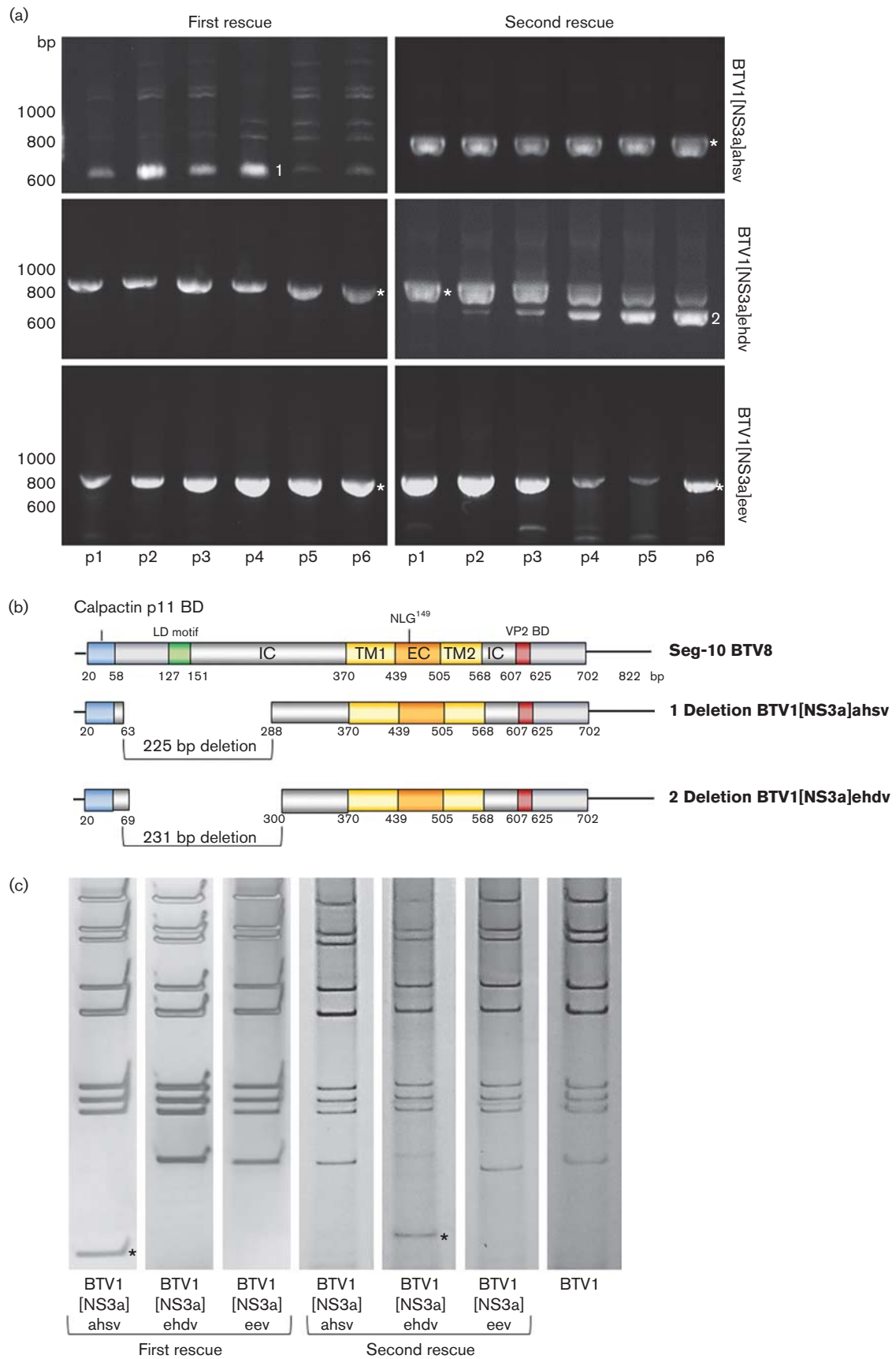


Fig. 3. (a) Seg-10 analysis of six subsequent passages of BTV1[NS3a]ahsv, BTV1[NS3a]ehdv and BTV1[NS3a]eev for two independent virus rescues. Indicated bands were isolated and sequenced. Bands indicated by * contained unchanged full-length Seg-10, and numbered bands contained mutated Seg-10 as shown in (b). (b) Schematic representation of full-length Seg-10 with NS3/NS3a domains indicated. The identified Seg-10 deletion mutants indicated by numbers corresponding to (a) are depicted below, with the size and location of the deletion indicated. (c) Deletions in Seg-10 were confirmed by dsRNA isolation from the sixth virus passage followed by gel electrophoresis analysis. Bands corresponding to Seg-10 with a deletion are indicated by *.

AHSV variant was now also stable during six passages, whereas Seg-10 of BTV1[NS3a]ehdv was not. This was confirmed by sequencing of Seg-10, revealing an in-frame deletion of 231 bp (69–300) (Fig. 3b). This deletion was also observed by examining the dsRNA of this virus (Fig. 3c). Since BTV1[NS3a]ahsv did not induce CPE despite a stable chimeric Seg-10 and thus putative protein expression, AHSV NS3/NS3a expression was examined (Fig. 2b). Immunostaining with AHSV NS3 specific mAbs was observed, indicating that expression of AHSV NS3/NS3a leads to a phenotype without CPE in the BTV background.

Growth and release of the stable BTV mutants with chimeric Seg-10 in both BSR (mammalian) and KC (*Culicoides*) cells were determined to study *in cis* complementation of NS3/NS3a of different orbiviruses (Fig. 4). In BSR cells, no obvious differences in growth between BTV1 and BTV1[NS3a]ehdv were shown. BTV1[NS3a]eev showed a slight but insignificant reduction of growth and release. The virus titre of BTV1[NS3a]ahsv in the cell fraction was also slightly but not significantly reduced. Release of this virus, however, was significantly reduced by >100 times at the end of the experiment compared with BTV1 (Fig. 4a).

In KC cells, all three chimeric viruses reached a significantly lower virus titre compared with BTV1 ($P < 0.05$) in both the cell fraction and the medium ($P < 0.01$) (Fig. 4b). No significant difference between the chimeric viruses was measured.

BTV reassortants with Seg-10 from BTV25 and BTV26

BTV1 and BTV8 with Seg-10 from BTV25 and BTV26 were generated. These BTV reassortants were stable as checked by PCR amplification of entire Seg-10 after several virus passages (data not shown). Infection of BSR cells induced obvious CPE, which was very similar to CPE induced by the ancestor BTV (Fig. 5a). However, the plaques seemed to be slightly smaller and denser for both BTV1- and BTV8-based Seg-10 reassortants.

Growth and release of the Seg-10 mono-reassortants were determined in both BSR (mammalian) and KC (*Culicoides*) cells, and compared with those in ancestor BTV and BTV without NS3/NS3a expression (Fig. 5b–e). BTV1[NS3]25 and BTV1[NS3]26 showed no significant differences in virus titres for both the cell-associated and secreted fraction in BSR cells (Fig. 5b). Virus titres for both fractions of KC cells were

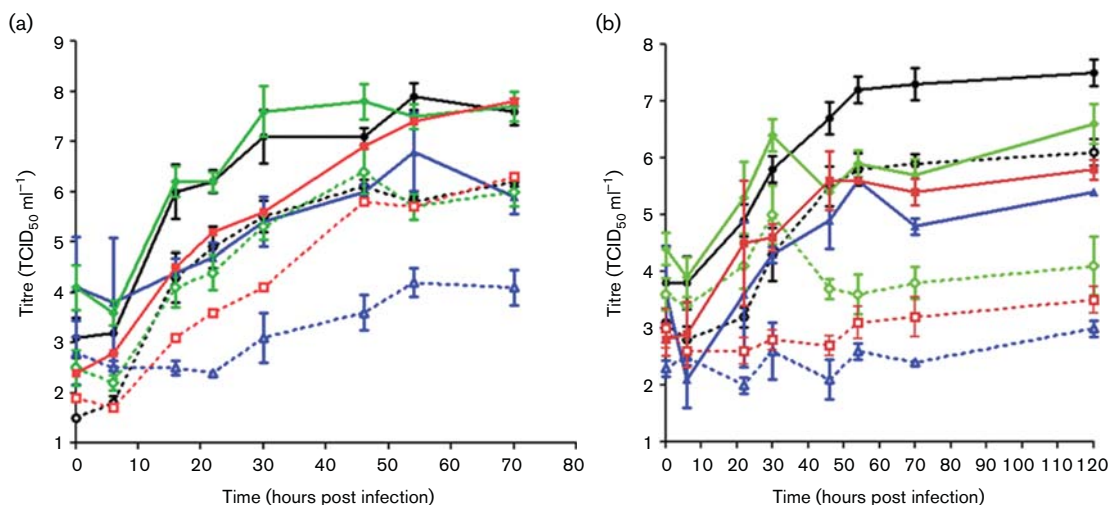


Fig. 4. Growth and release of BTV1 (black), BTV1[NS3a]ahsv (blue), BTV1[NS3a]ehdv (green) and BTV1[NS3a]eev (red) in BSR (a) and KC (b) cells. Dotted lines represent released virus and solid lines represent virus titre in the cell fraction. Error bars indicate SEM ($n=4$).

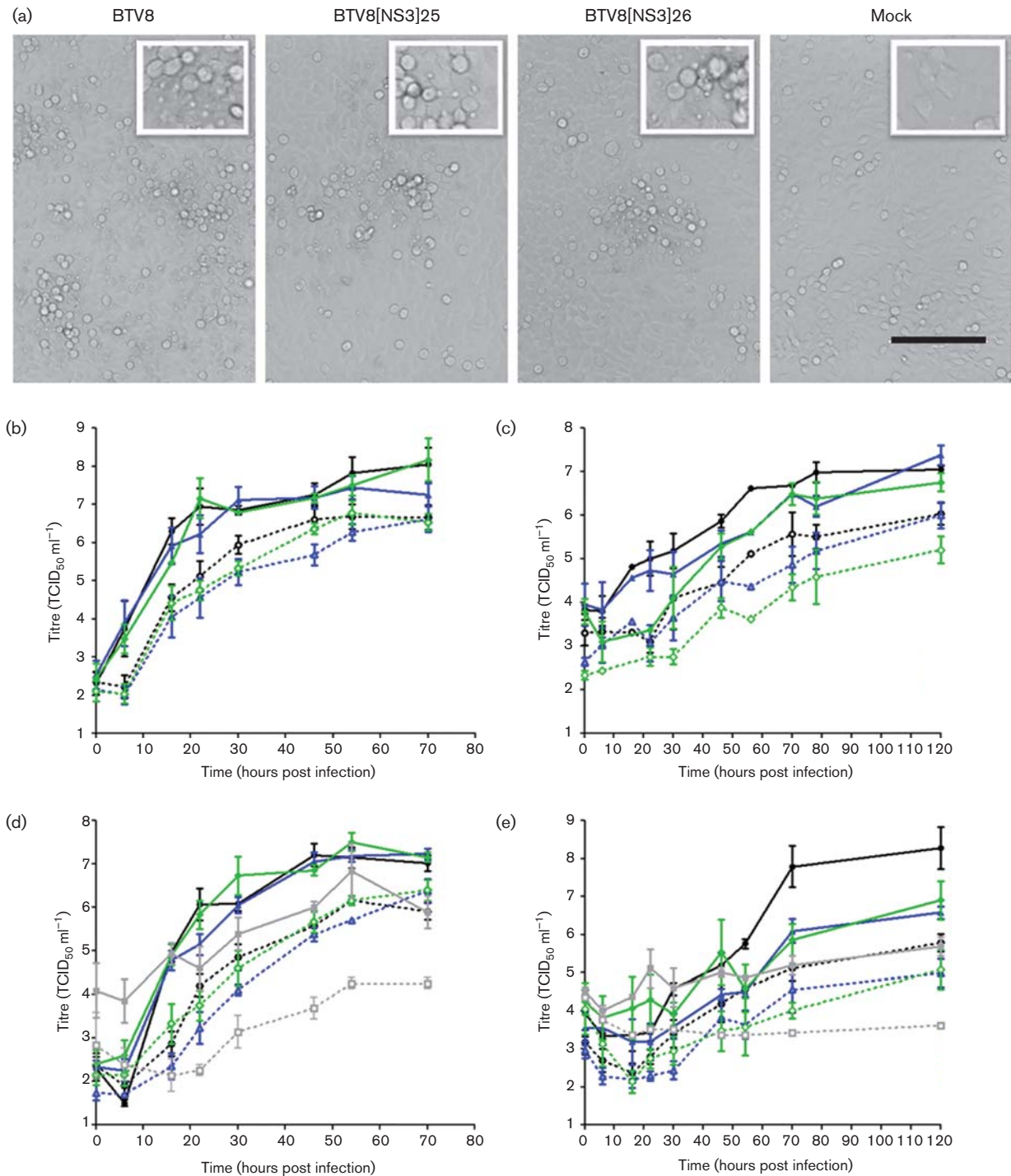


Fig. 5. (a) BSR cells infected with BTV8, BTV8[NS3]25 or BTV8[NS3]26 with an m.o.i. of 0.01 at 36 h post-infection. Enlarged pictures show apoptotic bodies characteristic for BTV CPE. Plaques induced by BTV8[NS3]25 and BTV8[NS3]26 seemed to be slightly smaller and denser compared with those induced by BTV8. Bar, 200 μ m. Growth and release of BTV1 (black), BTV1[NS3]25 (blue) and BTV1[NS3]26 (green) in BSR cells (b) and KC cells (c) were determined. Growth and release of BTV8 (black), BTV8[NS3]25 (blue), BTV8[NS3]26 (green) and BTV8[NS3]min (grey) were also measured in BSR cells (d) and KC cells (e). Solid lines represent growth in the cell fraction and dotted lines represent secreted virus. Error bars indicate SEM ($n=4$).

slightly but insignificantly lower for both BTV1-based reassortants during the course of the experiment (Fig. 5c).

In BSR cells, the growth of BTV8[NS3]25 and BTV8[NS3]26 was again not different from that of the ancestor virus BTV8. In this experiment, growth of the NS3/NS3a negative variant of BTV8 was also examined. This virus showed a significantly lower final titre compared with the other three viruses in the released fraction (Fig. 5d). Growth of all mutant viruses was clearly reduced in KC cells compared with growth of BTV8 (Fig. 5e). The highest titre of BTV8[NS3]25, BTV8[NS3]26 and BTV8[NS3]min was significantly lower compared with that of BTV8, in both the cell-associated fraction and the released fraction ($P < 0.05$).

DISCUSSION

Previously, it has been shown that NS3/NS3a is not essential for *in vitro* BTV replication by mutating both start codons (van Gennip *et al.*, 2014). RNA sequences within the NS3 ORF were shown to be essential for virus replication *in vitro*, independent of protein expression. Deleted RNA sequences were compensated by RNA inserts from other viral segments during virus rescue without restoring NS3/NS3a expression (Feenstra *et al.*, 2014b). Only small out-of-frame deletions in BTV Seg-10 were stable as long as NS3/NS3a-related protein was expressed and mutations disturbing NS3/NS3a expression were restored by point mutations (van Gennip *et al.*, 2014). Clearly, there is a strong selection for expression of NS3/NS3a, while so far unidentified RNA elements are also essential for virus replication. Here, this phenomenon was investigated in more detail by the introduction of multiple silent mutations in the NS3a ORF, therewith maintaining the translation of unchanged NS3/NS3a protein. Independently rescued variants with Seg-10(silent) showed that *in vitro* selection for RNA sequences in Seg-10 is stronger than for NS3/NS3a expression, since RNA sequences were deleted at the expense of protein expression. Since NS3/NS3a is not essential, a stronger selection for RNA sequences is explainable, also since selection on RNA takes place during packaging, which occurs before the selection on NS3/NS3a protein expression.

NS3/NS3a proteins are conserved among orbivirus species (Huismans *et al.*, 2004; van Niekerk *et al.*, 2003; van Staden & Huismans, 1991) and their function is likely similar (Jensen *et al.*, 1994; Meiring *et al.*, 2009; van de Water *et al.*, 2015; van Staden *et al.*, 1995). However, reassortants consisting of genome segments of different orbivirus species have never been isolated and virus rescue of such Seg-10 mono-reassortants using reverse genetics has failed. Likely, the highly conserved UTRs are specific for virus species and essential for RNA replication and recruitment of viral RNA for packaging. Indeed, BTV with Seg-10 containing point mutations in the UTRs is not viable (Boyce & McCrae, 2015; Burkhardt *et al.*, 2014; van Rijn *et al.*, 2013). To investigate *in cis* complementation of the NS3/NS3a function and conservation of the RNA sequence

and structure between orbiviruses, BTVs with chimeric Seg-10 containing the NS3a ORF of AHSV, EHDV and EEV were rescued.

Chimeric Seg-10 was not stable during virus passages, except for BTV1[NS3a]eev (Fig. 3). Apparently, viral Seg-10 RNA sequences from other orbiviruses are not optimal and deletion mutants were therefore selected. No insertions from other segments were found, which suggests that RNA elements or structures in the NS3a ORF of EEV, EHDV and AHSV are functional in BTV replication, and are likely conserved for orbiviruses. More research is needed to identify these elements or structures.

Interestingly, both observed deletions in the chimeras were in-frame and putative expression of TM regions and the C-terminal cytoplasmic region were maintained (Fig. 3b). However, only BTV1[NS3a]ehdv and BTV1[NS3a]eev induced CPE and virus release in mammalian cells (Figs 2a and 4a). Moreover, BTV1[NS3a]ehdv with the in-frame deletion still induced CPE, which suggests that expression of TM regions is involved in CPE in BSR cells and this function of NS3 is exchangeable between orbiviruses. In contrast, BTV NS3 is not exchangeable by AHSV NS3, since BTV1[NS3a]ahsv did not induce CPE and release in mammalian cells, even though AHSV NS3 expression was confirmed by immunostaining (Fig. 2b). The more specific function of BTV NS3 in release from KC cells was, however, not retained, since virus release in these cells was significantly reduced for all three chimeric viruses (Fig. 4b).

We conclude that there is a delicate balance between expression of NS3/NS3a and the presence of RNA sequences/structures in Seg-10 for orbivirus replication *in vitro*. Apparently, both protein and RNA sequences have co-evolved in the field situation, and are both partly conserved. The function of NS3/NS3a in the non-specific release from BSR cells (Hyatt *et al.*, 1989) is highly conserved, whereas virus release from *Culicoides* cells (French *et al.*, 1989; Guirakhoo *et al.*, 1995; Noad & Roy, 2009; Wechsler & McHolland, 1988; Wechsler *et al.*, 1989) is orbivirus specific. Likely, binding of NS3/NS3a to VP2 has differentiated and became specific for each orbivirus species. However, VP2 of serotypes of each orbivirus species also exhibit a high variability (Maan *et al.*, 2007).

NS3/NS3a of BTV25 and BTV26 are clearly distinct from that of the classical 24 BTV serotypes, including a remarkable amino acid difference in the highly conserved late domain (PPRYSPAP to PPGYPSAP). Virus growth of BTV25 and BTV26 Seg-10 reassortants based on cell-culture-adapted BTV1 and virulent BTV8 showed remarkable differences. Exchange of Seg-10 affected growth and release of virus in *Culicoides* cells in the BTV8 background, but not in the BTV1 background (Fig. 5b, c). BTV1 is well adapted to cell culture, which could lead to loss of some wild type characteristics, such as virulence, as is often seen for cell-culture-adapted viruses. Therefore, the small effects of Seg-10 exchange could be less prominent in the cell-culture-adapted strain

and remained unnoticed. Similarly, the effect of NS4 was not significant in the BTV1 background, whereas the effect was obvious in the background of virulent BTV8 (Ratinier *et al.*, 2011). Seg-10 reassortants of the virulent BTV8 backbone did not affect virus growth in mammalian (BSR) cells. However, lower virus titres than for ancestor BTV8 were observed in *Culicoides* (KC) cells (Fig. 5d, e). This suggests that NS3/NS3a of BTV25 and 26 could be involved in the distinct characteristics of these new BTV serotypes (Batten *et al.*, 2012, 2014; Chaignat *et al.*, 2009; Planzer *et al.*, 2011).

The BTV Seg-10 mutants presented here show that orbiviruses have evolved both on the function of expressed viral proteins and viral RNA sequence/structure of genome segments. Apparently, both are important for virus replication *in vitro*. Previously, it has been shown that NS3/NS3a is required for viraemia in sheep and virus propagation in *Culicoides* (Feenstra *et al.*, 2014a, 2015). Thus, NS3/NS3a is crucial *in vivo* for virus transmission between the insect vector and the ruminant host. However, NS3/NS3a protein is not essential for virus replication *in vitro* (van Gennip *et al.*, 2014), whereas RNA elements in the Seg-10 ORF are essential for BTV replication (Feenstra *et al.*, 2014b). Indeed, we observed here a strong selection on the Seg-10 RNA sequence at the expense of NS3/NS3a expression, when studying silent mutations in Seg-10. The functional Seg-10 RNA elements seem to be conserved among orbiviruses as these were exchangeable, shown by BTV chimeras containing NS3a ORFs of other orbivirus species. The exact function of these RNA elements is still unknown and no obvious sequence or structure homology between inserted sequences could be recognized. Still, it might be possible that these RNA inserts contain packaging signals or will form RNA structures to enable packaging. More studies are needed to elucidate this phenomenon, since there is apparently a strong preference to insert these RNA sequences during rescue of BTV with Seg-10(silent) or with small deletions in Seg-10 (Feenstra *et al.*, 2014b). NS3/NS3a functions are only partially exchangeable between orbiviruses, since induction of CPE in mammalian cells was observed for some chimeras, whereas efficient release from insect cells was not observed. Seg-10 and NS3/NS3a sequences of two newly discovered BTV serotypes are probably involved in the distinct tropism of these viruses. More studies are needed to further elucidate the exact role of Seg-10 RNA as well as of its encoded NS3/NS3a protein in virus replication and virus release.

METHODS

Cell culture. BSR cells (clone of BHK-21 cells; Sato *et al.*, 1977) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 5% FBS, 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 2.5 µg amphotericin B ml⁻¹. KC cells (Wechsler *et al.*, 1989) derived from embryos of colonized *Culicoides sonorensis* (Jones & Foster, 1978) were grown in modified Schneider's Drosophila medium with 15% FBS, 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ at 27 °C.

Viruses. BTV1 (GenBank accession numbers FJ969719–FJ969727) with Seg-10 originating from BTV8 (FJ183383) and BTV8 (FJ183374–FJ183383) were used as virus backbones to generate BTV1 or BTV8 derivatives using reverse genetics as previously described (Boyce *et al.*, 2008; van Gennip *et al.*, 2012). In short, 10⁵ BSR cells were transfected with plasmids expressing VP1, VP3, VP4, NS1, NS2 and VP6. Eighteen hours post-transfection, BSR monolayers were transfected again with all 10 full-length RNA transcripts. Virus rescue was performed in duplicate. Wells were screened for CPE and VP7 expression by immunostaining of fixed monolayers with anti-VP7 mAb (ATCC CRL-1875) (American Type Culture Collection) according to standard procedures (Wensvoort *et al.*, 1986). Without signs of virus replication, cells in the duplicate well were repeatedly passaged to rescue virus mutants with delayed growth characteristics, until no VP7 expression could be identified any more using immunostaining. Attempts were repeated at least twice to be able to conclude that a virus containing a certain mutation could not be rescued. Virus stocks were prepared by infection of fresh BSR monolayers at low m.o.i., and harvested by freeze–thawing when > 50% of cells were immunostained or showed CPE in the duplicate well. Virus titres were determined by end-point dilution assays on BSR cells and expressed as TCID₅₀ ml⁻¹.

BTV8 without NS3/NS3a expression (BTV8[NS3]min) was generated by incorporation of BTV8 Seg-10 ΔC containing an out-of-frame deletion from position 102–263 (Feenstra *et al.*, 2014b). BTV8 Seg-10 with 180 silent mutations in the NS3a ORF was generated by GenScript Corporation, Piscataway, NJ, USA [Seg-10(silent); Fig. S1 available in the online Supplementary Material]. Virus with this mutated BTV8 Seg-10 was named BTV1[NS3a]silent. Seg-10 with sequences of AHSV, EHDV and EEV were based on GenBank accession numbers KM820858, HM636906 and AY115878, respectively, and were also generated by GenScript. BTVs with Seg-10 containing the NS3a ORFs of AHSV, EHDV or EEV were named BTV1[NS3a]ahsv, BTV1[NS3a]ehdv and BTV1[NS3a]eev, respectively. BTV1 with Seg-10 from BTV25 and BTV26 (EU839846 and JN255162) were named BTV1[NS3a]25 and BTV1[NS3a]26. Note that the NS3 ORF of BTV25 was flanked by UTRs of BTV8 Seg-10, since UTR sequences of BTV25 Seg-10 were not available.

Analysis of mutants and reassortants by PCR, sequencing and immunostaining. Stability of rescued virus mutants was investigated during six subsequent passages on BSR cells. RNA of passaged virus was isolated using the High Pure Viral RNA kit (Roche) according to manufacturer's protocol. Entire Seg-10 was reverse transcribed and amplified using the one-step RT-PCR kit (Qiagen) with Seg-10 primers (F-full-S10* 5'-GTTAAAAAGTGTGCGTGCC-3' and R-full-S10 5'-GTAAGTGTGTAGTGTGCGGCAC-3') as described previously (Feenstra *et al.*, 2014b). Purified amplicons were sequenced using the same primers and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The complete consensus sequence was assembled and determined using Lasergene SeqMan Pro Software (DNASTAR, version 12).

CPE of infected BSR monolayers was examined microscopically and infection was confirmed by immunostaining with VP7-specific mAb ATCC CRL-1875. Expression of AHSV NS3a by infection with BTV1[NS3a]ahsv was similarly immunostained with AHSV NS3-specific mAb 4D3 (Ingenasa). Transient expression of NS3 by Seg-10 with 180 silent mutations was confirmed by transfection of BSR cells with Seg-10(silent) RNA using Lipofectamine 2000 (Invitrogen), followed by immunostaining using BTV NS3 mAbs 32B6 and 33H7 (Ingenasa).

Analysis of viral dsRNA. Viral dsRNA was analysed to detect significant changes in Seg-10 of mutant viruses in order to exclude PCR artefacts. BSR monolayers were infected with six-times-passaged mutant BTV. Medium was discarded at 48 h post-infection and

0.1 ml Trizol cm^{-2} was added to the cells and incubated for 5 min at room temperature. Then, 0.2 ml chloroform $(\text{ml Trizol})^{-1}$ was added and the mixture was centrifuged for 10 min at 6240 g. The water phase was isolated and RNA was precipitated with 0.8 ml 2-propanol ml^{-1} , and centrifuged for 30 min at 16 000 g, 4 °C. The pellet was washed with 70 % ethanol and dissolved in 100 μl RNase-free water. To precipitate ssRNA, 50 μl of 7 M LiCl was added, and incubated for 30 min at -20 °C. After centrifugation for 15 min (16 000 g, 4 °C), dsRNA in the supernatant was further purified using the RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol. Approximately 200 ng dsRNA was separated by 4–12 % PAGE and visualized using the Silverexpress silver staining kit (Invitrogen) according to the manufacturer's protocol.

Virus growth and release assays. Monolayers of KC cells (5×10^6) or BSR cells (5×10^5) in 2 cm^2 wells were infected with virus at an m.o.i. of 0.01. Virus was attached for 1.5 h at 27 °C or 37 °C for KC and BSR cells, respectively. Unattached virus was removed by washing with PBS, and fresh medium was added. This time point was set as 0 h post-infection. Incubation was continued and cells and culture media were harvested at the indicated time points. To release virus from cell fractions, cells were lysed by freeze–thawing at -80 °C. Virus titres in cell and culture medium were determined using end-point dilution in BSR cells. Assays were repeated independently four subsequent times with two independently generated virus stocks. The highest titres reached for different viruses in the cell fraction and the culture medium were compared using a one-way ANOVA with post hoc Tukey's test.

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