Structural Protein VP2 of African Horse Sickness Virus Is Not Essential for Virus Replication *In Vitro*

René G. P. van Gennip,^a Sandra G. P. van de Water,^a Christiaan A. Potgieter,^{b,c} Piet A. van Rijn^{a,b}

Department of Virology, Central Veterinary Institute of Wageningen University and Research Center, Lelystad, The Netherlands^a; Department of Biochemistry, Centre for Human Metabolomics, North-West University, Potchefstroom, South Africa^b; Deltamune, Lyttelton, South Africa^c

ABSTRACT The *Reoviridae* family consists of nonenveloped multilayered viruses with a double-stranded RNA genome consisting of 9 to 12 genome segments. The *Orbivirus* genus of the *Reoviridae* family contains African horse sickness virus (AHSV), bluetongue virus, and epizootic hemorrhagic disease virus, which cause notifiable diseases and are spread by biting *Culicoides* species. Here, we used reverse genetics for AHSV to study the role of outer capsid protein VP2, encoded by genome segment 2 (Seg-2). Expansion of a previously found deletion in Seg-2 indicates that structural protein VP2 of AHSV is not essential for virus replication *in vitro*. In addition, in-frame replacement of RNA sequences in Seg-2 by that of green fluorescence protein (GFP) resulted in AHSV expressing GFP, which further confirmed that VP2 is not essential for virus replication. In contrast to virus replication without VP2 expression in mammalian cells, virus replication in insect cells was strongly reduced, and virus release from insect cells was completely abolished. Further, the other outer capsid protein, VP5, was not copurified with virions for virus mutants without VP2 expression. AHSV without VP5 expression, however, could not be recovered, indicating that outer capsid protein VP5 is essential for virus replication *in vitro*. Our results demonstrate for the first time that a structural viral protein is not essential for orbivirus replication *in vitro*, which opens new possibilities for research on other members of the *Reoviridae* family.

IMPORTANCE Members of the *Reoviridae* family cause major health problems worldwide, ranging from lethal diarrhea caused by rotavirus in humans to economic losses in livestock production caused by different orbiviruses. The *Orbivirus* genus contains many virus species, of which bluetongue virus, epizootic hemorrhagic disease virus, and African horse sickness virus (AHSV) cause notifiable diseases according to the World Organization of Animal Health. Recently, it has been shown that nonstructural proteins NS3/NS3a and NS4 are not essential for virus replication *in vitro*, whereas it is generally assumed that structural proteins VP1 to -7 of these nonenveloped, architecturally complex virus particles are essential. Here we demonstrate for the first time that structural protein VP2 of AHSV is not essential for virus replication *in vitro*. Our findings are very important for virologists working in the field of nonenveloped viruses, in particular reoviruses.

KEYWORDS reovirus, orbivirus, AHSV, structural protein VP2, virus replication, reverse genetics

Reoviruses are nonenveloped icosahedral symmetric viruses of several layers of proteins with a segmented genome consisting of 9 to 12 segments of double-stranded RNA. The *Reoviridae* family consists of several genera, of which the *Orbivirus* genus contains many virus species (1, 2), including African horse sickness virus (AHSV),

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Address correspondence to René G. P. van Gennip, rene.vangennip@wur.nl.

bluetongue virus (BTV), and epizootic hemorrhagic disease virus (EHDV), which cause notifiable animal diseases. These diseases are noncontagious, infectious, arthropod-borne viral diseases transmitted by biting *Culicoides* midges (3).

AHSV forms a distinct virus species with nine serotypes (AHSV1 to -9) within the genus *Orbivirus* (4, 5). The mortality rate caused by AHSV infection is over 90% in fully susceptible domestic horses, whereas zebras and African donkeys rarely show clinical signs (6). AHS is endemic to sub-Saharan Africa and has periodically caused epidemics in other areas, such as India, Pakistan, Spain, and Portugal (7–12). *Culicoides imicola* and *C. bolinotos* are considered the most important midge species for transmission of AHSV in South Africa (10, 13–15). The geographical distribution and seasonal incidence of AHS are largely determined by the presence of these competent *Culicoides* vectors.

The orbivirus genome consists of 10 genome segments encoding seven structural proteins, VP1 to -7, and at least four nonstructural proteins, NS1 to -4. The structural proteins VP2, VP3, VP5, and VP7 form a triple-layered virus particle (16), whereas VP1, -4, and -6 are located inside this particle and form the replication complex required for replication and transcription of viral RNA (17, 18). Nonstructural proteins are involved in virus replication, morphogenesis, and release from the infected cell (18–21). Recently, an additionally translated open reading frame (ORF) has been identified in genome segment 10 (Seg-10 ORF2), the protein function of which is still unknown (22).

NS3/NS3a, NS4, and the proposed Seg-10 ORF2 of BTV are not essential for virus replication *in vitro* (19, 20, 23), whereas NS1 and NS2 are not needed for reconstitution of infectious core particles of BTV (24). As is the case for BTV, NS3/NS3a expression of AHSV could be abolished, leading to promising vaccine candidates, named disabled infectious single animal (DISA) vaccine (25).

The DISA vaccine platforms for BTV and AHSV have been applied for many serotypes by exchange of Seg-2 expressing the serotype-determining VP2 protein (25, 26). Outer capsid protein VP2 is the major target for serotype-specific neutralizing antibodies (27–38) inducing serotype-specific protection (39, 40). VP2 is the most variable protein of orbiviruses (41, 42) and is involved in attachment to cells by binding to sialic acid moieties of cellular receptors prior to internalization of the virus particle (43, 44). Further, VP2 is involved in virus assembly and egress by interacting with vimentin and a component of the cellular ESCRT pathway via binding to nonstructural viral protein NS3/NS3a (45, 46). Proteolytic cleavage of orbivirus led to infectious subviral particles with similar specific infectivity for BHK-21 cells. Core particles, i.e., orbivirus particles from which the outer shell proteins were removed, are infectious, indicating that VP2 and VP5 are not essential for infection of mammalian and insect cells (47).

More recently, AHSV serotype 7 with an in-frame deletion in Seg-2 corresponding to 225 amino acids (amino acid positions 279 to 503) has been extensively studied (48). AHSV7 expressing this truncated VP2 (tVP2) exhibits a growth advantage in tissue culture, and induction of cytopathic effect (CPE) is faster than for AHSV4 with full-length VP2 protein. Remarkably, the region from amino acids 340 to 360 has been associated with tissue tropism and virulence (49), and several regions within this deletion are most probably located on the tips of VP2 triskelions and at the outside to the top of the triskelion hub (central domain) (48). More remarkably, the deleted region is very immunogenic (31, 50), and this part of structural protein VP2 is not essential for virus replication *in vitro*. Here we studied additional deletions in Seg-2 of VP2 (Seg-2[VP2]) to study the function of structural protein VP2 in AHSV replication *in vitro*.

RESULTS

AHSV4LP expressing truncated VP2 protein from different serotypes is viable.

Reverse genetics of virus strain AHSV4LP has been used to exchange Seg-2 of all nine AHSV serotypes (25). Here, we studied mutated Seg-2 expressing truncated VP2 (tVP2) of different serotypes. Seg-2[tVP2] of serotype 7 contains the in-frame deletion of 225 amino acid codons from residue 279 to 503 (48). This Seg-2[tVP2] of serotype 7 was incorporated in the virus background of AHSV4LP. Similar deletions in Seg-2[tVP2] of serotypes 4 (Fig. 1) and 5 (from amino acids 285 to 509, a deletion of 224 amino acids)

FIG 1 Schematic overview of mutations in genome segment 2. AHSV4LP strains with mutated Seg-2 were rescued (+) or were not viable (–). Putatively translated, untranslated, and deleted RNA sequences are indicated by boxes, lines, and dashed lines, respectively. Putatively expressed GFP ORF is indicated by striped boxes. AUG→GCC mutations of putative in-frame start codons are indicated by asterisks at nucleotide positions 13 to 16, 37 to 39, 196 to 198, 214 to 216, 343 to 345, 279 to 281, 529 to 531, 658 to 660, and 661 to 663. Nucleotide positions are indicated with respect to full-length Seg-2 (at the top, with the start at position 13 and the stop at position 3192), and the lengths of Seg-2 of mutants are shown at the right. Amino acid positions of interest are indicated with respect to AHSV4LP VP2 on the second bar. Expression of the mutated VP2s of the respective viruses was determined by immunostaining with antibodies directed against VP2 of serotype 4.

were constructed and were successfully incorporated in AHSV4LP. AHSV4 strains expressing tVP2 from different serotypes were detected by immunostaining (immunoperoxidase monolayer assay [IPMA]) of infected monolayers with anti-VP5 (α -VP5) monoclonal antibody (MAb) and serotype-specific α -VP2 GP sera (not shown). Similar to the case for tVP2 of serotype 7, these results indicate that the corresponding parts of VP2 of serotypes 4 and 5 are not essential for virus replication *in vitro*. Presumably, tVP2 proteins of other AHSV serotypes are also functional, but these were not investigated.

VP2 expression is not essential for *in vitro* replication of AHSV4LP. To abolish translation of the N-terminal part of VP2, AUG→GCC mutations were introduced for all putative in-frame start codons upstream of the deletion in Seg-2[tVP2] of serotype 4 (mutVP2) (Fig. 1). AHSV4LP with Seg-2[mutVP2] was rescued, demonstrating that the N-terminal part of VP2 is not essential (Fig. 1). Then, unique restriction sites in Seg-2 were used to expand the previously studied deletion in Seg-2[tVP2] (Fig. 1). Δ A and Δ B contain expanded deletions adjacent to the 3' and 5' termini of the deletion in Seg-2[tVP2], respectively. The first deletion (Δ A) resulted in an out-of-frame deletion of a further 650 nucleotides (nt) downstream the original deletion (see Fig. 1), where only the N-terminal 284 amino acids were translated. The second deletion (Δ B) also resulted in an out-of-frame deletion of a further 700 nucleotides mainly upstream of the original deletion, where only the N-terminal 69 amino acids were translated. AHSV4LP with these deletions (AHSV4 Δ A and AHSV4 Δ B) was rescued, indicating that these regions are also not essential (Fig. 1). Since deletions in AHSV4 Δ A and AHSV4 Δ B are out-of-frame deletions, putative translation of the remaining C-terminal part of VP2 was aborted, indicating that the entire C-terminal part of the VP2 protein is not essential.

The viability of AHSV4-mutVP2, AHSV4 Δ A, and AHSV4 Δ B was confirmed by infection of fresh BSR monolayers followed by induction of CPE and immunostaining with α -VP5 MAb. AHSV4 mutants with AUG→GCC mutations or with deletions in Seg-2 of serotype 4 were negative for immunostaining with antibodies directed against VP2 of serotype 4, except for AHSV4 with tVP2 (Fig. 1). Altogether, these results show that VP2 protein

FIG 2 AHSV4LP expressing GFP in Seg-2. (A) Cytopathogenic effect (CPE) (top panels) and coinciding GFP expression (bottom panels) in BSR monolayers were observed at 2 days postinfection with AHSV4ΔC-GFP for successive virus passages 4, 5, and 6. Magnified pictures indicate apoptotic bodies characteristic for CPE induced by AHSV. Bars represent 400 μm. (B) Genetic stability of Seg-2 of AHSV4ΔC-GFP was studied by reverse transcription-PCR (RT-PCR) amplification of specific overlapping regions in ΔC-GFP for successive virus passages 4, 5, and 6.

is not expressed by the indicated AHSV4LP mutants, and we therefore conclude that structural VP2 protein is not essential for virus replication *in vitro*.

Viral RNA elements in the ORF of Seg-2[VP2] are essential for virus rescue. To study the role of RNA sequences of Seg-2 in virus replication, the deletion in the ORF of VP2 was further expanded to 2,434 bp (ΔC) (Fig. 1). The deletion ΔC comprised an out-of-frame deletion of a further 1,800 nucleotides, resulting in a C-terminally truncated VP2 where only 70 amino acids are translated. AHSV4ΔC was rescued and showed CPE and immunostaining with α-VP5 MAb, demonstrating that the region from nt 215 to 2648 in the ORF of Seg-2 is not essential for virus replication. Sequences of untranslated regions (UTRs) of genome segments are considered essential and were not investigated. Deletion ΔC was the largest deletion in Seg-2 tested, but it still contained RNA sequences of the ORF from the start codon to nt 215 and from nt 2648 to the stop codon. Aiming to investigate the RNA sequences from nt 13 to 215 and 2648 to 3192, we first inserted foreign RNA sequences (ORF of green fluorescent protein [GFP]) in frame in ΔC (ΔC-GFP). AHSV4LP with ΔC-GFP was rescued (Fig. 1, AHSV4ΔC-GFP). Segment ΔC-GFP was extensively studied by sequencing of overlapping amplicons covering the entire Seg-2 and inserted GFP ORF, but no insertions or other modifications were found. GFP was stably expressed for at least six consecutive virus passages (Fig. 2). Clearly, GFP expression colocalized with CPE, and GFP ORF was stably inserted in AHSV4ΔC-GFP. Then, the GFP ORF was inserted between the UTRs (ΔD-GFP) resulting in deletion of RNA sequences from nt 13 to 215 and 2648 to 3192 compared to ΔC-GFP (Fig. 1). Despite several attempts, AHSV with ΔD-GFP was not rescued (Fig. 1, AHSV4ΔD-GFP), which indicates the importance for virus rescue of the RNA sequences adjacent to one or both UTRs of Seg-2. Apparently, RNA sequences within the ORF and flanking the UTRs are crucial for virus replication; however, no conclusions can be drawn about the minimal length of these RNA sequences. Still, the RNA sequences adjacent to start and stop codons in the VP2 ORF seem to be specific, since these could not be replaced by foreign RNA sequences such as the GFP ORF sequence tested here.

FIG 3 Constellation of Seg-2 deletion mutant AHSVs. Viral dsRNA was isolated from infected cells after four virus passages for AHSV4LP (wt) and the indicated virus mutants (see Fig. 1). Genome segments 1 to 10 (S1 to S10) of AHSV4LP and their lengths (bp) are indicated at the right. Asterisks indicate Seg-2 of AHSV mutants.

AHSV4LP mutants with deletions in Seg-2 are genetically stable. AHSV4LP mutants were once more passaged on BSR cells, and genomic double-stranded RNA (dsRNA) was studied (Fig. 3). Obviously, Seg-1 and Seg-3 to Seg-10 were identical to those of the ancestor virus AHSV4LP. Seg-2 of the deletion variants migrated faster than the entire Seg-2, as expected because of their reduced lengths. The length of whole Seg-2 of AHSV4LP is 3,229 bp, and Seg-2[tVP2] and ΔA migrated as segments of 2,554 and 1,893 bp in length, respectively (Fig. 3). ΔB (1,846 bp) comigrated with Seg-4 (1,978 bp), which is slightly slower than expected. Sequencing of the entire ΔB , however, confirmed the deletion in ΔB , and no additional modifications were found (not shown). ΔC (765 bp) migrated slightly slower than Seg-10, which is 757 bp in length. AHSV4 ΔC -GFP contains a Seg-2 band of approximately 1,560 bp, which was expected because of the insert of GFP ORF in ΔC .

AHSV without VP2 expression forms infectious particles. The composition of AHSV particles with different deletions in Seg-2[VP2], AHSV4LP, AHSV4-tVP2, and AHSV4 ΔC was studied by Western blot analysis of proteins of partially purified virions with antibodies specific for VP7, VP5, and VP2 (Fig. 4). VP7 protein was detected for all three studied AHSV4LP variants, confirming the isolation of virions (lower panel). VP5 and VP2 were detected for AHSV4LP and AHSV-tVP2, although detection of tVP2 and VP5 was much weaker, but tVP2 is indeed represented as a smaller protein (upper and middle panels). No VP2 or VP5 was detected in purified particles of AHSV4 ΔC (Fig. 4, right lanes), indicating that these purified particles are similar to core particles. This could suggest that VP5 is not essential either, but AHSV with small mutations abolishing VP5 expression could not be rescued (not shown). VP5 protein was expressed in cells infected by AHSV4 ΔC as shown by IPMA with α -VP5 MAb, and this VP5 expression is apparently essential for virus rescue (Fig. 5). We conclude that VP5 protein is essential for virus replication *in vitro*, although the protein was not copurified with the AHSV4 ΔC particles. In contrast, AHSV4 ΔC without VP2 expression is viable and forms virion particles that are infectious for BSR cells.

VP2 is involved in growth and release of AHSV. Virus growth of AHSV4LP, AHSV4-tVP2, and AHSV4 ΔC was studied in BSR (mammalian) and KC (insect) cells (Fig. 5). AHSV4LP and virus mutants AHSV4-tVP2 and AHSV4 ΔC showed plaques of similar size in BSR monolayers immunostained with α -VP5 MAb (Fig. 5A, upper panels). In KC

FIG 4 Western blot analysis. Proteins VP2, VP5, and VP7 of purified virus particles for mock infection and AHSV4LP, AHSV4-tVP2, and AHSV4ΔC were analyzed. Virus proteins were detected using VP2-, VP5-, and VP7-specific antibodies as shown. The weak detection of the smaller tVP2 is indicated by an asterisk. The positions of protein molecular mass standards are indicated at the left in kDa.

cells, AHSV4LP and AHSV4-tVP2 formed groups of immunostained cells, indicating virus spread, whereas AHSV4ΔC infection resulted in single immunostained cells, indicating that this mutant was not spreading to neighboring cells (lower panels). It is likely that AHSV4ΔC is not released from KC cells after initial infection.

Virus replication and virus release in BSR and KC cells was studied in more detail (Fig. 5B). In BSR cells, growth of AHSV4LP, AHSV4-tVP2, and AHSV4ΔC confirmed that virus replication was not hampered by lack of VP2 protein (left panel). The growth of all three viruses on BSR cells is very similar for cell-associated virus and released virus. AHSV4ΔC seemed to be slightly delayed, but this likely reflects the lower percentage of initially attached virus. The growth kinetics of AHSV4LP, AHSV4-tVP2, and AHSV4ΔC were very similar, and the virus titer was not significantly different after 48 to 72 h postinfection (hpi) in BSR cells.

In KC cells, the initial virus titer of AHSV4ΔC after attachment to KC cells ($t = 0$) was also lower than that for AHSV4LP and AHSV4-tVP2, as observed for BSR cells. AHSV4LP and AHSV4-tVP2 showed very similar growth kinetics for the cell-associated and the released virus (Fig. 5B, right). AHSV4ΔC grew significantly slower than AHSV4LP and AHSV4-tVP2, as observed for the KC cell-associated fraction with maximum virus titers of $4.46 \log_{10}$ 50% tissue culture infective dose ($TCID_{50}$)/ml for AHSV4ΔC and 7.81 and $7.95 \log_{10}$ $TCID_{50}$ /ml for AHSV4LP and AHSV4-tVP2, respectively ($P < 0.05$). Also, the virus titer of AHSV4ΔC in the medium of infected KC cells did not increase. Apparently, release of AHSV4ΔC from KC cells is completely blocked due to the lack of VP2 protein.

DISCUSSION

Previously, it has been shown that nonstructural proteins NS3/NS3a and NS4 are not essential for BTV replication *in vitro* (19, 20, 23). Further, nonstructural proteins NS1 and NS2 are not needed to reconstitute infectious BTV core particles (24). The recently discovered Seg-10 ORF2 is also dispensable (22, 51). Regarding structural proteins VP1 to -7 of orbiviruses, it has been assumed that these are all essential for virus replication. Surprisingly, a region in VP2 of AHSV7, probably located at the outside of the virus particle, is not essential for virus replication *in vitro* (48). We show here that corresponding regions in VP2 of other serotypes are also dispensable. More importantly, we show for the first time that the structural VP2 protein, one of the outer capsid proteins of AHSV, is not essential for *in vitro* virus replication in BSR (mammalian) cells (Fig. 1 and 5).

FIG 5 Phenotype and virus growth of AHSV4LP, AHSV4-tVP2, and AHSV4ΔC on mammalian and insect cells. (A) Monolayers were infected at an MOI of 0.1. Infection was visualized by immunostaining with α -VP5 MAb. Upper row, infected BSR monolayers at 1 day postinfection. Bars 1,000 μ m. Lower row, infected KC monolayers at 2 days postinfection. Bar, 200 μ m. Note that KC monolayers infected with AHSV4ΔC show single immunostained cells. (B) Virus titers in medium and cell-associated fractions after infection of BSR or KC cells were examined at the indicated time points. Values represent means and standard deviations from three independent experiments.

Since expression of VP2 protein is not essential, we investigated the requirement of RNA sequences of Seg-2 for virus replication. Presumably, nontranslated regions are essential, and these were excluded for further studies. A major part of the VP2 ORF up to 2,434 bp in length was dispensable (Fig. 1, AHSV4ΔC). To study the importance of RNA sequences adjacent to the UTRs, the GFP ORF was first inserted in the largest tested deletion, ΔC, since foreign RNA inserts were unstable in Seg-10 of BTV (51, 52). AHSV4ΔC-GFP was genetically stable for up to six virus passages, and GFP was still expressed. Thus, in agreement with previous findings for deletions in Seg-10 of AHSV (25), large deletions in Seg-2 are stable and the foreign GFP gene was stably expressed.

Subsequently, the GFP ORF was inserted between exclusively UTR sequences of Seg-2 (ΔD-GFP), but the attempted AHSV4ΔD-GFP virus could not be rescued (Fig. 1). We conclude that viral RNA sequences adjacent to either of the UTRs of Seg-2 of AHSV4LP as present in ΔC-GFP are essential for virus replication (Fig. 1) and cannot be replaced by foreign RNA sequences or compensated for by viral *cis* RNA sequences. In addition to UTR sequences, these adjacent RNA sequences within the ORF are essential for virus rescue, as previously described for mutations adjacent to the 5' UTR of the NS3 ORF in Seg-10 of BTV (53–55). More research is needed to elucidate the minimal RNA sequences required for replication of AHSV4LP.

Mutant AHSV7-tVP2 forms RNA-filled, spherical, intact virus particles containing a smaller VP2 protein as shown by cryo-electron microscopy analysis (48). In our exper-

iments, the protein band of tVP2 of serotype 4 is very weak as observed by Western blot analysis (Fig. 4), suggesting a smaller amount of this protein in partially purified particles. On the one hand, binding of tVP2 protein to core particles could be weaker than for VP2, and therefore a part of tVP2 would have been lost during purification of AHSV4-tVP2 particles. On the other hand, detection of transferred tVP2 with MABs could be less sensitive because of small conformational changes. Nevertheless, tVP2 protein binds to the core particles together with VP5, whereas VP5 was not copurified without VP2 (Fig. 4, middle panel, lane AHSV4 Δ C). This could suggest that VP5 protein is not essential either; however, AHSV not expressing VP2 and small mutations in Seg-6 abolishing VP5 expression is not viable (not shown). Interactions between VP5 trimers and VP7 on the subcore are predicted to be weak (44). VP5 probably was dissociated from the virus particles by the purification method used. To further investigate this, attempts to rescue AHSV4 with mutated Seg-6 were performed, but this single Seg-6 mutant was not rescued (not shown). Taking the results together, the detailed role of VP5 in virus replication is unclear. VP5 could act as protein bound to the virus particle or as free protein in the infected cell. Further studies, such as electron microscopic images, are required to elucidate the location of VP5 for AHSV mutants lacking VP2 protein. We conclude that, in contrast to the dispensable VP2, AHSV VP5 protein is essential for *in vitro* virus replication.

Removing the outer shells of orbiviruses has resulted in core particles that are infectious for both mammalian and insect cells (56). In addition, infectious BTV core particles can be reconstituted *in vitro* using 10 synthesized runoff RNA transcripts and artificially produced proteins VP1, -3, -4, -6, and -7 (24). For both derived core particles, a complete set of 10 dsRNA segments is present in the core particle. Therefore, infection of cells with these core particles resulted in a complete orbivirus, including the outer shell. Mutant AHSV4 Δ C is not able to reconstitute complete orbivirus, as infectious particles without VP2 are assembled. The presence of VP5 on this particle is unclear, but expression in the infected cell is essential. Virus is secreted from mammalian cells by a nonspecific process independent from the NS3/NS3a-VP2-mediated release process. VP5 protein might play a role in this process, since VP5 possesses a conserved domain involved in membrane targeting. Further, VP5 interacts with membrane-associated NS3, which might be important for virus assembly (57).

In contrast to virus release from BSR cells, release of AHSV4 Δ C from KC cells is completely blocked by the deletion of VP2 (Fig. 5). AHSV4-tVP2 is released from KC cells similarly to AHSV4LP. Apparently, tVP2 protein is fully functional in this specific release process. Consequently, the binding site on VP2 of NS3/NS3a is not located in the region from amino acid 283 to 508 of VP2 protein of AHSV4LP.

Previously, we have also shown that virus release is completely blocked for NS3/NS3a knockout mutants of AHSV and BTV (23, 25, 51). BTV release is proposed to occur by binding of the C terminus of NS3/NS3a to VP2, suggesting NS3/NS3a as a bridging molecule facilitating virus engagement with the host cell membrane trafficking machinery (45, 58). This process has been proposed as the major mechanism of virus release from KC (insect) cells (59–61). Our results support the proposed highly specific NS3/NS3a-VP2-mediated mechanism for release of orbivirus particles.

Generally, RNA viruses have a condensed and compressed genome, including overlapping open reading frames, suggesting that dispensable genetic capacity has been removed during evolution. Nonstructural protein NS3/NS3a of orbiviruses is dispensable *in vitro* but highly conserved, which suggests a crucial role in the life cycle of these insect-borne viruses. Structural virus proteins have been assumed to be essential for *in vitro* and *in vivo* replication, in particular structural proteins of nonenveloped viruses such as reoviruses, with their architectural complex virus structure. Remarkably, tVP2 lacks dispensable domains at the outside of the virus particle. These domains likely interact with the cellular receptor as one of the first steps in virus infection and have been implicated in tissue tropism and virulence (49). Furthermore, VP2 protein is the major target for neutralizing antibodies (29–32), suggesting an essential role in infection of horses.

We here show that the entire structural VP2 protein appears to be dispensable for virus replication *in vitro*. It will be very interesting to study the virulence, pathogenesis, and tissue tropism of these AHSV VP2 mutants by experimental infection of horses. Our findings also have implications for vaccine development for AHS and open new possibilities for research on members of the *Reoviridae* family.

MATERIALS AND METHODS

Cell lines and viruses. BSR cells (a clone of baby hamster kidney cells [62]) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 5% fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B). *Culicoides variipennis* (KC) cells were grown in modified Schneider's *Drosophila* medium with 15% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (63).

Virus strain AHSV4LP has been generated by passage of spleen isolate HS32/62 in suckling mice and BHK-21 cells followed by selection of large plaques on Vero cells (6). All viruses described in this study are based on "synthetically" derived AHSV4LP (GenBank accession numbers KM820849 to KM820858) and were generated by reverse genetics (25). Virus stocks were obtained by infection of fresh BSR cells at a multiplicity of infection (MOI) of 0.1, harvested, and stored at 4°C.

cDNAs of AHSV genome segments. Plasmids with the complete genome segments of AHSV4LP for runoff transcription and expression plasmids with open reading frames (ORFs) encoding VP1, -3, -4, -6, and -7 and NS1 and -2 of AHSV4LP have been described previously (25). cDNAs with mutations or deletions in Seg-2 of AHSV4LP and Seg-2 of serotype 7 expressing tVP2 (GenBank accession number JQ742007.1) were synthesized by GenScript Corporation (Piscataway, NJ, USA) or were introduced by standard cloning procedures (Fig. 1). Alternatively, a plasmid with cDNA of the entire Seg-2 of serotype 5 (GenBank accession number KM886345) was amplified with divergent primers (AHSV5 VP2mut F [5'-P-TTCCCGCAGTTTAGGAAAGAAAG-3', nucleotide positions 1537 to 1558] and AHSV5 VP2mut R [5'-P-GATTTCTGAATAACTTTTCATCTAACTC-3', nucleotide positions 835 to 864] located up- and downstream of the mapped truncation followed by ligation, resulting in deletion Seg-2 expressing tVP2 of serotype 5. cDNAs with the inserted ORF of GFP (GenBank accession number AAB02576.1) in Seg-2 were synthesized by GenScript Corporation (Piscataway, NJ, USA) in appropriate plasmids for full-length runoff RNA transcription as previously described (64). Small mutations in Seg-6[VP5] of AHSV4LP to abort translation were introduced by filling in or removing sticky ends at nt 49 for BssHII and 174 for BsgI. All plasmids were transformed and maintained in *Escherichia coli* strain DH5 α (Invitrogen) and isolated using the High Pure plasmid isolation kit (Roche) or the QIAfilter plasmid midikit (Qiagen). Capped RNA runoff transcripts were synthesized after linearization of plasmid DNA, purified, and stored as previously described (65).

Rescue of AHSV4LP and mutants. Reverse genetics to recover AHSV and AHSV mutants has been described previously (25). Briefly, BSR monolayers were transfected with expression plasmids, followed by transfection with 10 runoff capped RNA transcripts in equimolar amounts after 24 h. At 22 h after RNA transfection, the transfection mix was replaced by 1 ml cell culture medium, and virus was rescued as previously described. Attempts were performed in duplicate and were repeated at least twice to conclude lethality associated with the respective mutation compared to rescue of control virus. Virus stocks were obtained by infection of fresh BSR cells with a multiplicity of infection (MOI) of 0.1, harvested, and stored at 4°C.

IPMA. Expression of virus proteins was determined by immunoperoxidase monolayer assay (IPMA) according to standard procedures as described previously (66) with α -VP5 MAb, α -VP2 MAb, or 500 $\times$ diluted α -VP2 guinea pig (GP) serum followed by conjugated rabbit α -mouse serum or conjugated rabbit α -GP serum (Dako). MAb 10AE12 is directed to VP5 of AHSV4LP. MAbs 6DG2, 10BE7, 8BG10, 6DF4, 10AE1, 8EA7, 8CG1, SMAA, 10BG7, 8EA6, 10BC12, and 8AF8 are against VP2 of AHSV4LP (generous gifts from Ingenasa, Spain). Monospecific polyvalent GP sera raised against baculovirus-expressed VP2 protein (α -VP2 GP serum) of different AHSV serotypes have been described recently (38). Generally, IPMA with α -VP5 MAb was performed to confirm infection or transfection, IPMA with 500 $\times$ diluted α -VP2 GP sera showed serotype-specific immunostaining, and IPMA with α -VP2 MAbs was used to study VP2 expression.

Analysis of dsRNA of AHSV mutants by PAGE. BSR monolayers were infected, and medium was discarded at total cytopathic effect (CPE). TRIzol (0.1 ml/cm²) was added to the cells and incubated for 5 min at room temperature. After harvesting, 0.2 ml chloroform/ml TRIzol was added, and the mixture was centrifuged for 10 min at 6,200 $\times$ g. The water phase was collected, and 0.8 ml isopropanol/ml was added. Precipitated RNA was centrifuged for 30 min at 4°C and 13,000 rpm. The pellet was washed with 70% ethanol and dissolved in 100 μ l RNase-free water. Fifty microliters of 7 M LiCl was added, followed by incubation for 30 min at -20°C to precipitate single-stranded RNA (ssRNA). After centrifugation for 15 min at 4°C and 13,000 rpm, dsRNA was purified from the supernatant using the RNA Clean and Concentrator-5 kit (Zymo Research) according to the manufacturer's protocol. Approximately 200 ng dsRNA was separated by 4 to 12% polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining using the SilverXpress kit (Invitrogen).

Growth curves on BSR and KC cells. Monolayers of 5 $\times$ 10⁵ BSR cells/well or 5 $\times$ 10⁶ KC cells/well in M 24-well plates were infected in triplicate at a multiplicity of infection (MOI) of 0.1. After virus attachment to cells for 1.5 h at 37°C (BSR cells) or 28°C (for KC cells), the medium was removed and monolayers were washed once. One milliliter of DMEM complete medium (BSR cells) or Schneider's complete medium (KC cells) was added, and incubation was continued (this is $t = 0$). Between 0 and 120

h postinfection (hpi), supernatants and cells were separated, harvested, and stored at -80°C . Experiments were repeated at least once. Virus titers were determined by endpoint dilution on BSR cells and expressed as $\log_{10}$ 50% tissue culture infective doses (TCID_{50})/ml. BSR cells were infected with 10-fold dilutions of samples, grown for 72 h, and studied for CPE and immunostaining. The highest titers in the cell fraction and the culture medium were compared using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test.

Isolation of virus particles. BSR cell monolayers (1.5×10^7 cells/150-cm² flask; 4 flasks in total) were infected at an MOI of 0.01 and incubated at 37°C . At 72 hpi, cells were collected by centrifugation at $3,000 \times g$ at 4°C for 15 min and washed twice in chilled $1 \times$ phosphate-buffered saline (PBS). Virions were purified using a slight modification of a method described previously (56, 67). Cells were lysed by incubation on ice for 30 min in 7 ml chilled 100 mM Tris-HCl (pH 8.8), 50 mM NaCl, 10 mM EDTA, and 0.1% (vol/vol) NP-40. To ensure lysis, cells were passed 10 times through a 28-gauge hypodermic needle fitted to a 20-ml syringe. Nuclei were removed by centrifugation at $1,000 \times g$ at 4°C for 15 min. *N*-Lauroyl sarcosine (Merck) was added to the cytoplasmic extract to a final concentration of 0.2% (wt/vol), followed by incubation at 25°C for 1 h. Four milliliters of the cytoplasmic extract was loaded onto a 1-ml sucrose cushion (40% [wt/vol] sucrose prepared in 600 mM MgCl_2 –20 mM Tris-HCl [pH 8.0]) and subjected to ultracentrifugation at $141,000 \times g$ (36,000 rpm) at 20°C for 2 h in a Beckman Optima L-70K ultracentrifuge using an SW40 Ti swing-bucket rotor. The pellet with AHSV particles was resuspended in 25 μl of 20 mM Tris-HCl (pH 8.0) and stored at 4°C .

Western blot analysis. Samples (1.5 μl) with virions in $1 \times$ LDS buffer (NuPAGE) with reducing agent (NuPAGE) were heated for 2 min at 70°C and separated by electrophoresis on a 12% BIS-TRIS polyacrylamide-gel in $1 \times$ MOPS (morpholinepropanesulfonic acid)-SDS buffer using the XCell-Surelock system. Separated proteins were transferred to nitrocellulose paper. Transferred proteins were detected with the indicated MABs, followed by binding of horseradish peroxidase (HRPO)-conjugated rabbit α -mouse Abs (Dako) and incubation with 3-amino-9-ethylcarbazole (AEC).

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