Directed genetic modification of African horse sickness virus by reverse genetics

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African horse sickness virus (AHSV), a member of the *Orbivirus* genus in the family Reoviridae, is an arthropod-transmitted pathogen that causes a devastating disease in horses with a mortality rate greater than 90%. Fundamental research on AHSV and the development of safe, efficacious vaccines could benefit greatly from an uncomplicated genetic modification method to generate recombinant AHSV. We demonstrate that infectious AHSV can be recovered by transfection of permissive mammalian cells with transcripts derived in vitro from purified AHSV core particles. These findings were expanded to establish a genetic modification system for AHSV that is based on transfection of the cells with a mixture of purified core transcripts and a synthetic T7 transcript. This approach was applied successfully to recover a directed cross-serotype reassortant AHSV and to introduce a marker sequence into the viral genome. The ability to manipulate the AHSV genome and engineer specific mutants will increase understanding of AHSV replication and pathogenicity, as well as provide a tool for generating designer vaccine strains.

Introduction

African horse sickness, of which African horse sickness virus (AHSV) is the aetiological agent, is a non-contagious disease of equids that is transmitted by *Culicoides* biting midges. African horse sickness is the most devastating of all equine diseases, with a mortality rate that can exceed 90% in susceptible horse populations.[1] Although endemic to sub-Saharan Africa, AHSV sporadically escapes from its geographical area and outbreaks of the disease have occurred in North Africa, the Middle East, the Arabian Peninsula and Mediterranean countries.[2,3] As a consequence of its severity, economic impact and ability to spread rapidly from endemic regions, African horse sickness is listed by the World Organization for Animal Health as a notifiable equine disease.

AHSV is a member of the *Orbivirus* genus in the Reoviridae family and has a genome composed of 10 segments of linear double-stranded RNA (dsRNA), designated from segment 1 (S1) to S10 in decreasing order of size.[4] The viral genome encodes seven structural proteins and at least three non-structural proteins (NS1 to NS3). Structural proteins VP1, VP3, VP4, VP6 and VP7 form the virus core particle, which is surrounded by an outer capsid layer composed of VP2 and VP5.[5] Shortly after cell entry by the virus, the outer capsid proteins are removed and the transcriptionally active core particle is released into the host cell cytoplasm. Within the core particle, each of the dsRNA genome segments is repeatedly transcribed by the core-associated enzymes VP1 (RNA-dependent RNA polymerase), VP4 (capping enzyme) and VP6 (helicase), resulting in extrusion of newly synthesised, capped, viral single-stranded RNA (ssRNA). The extruded transcripts, in turn, function as templates for the synthesis of viral proteins and also act as templates for the synthesis of genomic dsRNA following their encapsidation in progeny viral cores.[6,7]

Although a combination of mutagenesis and re-expression of AHSV proteins in heterologous hosts has allowed progress to be made regarding the structure–function relationships of selected AHSV proteins,[8-13] definitive roles for many of these proteins in the context of a replicating virus remain unresolved. Indeed, a major obstacle to studies aimed at understanding AHSV biology has been the inability to genetically manipulate the segmented dsRNA viral genome. However, recent technological advances for members of the Reoviridae family have demonstrated that it is possible to recover genetically engineered recombinant viruses through a reverse genetics approach. Despite variations in their molecular design and methodology, these reverse genetics systems all share a common feature, which is the availability of cloned complementary DNA (cDNA) copies of the viral genomes that can be genetically modified and manipulated to generate live viruses containing precisely engineered changes in their genomes.[14-17] Recently, a plasmid-based reverse genetics system was reported for AHSV.[18] This system requires the construction of recombinant plasmids in which cDNA copies of the 10 viral genome segments are each cloned under the control of a T7 RNA polymerase promoter in order to synthesise synthetic T7 viral transcripts. Transfection of permissive cells with these in–vitro synthesised synthetic T7 transcripts resulted in the recovery of infectious AHSV, indicating that the synthetic T7 transcripts can serve as functional substitutes of the authentic viral transcripts. A variation of this approach requires the construction of additional expression helper plasmids to synthesise the AHSV inner core proteins and two non-structural proteins in permissive cells, followed by transfection of these cells with all 10 synthetic T7 transcripts.[18] It can be envisaged that a reverse genetics approach which is not dependent on extensive cloning procedures may represent an attractive alternative to these plasmid-based reverse genetics systems.

Our objective in this study was to establish an efficient, broadly applicable method to generate recombinant AHSV. Here we show that transfection of permissive mammalian cells with a mixture of purified AHSV core-derived transcripts and an in-vitro synthesised T7 transcript derived from a polymerase chain reaction (PCR) amplicon leads to the production of recombinant AHSV. The performance of this system was validated by isolation of both a viable cross-serotype single-gene reassortant virus and a mutant virus containing a defined mutation in the replicating viral genome. The implications of targeted alterations of the AHSV genome are highly significant to research focused on basic studies of AHSV biology and the development of recombinant vaccine strains.

Materials and Methods

Cells and viruses

BSR cells (a clone of baby hamster kidney-21 cells) were cultured at 37 °C and 5% CO_2 in Eagle's minimum essential medium (EMEM) supplemented with Earle's balanced salt solution, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 5% (v/v) foetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B) (HyClone Laboratories, Logan, UT, USA).

AHSV serotypes 3 (AHSV-3) and 4 (AHSV-4) were used for cell infections. Cell monolayers were rinsed twice with incomplete EMEM (lacking FBS and antibiotics) and then infected with the virus at the desired multiplicity of infection (MOI). Virus adsorptions were performed at 37 °C for 1 h, followed by incubation in complete EMEM.

Isolation of AHSV core particles

BSR cell monolayers were infected with AHSV-3 or AHSV-4 at a MOI of 0.08 plaque-forming units (pfu) per cell and harvested at 72 h post-infection. Core particles were purified using a modification of the method described by Mertens et al.[19] The virus-infected cells were lysed by incubation on ice for 30 min in chilled lysis buffer (100 mM Tris-HCl [pH 8.8], 10 mM ethylenediaminetetraacetic acid, 50 mM NaCl, 0.5% [v/v] NP-40), and the cell debris and nuclei were removed by centrifugation at 1000 *g* for 10 min at 4 °C. To remove the outer capsid proteins of virions, α-chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA) was added to the cytoplasmic extract to a final concentration of 60 μg/mL, followed by incubation at 37 °C for 1 h. *N*-lauroylsarcosine (Merck, Darmstadt, Germany) was then added to 0.2% (w/v) final concentration, and incubation was continued at 25 °C for 1 h. The sample was subjected to high-speed ultracentrifugation (141 000 *g* for 2 h at 20 °C) through a 1-mL cushion of 40% (w/v) sucrose, prepared in 600 mM $MgCl_2$ and 20 mM Tris-HCl (pH 8.0). The pelleted core particles were suspended in 20 mM Tris-HCl (pH 8.0).

Characterisation of AHSV core particles

Proteins from purified cores were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by staining of the gel with Coomassie brilliant blue (Merck, Darmstadt, Germany). Aliquots of the purified core particles were also adsorbed onto Formvar/carbon-coated 300-mesh copper grids for 90 s, rinsed with distilled water and then negatively stained with 2% (w/v) uranyl acetate. The grids were examined under a JEOL 2100F transmission electron microscope at 200 kV.

Synthesis and purification of AHSV core-derived transcripts

Core transcripts were synthesised in vitro at 30 °C for 6 h by incubating the AHSV cores (75 μg/mL) in AHSV core transcription buffer (100 mM Tris-HCl [pH 8.0], 4 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 500 μM S-adenosylmethionine, 6 mM DTT, 9 mM MgCl2, 1 U/μL of RiboLock RNase Inhibitor [Promega, Madison, WI, USA]). The sample was centrifuged at 20 000 *g* for 30 min at 4 °C and the supernatant was subjected to a second, identical centrifugation step to ensure removal of the core particles. The core particles were suspended in 20 mM Tris-HCl (pH 8.0) for re-use in subsequent in-vitro transcription reactions. The core transcripts were precipitated from the supernatant by addition of 8 M LiCl to a final concentration of 2 M, followed by incubation at 4 °C for 16 h. Following centrifugation as above, the pelleted core transcripts were suspended in 100 μL of sterile diethylpyrocarbonate (DEPC)-treated water and purified with the Nucleospin RNA Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Purified core transcripts were mixed with an equal volume of denaturing RNA loading buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and analysed by electrophoresis on a 1% (w/v) agarose gel.

Preparation of templates for synthesis of synthetic T7 transcripts

All molecular biology and cloning procedures were performed using standard methodology.[20] For reassortant virus studies, recombinant plasmid pJET/blunt-S10, which contains a cloned cDNA copy of the full-length S10 genome segment of AHSV-4, was used as a template in a PCR with the 5' end primer NS3T7F (5'-CCGGTAATACGACTCACTATAGTTAAAATTATCCC-3'; T7 RNA polymerase promoter underlined) and the 3' end-specific primer NS3R (5'-GTAAGTTGTTATCCCACTCCCTAGAAAACG-3'). The resulting amplicon was purified from an agarose gel with the Zymoclean Gel DNA Recovery kit (Zymo Research Corporation, Irvine, CA, USA) and used as the template in T7 transcription reactions.

A mutant version of the AHSV-4 S10 genome segment, containing an introduced *Eco*RI restriction enzyme recognition sequence at nucleotide position 270, was constructed by a megaprimer PCR-based mutagenesis strategy in which three primers and two PCRs were used.[21] In the first round of PCR, pJET/blunt-S10 was used as the template together with primer NS3T7F and an internal mutagenic primer (NS3mR: 5'-ACGTATGGGAATTCGTTCTGGATCAC-3'; *Eco*RI sequence underlined). The purified amplicon was then used as a 'megaprimer' along with primer NS3R in the second round of PCR to generate the full-length S10 genome segment containing the newly introduced sequence. The amplicon was purified from an agarose gel and then blunt-end cloned into the *Sma*I site of pUC19 to generate pUC-S10E. The mutation was subsequently verified by nucleotide sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Perkin Elmer, Foster City, CA, USA), followed by resolution on a Model 377 DNA sequencer (Perkin Elmer, Foster City, CA, USA). The AHSV-4 mutant S10 genome segment was PCR amplified from pUC-S10E with primers NS3T7F and NS3R, and used as the template in T7 transcription reactions. Transcription of the respective amplified products with T7 RNA polymerase is expected to yield synthetic T7 transcripts with authentic virus terminal sequences.

In-vitro T7 transcription reactions

T7 transcripts, containing a 5' cap analogue, were synthesised from the above purified amplicons with the MessageMAX T7 ARCA-capped Message Transcription kit (Epicentre, Madison, WI, USA) using the manufacturer's protocol. In these reactions, a ratio of 4:1 of anti-reverse cap analogue to rGTP was used. Following removal of the DNA template with RNase-free DNase I by incubation at 37 °C for 15 min, the T7 transcripts were purified with the Nucleospin RNA Clean-up kit (Macherey-Nagel, Düren, Germany).

Cell transfections and recovery of infectious virus

For recovery of AHSV from core transcripts, BSR cell monolayers at 80% confluence in 24-well tissue culture plates were transfected twice (16 h apart) with 800 ng of the core transcripts using Lipofectamine LTX reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The same protocol was used for recovery of directed reassortant AHSV, except that 400 ng each of AHSV-3 core transcripts and the AHSV-4 S10 genome segment T7 transcript were mixed prior to transfection of BSR cells. For recovery of a mutant AHSV-4 virus, BSR cells were likewise transfected with a mixture consisting of 400 ng each of AHSV-4 core transcripts and the AHSV-4 mutant S10 genome segment T7 transcript. At 72 h post-second transfection, the cells were lysed and used for infection of confluent BSR cell monolayers in 6-well tissue culture plates. Virus adsorption was performed at 37 °C for 1 h, after which the lysate was replaced with a 1-mL overlay consisting of complete EMEM and 0.5% (w/v) agarose. At 72 h post-infection, plaques were visualised by staining the cell monolayers with 0.1% (w/v) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Fresh BSR cell monolayers were infected with virus eluted from single plaques and the cells were incubated for 72 h to allow amplification of the virus.

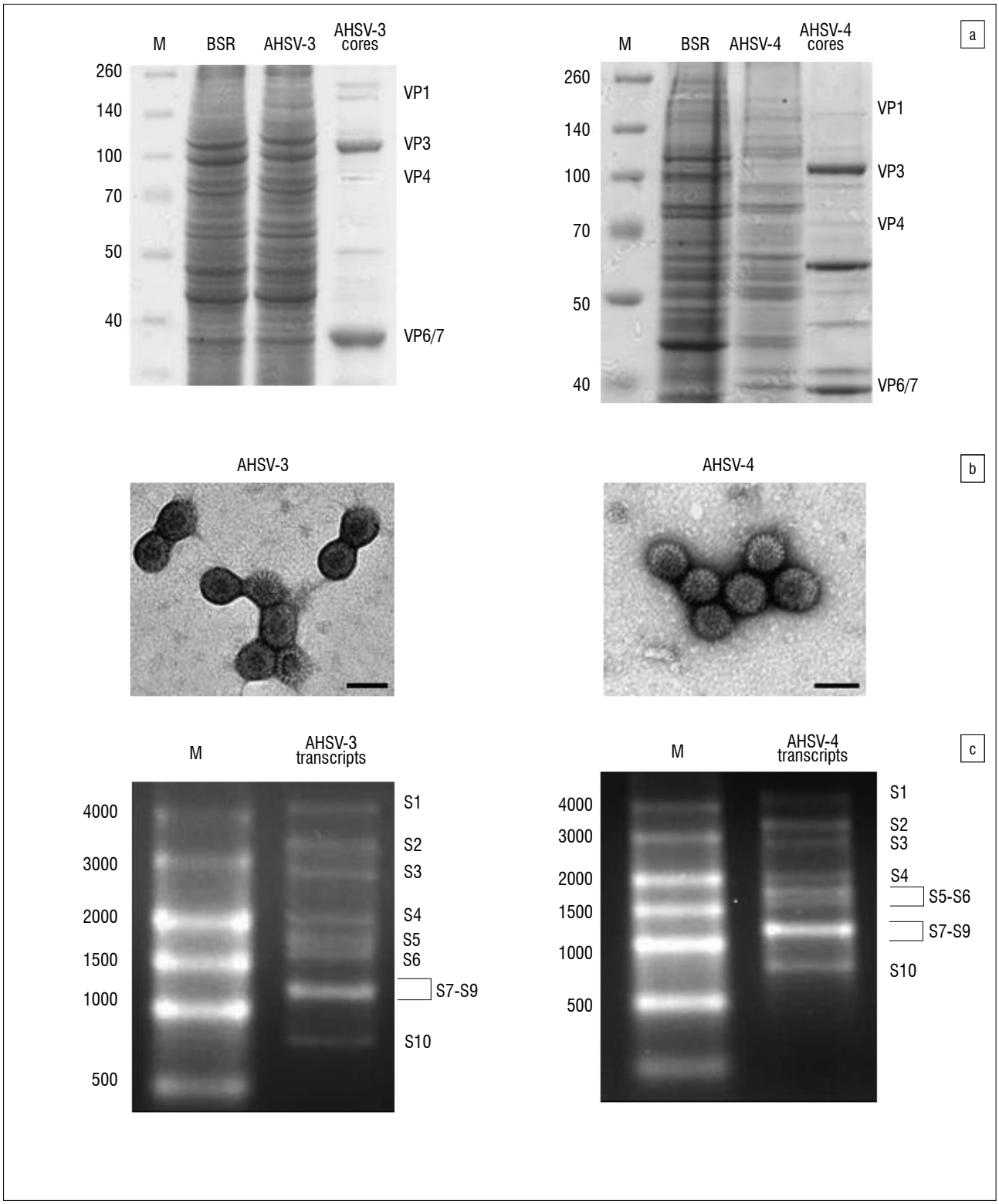

Figure 1: Characterisation of purified African horse sickness virus (AHSV) core particles and synthesis of core-derived transcripts. (a) SDS-polyacrylamide gel electrophoresis of purified AHSV-3 and AHSV-4 core particles. Uninfected (BSR) and virus-infected cells were included for comparative purposes. The sizes of reference protein markers (M, in kDa) are indicated on the left of the figure, and the AHSV core proteins on the right. (b) Transmission electron micrographs of negatively stained purified core particles (scale bar = 60 nm). (c) Agarose gels showing transcripts synthesised in vitro from purified core particles. The sizes of ssRNA markers (in nucleotides) are indicated on the left of the figure, and the core transcripts are indicated on the right.

RNase A and Proteinase K treatment of AHSV core transcripts and cores

BSR cell monolayers were transfected, as described above for the recovery of AHSV from core transcripts, with 800 ng of AHSV-4 core transcripts or 100 ng of AHSV-4 cores that had been pre-treated at 37 °C for 1 h with 100 μg/mL Proteinase K or 50 μg/mL RNase A.

Immunoblot analysis

Standard immunoblotting techniques[20] were used for detection of NS2 and VP7 protein expression at 12-h intervals over a time course of 72 h in BSR cells transfected with AHSV-4 core transcripts. Guinea pig polyclonal antiserum raised against NS2[8] or VP7[22] were used as primary antibodies and Protein A conjugated to horseradish peroxidase as the secondary antibody (Sigma-Aldrich, St. Louis, MO, USA).

Virus growth curves

Confluent BSR cell monolayers in 6-well tissue culture plates were infected with transfection-derived AHSV-3 or AHSV-4 at a MOI of 0.1 pfu/cell. At different time points post-infection, the virus titres were determined by serial dilution and plaque assays on BSR cells as described above.

Extraction of dsRNA

Total RNA was extracted from virus-infected cells with the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The ssRNA was removed by precipitation with 2 M LiCl and centrifugation at 17 000 *g* for 30 min at 4 °C. The dsRNA was subsequently precipitated from the collected supernatant with 0.1 volume of NaOAc and 2 volumes of absolute ethanol. Following centrifugation as above, the pelleted dsRNA was washed twice with 70% ethanol and then suspended in DEPC-treated water. The purified dsRNA samples were analysed by agarose gel electrophoresis or used for cDNA synthesis.

Screening of transfection-derived viruses for reassortant and mutant AHSV

Purified dsRNA was used as a template to synthesise cDNA copies of the respective AHSV genome segments using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. To identify AHSV reassortants, PCR amplification was subsequently performed using S10 genome segment-specific primer pairs for either AHSV-3 or AHSV-4. The primer pairs used for AHSV-3 and AHSV-4 were: A3S10-186F (5'-GATACTTAACCAAGCCATGTC-3') and A3S10-700R (5'-GTTTGATCCACCCAACACTG-3'), and A4S10-239F (5'-CTATGGCGGAAGCATTGC-3') and A4S10-742R (5'-GTATGTTGTTATCCCACTCC-3'), respectively. The reaction mixtures were analysed by agarose gel electrophoresis. To identify mutant AHSV-4, the PCR amplification was performed with primers NS3F (5'-GTTAAAATTATCCCTTGTCATGAATCTAGCTAC-3') and NS3R. The amplicons were subjected to *Eco*RI restriction endonuclease digestion, followed by analysis of the digestion products on an agarose gel. As a final confirmation, the nucleotide sequences of the amplified S10 genome segments from putative reassortant and mutant viruses were also determined.

Results

Purification of AHSV cores and in-vitro synthesis of core transcripts

AHSV-3 and AHSV-4 core particles were purified from virus-infected cells and the protein composition of the core particles was verified by SDS-PAGE. The core proteins VP1, VP3 and VP4 were readily visualised in the stained SDS-polyacrylamide gels, whereas the core proteins VP6 and VP7 co-migrated as a single band as a result of their similar molecular masses (38.4 kDa and 37.9 kDa, respectively) (Figure 1a). To furthermore confirm the absence of both of the outer capsid proteins VP2 and VP5, the AHSV core particles were examined by negative-staining transmission electron microscopy. Large quantities of core particles with a diameter of 65 nm were observed (Figure 1b). These particles displayed the characteristic capsomeres of orbivirus particles and were smaller in size to intact virion particles (80 nm in diameter).[5,23] To confirm that the purified AHSV-3 and AHSV-4 core particles were transcriptionally active, the cores were used for in-vitro synthesis of AHSV ssRNA. Agarose gel electrophoretic analysis of the purified core transcripts indicated that all 10 viral transcripts were synthesised, with no obvious evidence of premature termination or degradation of the transcripts (Figure 1c).

AHSV protein expression in transfected BSR cells

To determine whether the in-vitro synthesised AHSV core transcripts could serve as templates for viral protein synthesis in vivo, BSR cells were transfected with purified AHSV-4 core transcripts and harvested at different time intervals after infection. As markers through which the expression of viral proteins could be assessed over time, the non-structural protein NS2 and the structural protein VP7 were used. As shown in Figure 2, immunoblot analysis of cell lysates prepared from the transfected BSR cells confirmed that the respective proteins were synthesised from 60 h post-transfection onwards. These results thus indicate that transfection could effectively deliver in-vitro synthesised AHSV-4 core transcripts to the cytoplasm of BSR cells where they serve as templates for virus protein synthesis.

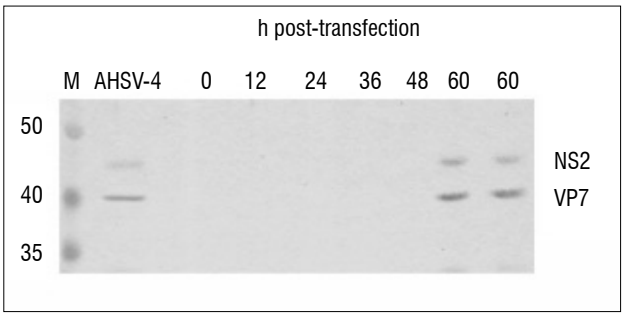

Figure 2: Viral protein expression in transfected BSR cells. Expression of the NS2 and VP7 proteins in BSR cells transfected with purified AHSV-4 core transcripts was determined at 12-h intervals by immunoblotting using appropriate antibodies. BSR cells infected with AHSV-4 served as a positive control. The sizes of reference protein markers (M, in kDa) are indicated on the left of the figure.

Recovery of infectious AHSV from transfected BSR cells

To determine whether infectious AHSV could be recovered from in-vitro synthesised core transcripts, plaque assays were performed of lysates prepared from BSR cells transfected with purified AHSV-3 or AHSV-4 core transcripts. Clear, well-defined plaques were observed, suggesting the presence of replicating virus (Figure 3a). To confirm virus recovery, viruses from individual plaques were amplified, after which dsRNA was extracted and analysed by agarose gel electrophoresis. The electrophoresis pattern of dsRNA segments extracted from transfection-derived AHSV-3 (Figure 3b) and AHSV-4 (Figure 3c) were identical to that of the corresponding parental AHSV serotype derived from cell infection. These results not only confirmed that AHSV-3 and AHSV-4 were recovered successfully from their corresponding core transcripts, but also that the core transcripts had been replicated to produce new genomic dsRNA. Moreover, the parental and transfection-derived AHSV-3 and AHSV-4 displayed similar growth curves in BSR cells, indicating that the recovered viruses had no gross replication or growth defects (Figure 3d).

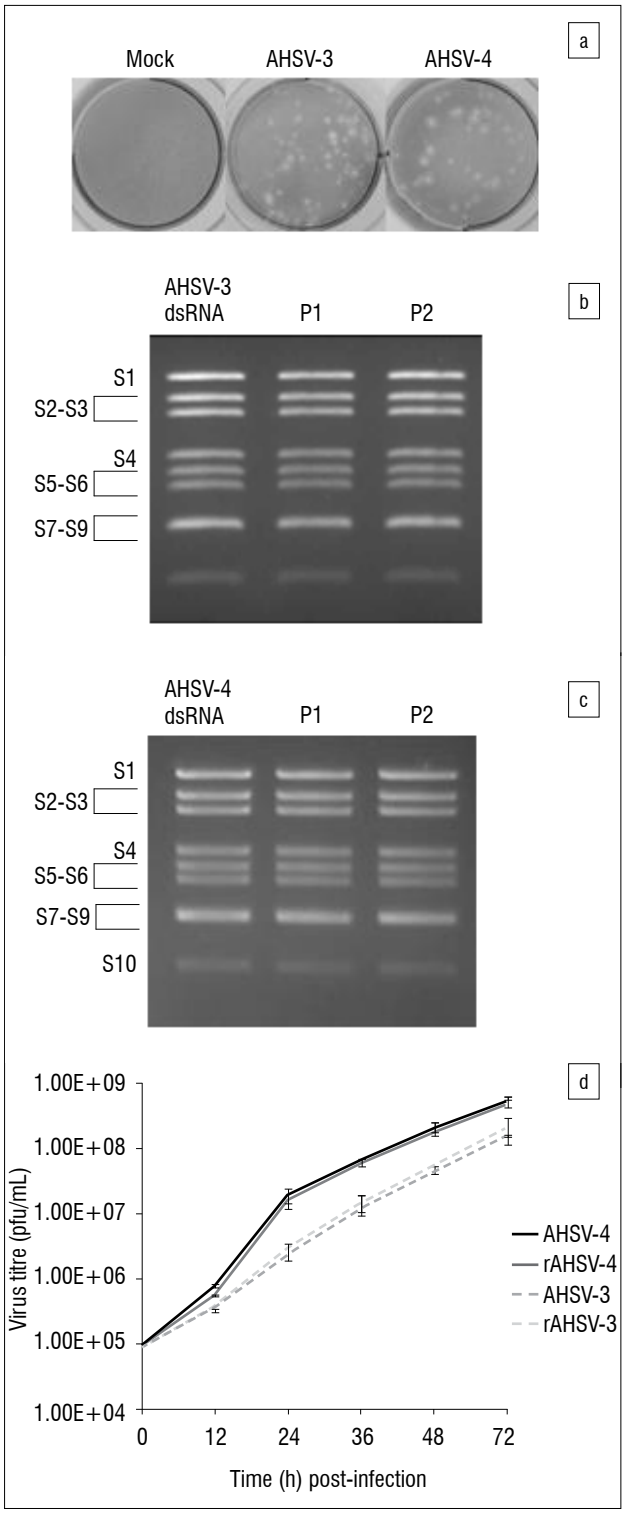

Figure 3: Recovery of African horse sickness virus (AHSV) following transfection of purified core transcripts into BSR cells. (a) Plaque assays performed on lysates of BSR cells transfected with purified AHSV-3 or AHSV-4 core transcripts. Mock-transfected BSR cells were included as a control. (b and c) Agarose gels showing the migration patterns of AHSV-3 and AHSV-4 dsRNA compared to the dsRNA extracted from transfection-derived viruses (indicated as P1 and P2 in each figure). (d) Growth curves of the transfection-derived AHSV-3 (rAHSV-3) and AHSV-4 (rAHSV-4) in BSR cells. For comparative purposes, the BSR cells were also infected with the parental AHSV-3 and AHSV-4 serotypes. The titre of the respective viruses at different times post-infection, as indicated in the figure, was determined by serial dilution and plaque assays on BSR cells. Each data point represents the mean±standard deviation from three independent experiments.

Confirmation that AHSV core transcripts are infectious

To characterise the infectious material present in the AHSV core transcript preparations, the sensitivity of the in-vitro synthesised AHSV-4 core transcripts to RNase A and Proteinase K was compared with that of AHSV-4 cores. Following transfection of BSR cells and plaque assays, the results indicated that the infectivity of the purified core transcripts was eliminated by the RNase A treatment, whereas the cores retained their infectivity. In contrast, Proteinase K treatment of core particles completely inhibited their ability to cause infection, whereas the infectivity of purified core transcripts was unaffected (Figure 4). These results confirmed that core transcripts are the sole source of infectivity in the purified core transcript preparations.

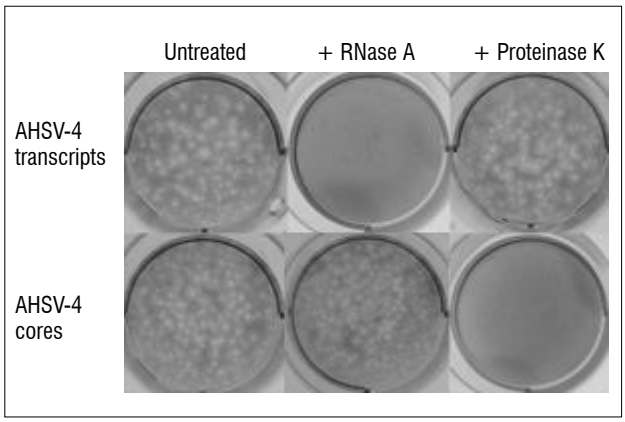

Figure 4: Confirmation that cores are not the source of infectivity in purified African horse sickness virus (AHSV) transcript preparations. Plaque assays were performed on lysates prepared from BSR cells that had been transfected with core transcripts and cores of AHSV-4, following treatment with RNase A or Proteinase K. Untreated samples were included as controls.

Generation of a targeted cross-serotype reassortant virus

After the infectivity of the purified AHSV core transcripts was established and the recovery of virus following their transfection into BSR cells was shown, we attempted to modify the AHSV genome by replacing the S10 genome segment of AHSV-3 with an in-vitro synthesised synthetic T7 transcript of the AHSV-4 S10 genome segment. For this purpose, purified AHSV-3 core transcripts were mixed with the AHSV-4 S10 genome segment T7 transcript (Figure 5a) and transfected into BSR cells. To screen for reassortant viruses, viruses from randomly selected plaques were amplified, dsRNA was extracted from the virus-infected cells and cDNA was synthesised. The origin of the S10 genome segment was then determined by PCR using primers specific for the S10 genome segment of AHSV-3 and AHSV-4. The amplicons were analysed by agarose gel electrophoresis and the identity of the amplicons obtained by PCR amplification (using the AHSV-4 S10 genome segment-specific primers) was confirmed by nucleotide sequencing. The results, presented in Figure 6, indicate that a cross-serotype reassortant AHSV could be recovered successfully using this approach.

Generation of a mutant AHSV-4

To determine if a specific mutation could be introduced into the AHSV genome using the above approach, we attempted to incorporate a unique *Eco*RI restriction enzyme site into the S10 genome segment of AHSV-4. To recover this mutant virus, purified AHSV-4 core transcripts and an AHSV-4 mutant S10 genome segment T7 transcript (Figure 5b) were transfected into BSR cells. Viruses resulting from the transfection were amplified and the dsRNA extracted from virus-infected BSR cells was used for cDNA synthesis. To screen for viruses containing the introduced mutation, the S10 genome segment was PCR amplified, digested with *Eco*RI and the reaction mixtures analysed by agarose gel electrophoresis.

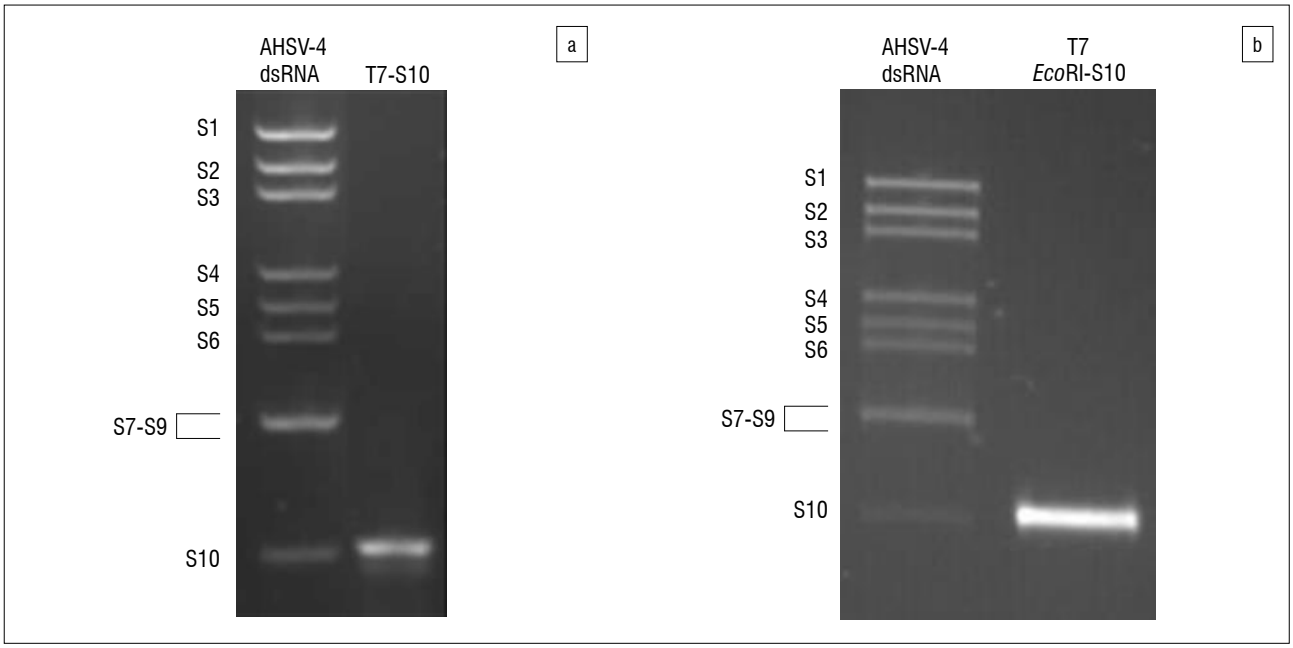

Figure 5: Agarose gels showing the in-vitro synthesised T7 transcripts of (a) African horse sickness virus (AHSV)-4 S10 genome segment (T7-S10) and (b) AHSV-4 mutant S10 genome segment (T7-*Eco*RI-S10) that were used in cell transfections to recover directed reassortant and mutant AHSV, respectively.

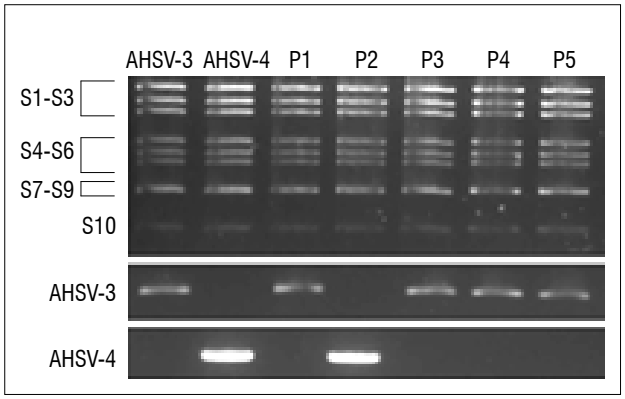

Figure 6: Recovery of cross-serotype reassortant African horse sickness virus (AHSV) after transfection of BSR cells with a mixture of AHSV-3 core transcripts and an AHSV-4 S10 genome segment T7 transcript. Agarose gels show viral dsRNA extracted from individual recovered viruses (indicated as P1 through P5 in top panel) and used for subsequent cDNA synthesis. The origin of the S10 genome segment in the recovered viruses was determined by PCR using primers specific for the S10 genome segment of AHSV-3 (middle panel) or AHSV-4 (bottom panel). BSR cells infected with AHSV-3 or AHSV-4 served as positive controls.

The results indicated that, in contrast to the non-mutated S10 genome segment which was not cleaved by the restriction enzyme, the S10 genome segments of mutant AHSV-4 viruses were cleaved and yielded two DNA fragments of the expected sizes (270 and 493 base pairs) (Figure 7). The nucleotide sequences of the purified amplicons were also determined, the results of which confirmed the presence of the unique *Eco*RI restriction enzyme site.

Discussion

The greatest obstacle preventing translation of basic research to AHSV biology has been the inability to manipulate the 10-segmented dsRNA viral genome through reverse genetics. Moreover, AHSV is a significant equine pathogen, and an unencumbered reverse genetics system would allow the development of novel recombinant vaccine strains. Thus, towards establishing a simple, broadly applicable genetic modification system for AHSV, we first examined whether transcripts synthesised in vitro from purified AHSV core particles have the same coding potential as those produced from transcribing core particles in virus-infected cells. As is the case for viral transcripts produced during natural infection, the in-vitro synthesised core transcripts were shown to be infectious when transfected into the cytoplasm of permissive BSR cells, resulting in the recovery of viable viruses that were identical to the parental AHSV serotypes in terms of growth characteristics in the mammalian cells. These findings were subsequently extended to demonstrate the genetic manipulation of the AHSV genome. Targeted reassortant and mutant viruses were generated successfully by making use of a combination of core-derived transcripts and a synthetic T7 viral transcript, which was derived from a PCR amplicon consisting of either a wild-type or a mutant cDNA copy of the S10 genome segment of AHSV-4 under the control of an upstream T7 promoter sequence. The approach used in this study is especially suited to investigations focusing on a single viral genome segment and may be applied to any one of the 10 AHSV genome segments. Although not investigated in this study, the approach may furthermore be suitable to generate reassortant AHSV in which more than one genome segment has been exchanged.

Although a reverse genetics system has been described recently for AHSV that makes it possible to generate strains with a single recombinant gene, this system relies on cloning cDNA copies of the entire viral genome and a modified version requires the construction of additional expression helper plasmids.[18] Compared to these systems, the approach used in this study is relatively easy to perform. Our approach obviates the need to construct a full set of 10 cDNA clones as only a single PCR product or cDNA clone needs to be generated, and the approach is sufficiently robust to enable recovery of targeted reassortant and mutant AHSV. Moreover, the time and labour involved in the isolation and purification of core particles can be reduced through 'recycling' of the core particles. In this study, we found that the same sample of core particles could be re-used up to four times in in-vitro transcription reactions without any significant loss in the yield and quality of synthesised transcripts. It should, however, be noted that screening is required to identify recombinant AHSV. Reassortant viruses were recovered at a 20–50% frequency, and mutant viruses at a frequency of between 70% and 90%. The differences in virus recovery may reflect variations in the degree of capping of the T7 transcript

preparations.[24] In addition to being poorly translated, the 5' triphosphate moiety of uncapped transcripts is known to be a pathogen-associated molecular pattern recognised by RIG-I, which may lead to the induction of antiviral responses and thus also negatively influence the recovery of the virus.[25,26] The lower efficiency associated with the recovery of reassortant AHSV compared to mutant AHSV might be because of serotype-specific differences in genome segment sorting and packaging signals, but requires further investigation. Nevertheless, the efficiencies are sufficiently high so that mass screening of the transfection-derived viruses can be easily performed depending on the targeted gene and available tools, for example, by making use of discriminating monoclonal antibodies and/or discriminating PCR assays.

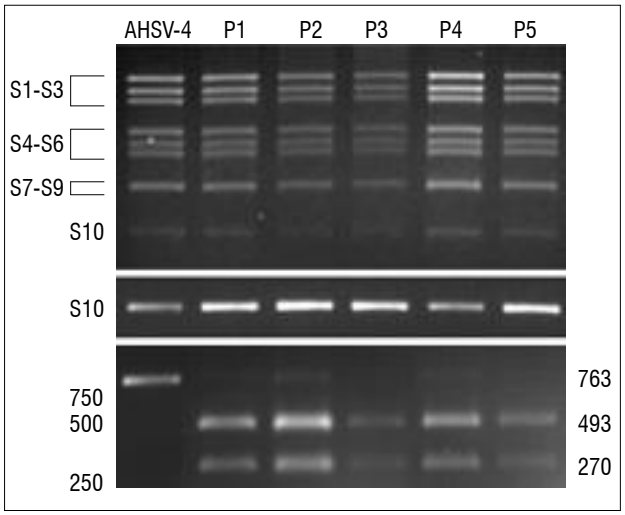

Figure 7: Recovery of mutant African horse sickness virus (AHSV)-4 after transfection of BSR cells with a mixture of AHSV-4 core transcripts and an AHSV-4 mutant S10 genome segment T7 transcript containing an *Eco*RI marker mutation. Agarose gels showing viral dsRNA extracted from individual recovered viruses (indicated as P1 through P5 in top panel), cDNA of the viral S10 genome segments (middle panel) and digestion of the cDNA products with *EcoRI* (bottom panel). BSR cells infected with AHSV-4 served as a control. The sizes of DNA markers (in base pairs) are indicated on the left of the figure, and the sizes of the digestion products are indicated on the right.

The ability to generate directed reassortant and mutant AHSV may have important implications for vaccine development. No effective treatment exists for African horse sickness and control of the disease relies on preventative vaccination. The current commercially available vaccine comprises a cocktail of live attenuated strains of only seven of the nine different AHSV serotypes, and the shortcomings of this vaccine have motivated efforts to develop alternative safe vaccines.[27,28] As opposed to random attenuation by serial passage of the virus, a reverse genetics approach may enable the rational design of attenuated vaccine strains via directed mutagenesis and/or by engineering of disabled infectious single-cycle (DISC) vaccine strains. DISC strains lack an essential gene product, and consequently are capable of replicating only once in infected cells, but they are nevertheless capable of eliciting both cellular and humoral immune responses.[29-31] Unlike the current commercial AHSV vaccine, DISC vaccines would make it possible to differentiate between animals that have been vaccinated and animals that have been infected with replicating virus (DIVA principle). In addition, the potential capability of replacing the antigenically important outer capsid proteins VP2 and VP5[32,33] from one AHSV serotype with those of another can be used to generate 'serotyped' vaccines. This capability could represent a means to rapidly produce vaccines against the different AHSV serotypes and create the option to custom engineer vaccines in order to protect against emerging epidemic strains.

In conclusion, transfection of cultured mammalian cells with a combination of viral core transcripts and a synthetic T7 transcript represents a feasible genetic modification system for AHSV. As such, it may provide a means by which safe designer live attenuated AHSV vaccine strains could be generated and also represents a potentially powerful tool for studies aimed at understanding AHSV biology, including aspects pertaining to replication, transmission, pathogenesis and immunity.

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Authors' contributions

The initiation, conception and planning of the study involved E.V., D.J.P. and J.T. The experiments were performed by E.V., D.J.P. and A.C. All authors contributed to the data analysis and preparation of the manuscript.

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