Recovery of African horse sickness virus from synthetic RNA

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INTRODUCTION

African horse sickness is an insect-borne viral disease of equids, predominantly endemic in sub-Saharan Africa, caused by African horse sickness virus (AHSV) (Mellor & Hamblin, 2004). The effects of the disease, particularly in susceptible populations of horses, can be devastating and mortality rates may exceed 90% (Mellor & Hamblin, 2004). As a consequence of its severity and its ability to expand rapidly due to the large population of insect vectors (Culicoides biting midges) in many parts of the world, African horse sickness has been allocated Office International des Epizooties List A status (Diouf et al., 2013; Gale et al., 2010; Mellor & Hamblin, 2004; Purse et al., 2005).

AHSV belongs to the genus Orbivirus within the family Reoviridae and, like the prototype species bluetongue virus (BTV), has a genome of 10 dsRNA segments (S1–S10) (Grubman & Lewis, 1992; Roy et al., 1994). The virus particle has a layered structure, the outer layer of which is lost before the remaining inner core particle enters the cytoplasm of the host cell (Martin & Zweerink, 1972). While the viral genomic dsRNAs never leave the core particle, the core particle itself is transcriptionally active, synthesizing and extruding multiple capped single-stranded transcripts (mRNAs) of each genomic RNA segment into the host cell cytoplasm (Martin & Zweerink, 1972; Verwoerd et al., 1972). For the purposes of clarity we refer to these mRNAs as ‘core transcripts’. These transcripts not only synthesize the viral proteins, but also act as templates for the synthesis of the new genomic dsRNA segments of progeny virus particles. The overall morphology, coding strategy and virus replication cycle are similar to that of BTV; however, AHSV is distinct from BTV both at the genetic level and in the proteins it encodes. In addition, the AHSV host range is different from that of BTV: disease is caused by AHSV in horses and its pathogenesis is very different from that of BTV in sheep.

Currently, plasmid-based reverse genetics systems exist for all major groups of animal RNA viruses, including segmented negative-strand viruses (Bridgen & Elliott, 1996; Neumann & Kawaoka, 2004; Neumann et al., 2002; Radecke et al., 1995; Taniguchi et al., 1978). In contrast, developing a plasmid-based reverse genetics system for the members of the family Reoviridae with 10 or more dsRNA segments has proven to be more challenging and only recently have two different reverse genetics systems been reported – one for mammalian reoviruses in which viable viruses are generated from cloned cDNAs and the other for BTV that uses a slightly different approach than that used for reoviruses (Boyce et al., 2008; Kobayashi et al., 2007). The BTV reverse genetics system is based on in vitro-generated RNA transcripts synthesized by T7 RNA polymerase of cDNA clones from BTV (Boyce et al., 2008). Using the system, we have shown that BTV utilizes a two-stage replication cycle with a primary and a secondary stage (Matsuo & Roy, 2009). The primary replication stage requires the BTV subcore (inner core) proteins (VP1, VP3, VP4 and VP6) and two non-structural proteins (NS1 and NS2), whereas a complete set of 10 ssRNAs is necessary to drive the downstream replication process (Matsuo & Roy, 2009).

In this paper we have demonstrated that AHSV could be recovered entirely from plasmid-derived T7 transcripts and
confirmed by introducing a marker sequence into the genome of AHSV. This method permitted the recovery of mutants in a consistent genetic background with no screening required to remove wildtype (WT) or helper virus. Furthermore, we showed that reassortant viruses exchanging a particular RNA segment could be recovered easily using T7 transcripts only. Our study also demonstrated that similar to BTV, expression of four transcription complex proteins of the AHSV subcore (inner core) plus two major non-structural proteins of AHSV sets the first stage of the replication cycle, which then triggers the full replication of the virus upon second transfection of a complete set of 10 transcripts. Exploiting the highly conserved nature of these core proteins between AHSV serotypes, promiscuity in the efficacy of conserved core proteins in driving replication of viral RNA transcripts of heterologous serotypes was demonstrated. The same serotype source of core proteins in the primary transfection could be used to rescue RNA transcripts of a heterologous serotype source of the second transfection, thus yielding virus with genetic properties of the RNA transcripts of the second transfection. This robust system can be applied for the rapid rescue of nine serotypes of AHSV. These findings have the potential to contribute not only to basic studies on AHSV virology, but also to the development of vaccines for AHSV that can be distinguished from WT AHSV strains.

RESULTS

Standardization of efficiency of virus recovery from RNA transcripts

The efficiency of virus recovery from AHSV transcripts was determined using a combination of DNA plasmids and WT viral transcripts generated in vitro from purified AHSV cores (‘core transcripts’). As recovery of BTV from T7 transcripts was most efficient when cells were transfected twice, first with only six transcripts (four encoding core proteins and two encoding two non-structural proteins), we mimicked the same procedure for the AHSV recovery assay (Matsuo & Roy, 2009). For the first transfection we generated mammalian expression plasmids (consisting of only coding regions) expressing the four core proteins (VP1, VP3, VP4 and VP6) and two NS proteins (NS1 and NS2) of AHSV serotype 6 (AHSV6) under the CAG promoter. The sequence of each inserted gene was verified. BSR cell monolayers were first transfected either with the six AHSV6 plasmids or a plasmid that expressed GFP instead of AHSV protein; cells were transfected a second time with AHSV4 core transcripts and overlaid with agarose as described previously (Boyce & Roy, 2007; Matsuo et al., 2010). After 3 days, cells were fixed, stained with 0.2 % (w/v) crystal violet (upper panel), and the number of plaques was counted and plotted (lower panel). NS, non-structural protein. (b) BSR cells were transfected with six plasmids derived from AHSV4 followed by a second transfection with 10 AHSV6 core transcripts and, in parallel to (a), plasmids from AHSV6 and 10 transcripts from AHSV4 were used for the two transfections. Virus titres (in p.f.u. ml⁻¹) rescued from each combination were calculated and plotted on a logarithmic scale.

Fig. 1. Efficiency of virus rescue using double transfection. (a) BSR cells were first transfected with six mammalian expression (pCAG/MCS-PM1) plasmids (Tani et al., 2007) expressing AHSV6 proteins (VP1, VP3, VP4, VP6, NS1 and NS2). Cells were then transfected a second time with AHSV4 core transcripts and overlaid with agarose as described previously (Boyce & Roy, 2007; Matsuo et al., 2010). After 3 days, cells were fixed, stained with 0.2 % (w/v) crystal violet (upper panel), and the number of plaques was counted and plotted (lower panel). NS, non-structural protein. (b) BSR cells were transfected with six plasmids derived from AHSV4 followed by a second transfection with 10 AHSV6 core transcripts and, in parallel to (a), plasmids from AHSV6 and 10 transcripts from AHSV4 were used for the two transfections. Virus titres (in p.f.u. ml⁻¹) rescued from each combination were calculated and plotted on a logarithmic scale.
transfection using pCAG-GFP as the first transfection instead of AHSV transcripts (Fig. 1a). This indicated that the first transfection with plasmids expressing the six AHSV proteins was sufficient to enhance the virus recovery from the complete set of core transcripts provided in the second transfection, similar to BTV. To confirm this, a second set of six plasmids of AHSV4 was generated in a similar manner and used for transfection together with AHSV6 core transcripts. This combination of six AHSV4 protein expression plasmids together with the complete set of AHSV6 core transcripts also generated AHSV plaques (Fig. 1b). These data suggest that the proteins in the first transfection, which could be from a different AHSV serotype, but not the second transfection, were responsible for setting the primary replication cycle, which then triggered the full replication of the virus upon second transfection. This further supported our hypothesis of a two-phase replication cycle for orbiviruses. In addition, the advantage of using the same source of plasmids for the first transfection and transcripts from different serotypes for the second transfection should enable us to alter serotypes rapidly.

Recovery of AHSV entirely from synthetic T7 transcripts

To recover AHSV entirely from synthetic T7 transcripts, an exact copy of each AHSV segment was cloned as described previously (Boyce et al., 2008). For each segment, a T7 promoter was introduced directly upstream of the genome segment and a restriction enzyme site inserted directly downstream. AHSV4 T7 transcripts synthesized using the restriction-digested plasmid clones were of the expected sizes when resolved on 1% denaturing agarose gels (Fig. 2a). BSR cell monolayers were first transfected with these six AHSV6 plasmids followed by a second transfection with a complete set of 10 T7 transcripts of AHSV4.

The combinations of protein-expressing plasmids and the exact-copy T7 RNA transcripts produced clear AHSV plaques on the transfected monolayer, indicating the recovery of infectious virions (Fig. 2b).

To confirm the authenticity of the rescued virus genome profiles, individual plaques were recovered, amplified in BSR cells and the genomic dsRNAs were isolated from infected cells as described previously (Boyce & Roy, 2007). The dsRNA migration patterns were identical to that of the WT AHSV4 but not AHSV6, the source of the primary replication proteins (Fig. 2c). These data suggested that AHSV4 could be rescued solely from T7 transcripts in the absence of any native viral transcripts or helper virus.

To investigate if specific mutant viruses could be rescued using this reverse genetics system, a single mutation at nt 442 of AHSV4 segment S10 was introduced to generate a novel AflII restriction site without affecting the encoded amino acid sequence (Fig. 3a). To rescue this mutant virus, we performed the same double-transfection protocol. However, the second transfection included nine WT AHSV4 T7 transcripts plus the mutant S10 transcript instead of the WT S10 transcript. Virus plaques were recovered, amplified further and genomic dsRNAs were purified from infected cells. The analysis of dsRNAs on a polyacrylamide gel showed the typical AHSV4 genomic dsRNA profile (Fig. 3b). To confirm the introduced mutation, S10 was amplified by reverse transcription (RT)-PCR, digested with AflII and the size of the fragments determined on agarose gel (Fig. 3c). In addition, the sequence of S10 showed the A→C mutation (data not shown). These data confirmed that the AHSV reverse genetics system is suitable for the direct introduction of mutations in the replicating viral genome.

Generation of reassortant viruses based on synthetic RNA transcripts

Reassortant viruses based on specific genes facilitate not only the functional mapping of the genes, such as virulence...
factors, but also allow for the rapid generation of vaccine strains of particular serotypes. Generation of such reassortants should be highly efficient based on RNA transfection as it would not require the screening of the virus plaques. As a proof of concept we generated the T7 transcript of AHSV6 S5 (encoding NS1 protein) and used it in the second transfection of cells together with the remaining nine AHSV4 T7 transcripts, while the first transfection still included the six plasmids of the primary replication complex of AHSV6 as described above. All plaques that were rescued from transfection had a characteristic AHSV plaque appearance without any visible difference (not shown). When the genomic dsRNAs were purified from infected cells of one of these reassortant viruses (RA4/6S5) and analysed on a polyacrylamide gel, it was not possible to distinguish the dsRNA migration patterns of the reassortant virus from that of the WT AHSV4 (Fig. 4a, compare lanes 1 and 2). Therefore, to verify that RA4/6S5 had AHSV6 segment S5, it was necessary to sequence the genomic segment, which confirmed that segment S5 of the reassortant virus was indeed the AHSV6 S5 segment (data not shown). Furthermore, since S5 of AHSV6, but not the AHSV4 S5, has a distinctive EcoRV site, S5 segments of AHSV4, AHSV6 and the reassortant RA4/6S5 were amplified by RT-PCR and digested with EcoRV. When analysed on agarose gel, it was confirmed that S5 segments of both AHSV6 and the reassortant virus were cleaved but not that of AHSV4 (Fig. 4b). These data further confirmed that reassortant virus could be rescued by the plasmid-based reverse genetics system.

**DISCUSSION**

The purpose of this study was to establish a reverse genetics system for AHSV entirely based on synthetic transcripts. Such a system is highly beneficial for the basic understanding of virus infection at the molecular level, including virus replication, host–virus interactions and viral pathogenesis. These studies could be exploited in the development of rationally designed attenuated vaccines and therapies. Indeed, the main outcome of recent success in the establishment of BTV reverse genetics systems has not been the generation of important information in relation to virus replication and its dependency on certain host factors and pathways, but more importantly, a set of highly attenuated vaccine strains have been generated successfully (Boyce et al., 2008; Matsuo & Roy, 2013).

Genetically, AHSV is significantly different from BTV, which has been studied most extensively and serves as a model system for AHSV. At the protein level, the polymerase VP1, one of the most conserved proteins, shares only 66.5% similarity. The serological determinant protein VP2 of the
outer capsid displays a marked difference to that of BTV, presenting less than 20% similarity between these two viruses. The inner core protein VP7, involved in overall structural stabilization, also shows low homology with only ~42% sequence similarity. These sequence differences are further emphasized by structural data, with VP2 (Manole et al., 2012) and VP7 (Basak et al., 1996) displaying conformations unique to AHSV. In addition, the non-structural proteins show distinct properties between the viruses. For example, the tubules formed by the NS1 proteins in virus-infected cells display a differential morphology in AHSV to that in BTV (Maree & Huismans, 1997), and NS3, which is a relatively conserved protein in BTV across different serotypes, is highly variable in AHSV (van Niekerk et al., 2001). These genetic and structural differences separate BTV and AHSV as individual virus species, as demonstrated experimentally by the lack of reassortment between these two viruses (Brown et al., 1988) and by the distinction in pathobiology.

Given that both viruses are members of the same genus, and that gross properties of viral architecture and replication are similar, the reverse genetics system presented here for AHSV in conjunction with that of BTV presented previously provide powerful systems to study the process of viral divergence and host restriction. Artificially creating AHSV/BTV reassortants or functional domain-switched viruses using the complementary reverse genetics systems could allow the investigation of specific viral factors that differentiate speciation and pathobiology.

We have reported previously that recovery of BTV from RNA requires two sets of transfections: the first transfection provides the primary replication complex consisting of four BTV proteins (VP1, VP3, VP4 and VP6) as well as NS1 and NS2 proteins. Interestingly, given genetic differences, this two-stage replication cycle is also applicable to AHSV replication. Similar to BTV, AHSV rescue was also improved by pre-expressing the structural VP1, VP3, VP4 and VP6 proteins together with the non-structural NS1 and NS2 proteins. The resulting rescue events increased almost 10-fold when the viral plasmids were used instead of a control plasmid in the first transfection (Fig. 1a). These data, taken together with those from the BTV reverse genetics system, suggest that a common two-stage replication mechanism is shared by members of the genus Orbiviruses and potentially other members of the family Reoviridae. Further study of such factors that influence replication could delineate minimum essential components as demonstrated for BTV (Matsuo & Roy, 2013), thus improving our biological understanding of the assembly and replication processes.

A reverse genetics system for AHSV can also contribute to vaccine development similarly to BTV. A new generation of vaccine that is replication-defective (i.e., a disabled infectious single-cycle virus) was developed and tested for BTV (Matsuo et al., 2011; Roy et al., 2009). A similar approach can be taken for AHSV to generate a defective virus with a faulty essential protein. The defective strain can only grow in complementing cells that express a functional version of the targeted protein but fails to replicate in either normal cells or animals. Here, we have presented evidence that a specific mutation and a reassortant virus can be generated using this system. These results have important implications for vaccine development as a segment with a lethal mutation can be introduced in a virus genome. Moreover, the two segments (S2 and S6) encoding the proteins responsible for the virus serotype (VP2 and VP5) can be introduced in a different genome and, for vaccine purposes, in the genome of a defective virus. Uniquely, the rescue of differing serotypes using the same origin of the primary transcription complex presented in this paper offers a particularly promising avenue for the rapid production of vaccines of various AHSV serotypes, allowing a swift response to emerging epidemic strains.

The data presented in this paper could also lead to the development of a better diagnostic system for AHSV, as a specific marker can be introduced in the virus genome. Detection of this marker or antibodies against it can be used to differentiate infected from vaccinated animals (the ‘DIVA’ concept). Current AHSV vaccines are based on live attenuated strains or inactivated virus and are therefore not DIVA compliant, as an infected animal cannot be differentiated from an animal that has been vaccinated with such a vaccine.

**METHODS**

**Cell lines and viruses.** BSR cells (a BHK-21 clone) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 5% (v/v) FBS (Invitrogen). AHSV4 and AHSV6 stocks were obtained by infecting BSR cells at an m.o.i. of 0.1 and harvesting 2–3 days p.i.

**Purification of AHSV cores.** BSR cells were infected with AHSV4 or AHSV6 at an m.o.i. of 0.05. AHSV cores were purified using a modification of methods described previously (Boyce & Roy, 2007; Mertens et al., 1987). Briefly, cells infected with AHSV were lysed at 2 days p.i. in a chilled lysis buffer [100 mM Tris/HCl (pH 8.8), 50 mM NaCl, 10 mM EDTA, 0.3% NP-40]. Nuclei and insoluble cell debris were removed by centrifugation at 1400 g at 4 °C for 15 min. The lysate was treated with 75 µg z-chymotrypsin ml−1 at 35 °C for 1 h and then with 0.2% (w/v) N-lauroyl sarcosine at 25 °C for 1 h. The samples were then centrifuged through a discontinuous sucrose gradient of 70% and 40% (w/v) sucrose in 20 mM Tris/HCl (pH 8.8), 0.6 M MgCl2 at 141 000 g at 16 °C for 1.5 h. The interface was collected and diluted down to 30% (w/v) sucrose. The diluted samples were centrifuged through a 40% (w/v) sucrose cushion in 20 mM Tris/HCl (pH 8.0) buffer at 141 000 g at 16 °C for 1 h. The pellets were resuspended in 20 mM Tris/HCl (pH 8.0).

**In vitro synthesis of AHSV ssRNA from cores and purification.** Core transcripts were synthesized and purified using a similar method used for BTV as described previously (Boyce & Roy, 2007) with some modifications. Briefly, 50 µg of AHSV cores were incubated at 30 °C for 4 h in 500 µl of transcription reaction mixture [100 mM Tris/HCl (pH 8.0), 4 mM rATP, 2 mM rGTP, 2 mM rCTP, 2 mM rUTP, 500 µM S-adenosylmethionine, 6 mM DTT, 9 mM MgCl2, 50 µM MnCl2] supplemented with 0.2 U RNasin Plus µl−1 (Promega). Cores were removed by centrifugation at 18 000 g at 4 °C for 45 min. The
supernatant was collected and incubated with 2 M LiCl at 4 °C for 16 h to precipitate core transcripts. Core transcripts were precipitated by centrifugation at 18,000 g at 4 °C for 15 min, resuspended into Opti-MEM (Invitrogen) containing 0.1 U RNasin Plus μl⁻¹ and purified using standard phenol/chloroform extraction methods, which can completely remove the core particles. Purified core transcripts were analysed by electrophoresis on a 0.8 % agarose gel in MOPS buffer in the presence of formaldehyde, using standard techniques.

**Sequence-independent cloning of AHV segments.** T7 plasmids for AHV transcripts used in the reverse genetics system were synthesized as described previously (Boyce et al., 2008). Briefly, dsRNA was extracted from purified core particles and cDNA from each segment was amplified using a sequence-independent method (full-length amplification of cDNAs) (Maan et al., 2007). Each segment was cloned and sequenced. A functional cassette was generated that introduced a T7 promoter directly upstream and a restriction enzyme site directly downstream.

For the plasmid-based AHV reverse genetics system, the protein genes required for AHV primary replication were cloned as described by Matsuo & Roy (2009). The coding region of each of the AHV S1, S3, S4, S7, S8 and S9 segments, encoding proteins VP1, VP3, VP4, VP6, NS1 and NS2, respectively, was inserted into the mammalian expression plasmid pCAG/MCS-PM1 (a generous gift from Y. Matsuura) (Tani et al., 2007). The sequence of each inserted gene was verified.

**T7 transcription.** Synthesis of capped AHV transcripts was performed as described previously (Boyce et al., 2008). All capped T7 transcripts were synthesized with a mMESSAGE mMACHINE T7 Ultra Kit (Ambion) following the manufacturer’s protocol.

**Transfection for recovery of infectious virus.** Confluent BSR cell monolayers were transfected once or twice with AHV core transcripts using Lipofectamine 2000 Transfection Reagent (Invitrogen) as described previously (Boyce et al., 2008). For the modified reverse genetics system, the first transfection was performed with 100 ng of primary replication that required protein expression plasmids (expressing VP1, VP3, VP4 and VP6) plus the non-structural proteins (NS1 and NS2), followed by a second transfection with 100 ng of each of the 10 T7 transcripts at 18 h post-first-transfection.

**Plaque assay.** At 6 h post-second-transfection for viral recovery or 2 h post-infection for virus titration, the culture medium was replaced with 1.5 ml overlay consisting of MEM, 2 % FBS and 1 % (w/v) agarose type VII (Sigma), and the plates were incubated at 35 °C in 5 % CO₂ for 3 days to allow plaques to appear. All picked plaques were amplified in BSR cells.

**Genomic dsRNA purification and electrophoresis.** For dsRNA purification, cells were infected and total RNA was extracted at 3–6 days p.i. using Tri-reagent (Sigma) as described previously (Boyece & Roy, 2007). Purified dsRNA samples were analysed using non-denaturing 8–11 % polyacrylamide gels.

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**REFERENCES**


