Arenavirus reverse genetics for vaccine development

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INTRODUCTION

Arenaviruses are important human pathogens with no Food and Drug Administration (FDA)-licensed vaccines available and current antiviral therapy being limited to an off-label use of the nucleoside analogue ribavirin of limited prophylactic efficacy. The development of reverse genetics systems represented a major breakthrough in arenavirus research. However, rescue of recombinant arenaviruses using current reverse genetics systems has been restricted to rodent cells. In this study, we describe the rescue of recombinant arenaviruses from human 293T cells and Vero cells, an FDA-approved line for vaccine development. We also describe the generation of novel vectors that mediate synthesis of both negative-sense genome RNA and positive-sense mRNA species of lymphocytic choriomeningitis virus (LCMV) directed by the human RNA polymerases I and II, respectively, within the same plasmid. This approach reduces by half the number of vectors required for arenavirus rescue, which could facilitate virus rescue in cell lines approved for human vaccine production but that cannot be transfected at high efficiencies. We have shown the feasibility of this approach by rescuing both the Old World prototypic arenavirus LCMV and the live-attenuated vaccine Candida#1 strain of the New World arenavirus Junin. Moreover, we show the feasibility of using these novel strategies for efficient rescue of recombinant tri-segmented both LCMV and Candida#1.

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Generation of recombinant viruses from cloned cDNAs has become possible for members of all negative-strand (NS) RNA viral families (Fodor et al., 1999; Neumann & Kawaoka, 2004; Neumann et al., 2002a). These plasmid-based reverse genetics techniques have revolutionized the study of NS RNA viruses by providing researchers with a powerful tool for investigating different aspects of viral biology both in cultured cells and animal models of infection (García-Sastre & Palese, 1993; Martínez-Sobrido & García-Sastre, 2010; Pekosz et al., 1999). Thus, reverse genetic approaches have facilitated the analysis of cis-acting viral sequences and trans-acting viral proteins required for virus replication and transcription (Durbin et al., 1997; Hass et al., 2004; Lee et al., 2000; López et al., 2001; Pinschewer et al., 2005; Wertz et al., 1994), assembly and budding (Gómez-Puertas et al., 2000; Li et al., 1993; Perez et al., 2003), and virus–host cell protein interactions (Campbell Dwyer et al., 2000; Hale et al., 2008; Mayer et al., 2007). Likewise, the use of reverse genetics approaches is playing a critical role in the identification and characterization of viral determinants of fitness (Grimm et al., 2007; Herfst et al., 2012; Steidle et al., 2010) and the investigation of mechanisms by which viruses counteract the host antiviral defences (Buettnner et al., 2010; Koehs et al., 2007; Mibayashi et al., 2007). Moreover, the ability to create recombinant viruses containing predetermined mutations within their genomes and analysis of their phenotype in vivo has provided investigators with novel and powerful approaches for the investigation of the cellular and molecular bases of viral pathogenesis (Bridgen et al., 2001; Morimoto et al., 2000; Nagai, 1999; Neumann et al., 2002b). In addition, reverse genetics has been instrumental in the generation of novel vaccine and vector platforms to combat viral infections (Davtyan et al., 2011; García-Sastre et al., 1998; Hai et al., 2008; Vigil et al., 2007; von Messling & Cattaneo, 2004).

 Arenaviruses are enveloped viruses with a bi-segmented NS RNA genome. Each RNA segment uses an ambisense strategy to direct the expression of two viral proteins in opposite orientations and separated by a non-coding intergenic region. The large segment (L; 7.2 kb) encodes the L protein, an RNA-dependent RNA polymerase, and the small RING finger protein Z that serves as a...
bona fide matrix protein. The small segment (S; 3.5 kb) encodes the viral nucleoprotein (NP) and surface glycoprotein (GP). Arenaviruses cause chronic infections in rodents across the world. Asymptomatically infected animals move freely in their natural habitat and humans can become infected by exposure to virus present in excretions of infected animals, which could result in severe disease (Buchmeier et al., 2007). Two Old World (OW) arenaviruses, Lassa (LASV) and Lujo (LUJV) viruses and six New World (NW) arenaviruses: Junin (JUNV), Machupo (MACV), Sabia (SABV), Guanarito (GTOV), Whitewater Arroyo (WWAV), and Chapare (CHPV) viruses, are known to cause haemorrhagic fever (HF) disease in humans (Buchmeier et al., 2007; Delgado et al., 2008). Among known HF arenaviruses, LASV, the causative agent of Lassa fever (LF), poses the highest impact in public health due to its vast endemic region within sub-Saharan Africa with a large population at risk. Notably, increased travelling to and from endemic regions has resulted in importation of cases of LF into non-endemic metropolitan areas around the globe (Buchmeier et al., 2007; Holmes et al., 1990; Isaácson, 2001). Moreover, evidence indicates that the globally distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is likely a neglected human pathogen of clinical significance in neonatal viral infections (Barton, 1996; Jahrling & Peters, 1992; Mets et al., 2000), as well as for immunocompromised individuals (Fischer et al., 2006; Palacios et al., 2008). Public health concerns about arenavirus infections in humans are further aggravated because no Food and Drug Administration (FDA) licensed arenavirus vaccines exist and current anti-arenaviral therapy is limited to an off-label use of the nucleoside analogue ribavirin, which is only partially effective, requires early and intravenous administration for its optimal efficacy, and is often associated with significant side effects (Kilgore et al., 1997; McKee et al., 1988; Mistry et al., 2009; Snell, 1988). Therefore, it is important to develop novel and effective antiviral strategies to combat human pathogenic arenaviruses.

The rescue of infectious arenavirus from cloned cDNAs was initially established for the prototypic member of the family, LCMV (Flatz et al., 2006; Sánchez & de la Torre, 2006). Subsequently, similar approaches were used to rescue recombinant JUNV (Albarino et al., 2009; Emonet et al., 2011b), LAVSV (Albarino et al., 2011a), PICV (Lane et al., 2009) and LUVJ (Bergeron et al., 2012). As with other NS RNA viruses, the development of arenavirus reverse genetics has allowed researchers to gain a better understanding of the arenavirus molecular and cell biology and virus-host interactions underlying arenavirus pathogenesis and associated disease, as well as to explore novel avenues for the development of antiviral drugs and vaccine design (Emonet et al., 2009, 2011a; Rodrigo et al., 2011).

Current arenavirus reverse genetics systems rely on the use of murine (m) RNA polymerase I (Pol-I) (Carne et al., 2011; Emonet et al., 2011b; Flatz et al., 2006) or bacteriophage T7 RNA polymerase (Albarino et al., 2011a; Bergeron et al., 2012; Hass et al., 2004; Lopez et al., 2001) promoters to direct the initial intracellular synthesis of the S and L genome or antigenome RNA species. These RNA species are subsequently recognized by the plasmid-supplied minimal viral trans-acting factors, L and NP, required for viral replication and transcription (Albarino et al., 2011b; Lan et al., 2009). These arenavirus rescue systems are based on the use of cell lines lacking FDA approval for vaccine development (Schiff, 2005). Therefore, the generation of recombinant arenavirus from cloned cDNAs in FDA-approved cell lines would facilitate the development of potential arenavirus vaccine seeds. Here we report the development of reverse genetic systems for the OW LCMV and the NW Candida#1 (live-attenuated vaccine strain of JUNV) arenaviruses based on the use of a human (h) RNA Pol-I promoter to direct the initial intracellular synthesis of the viral S and L antigenome RNA species. This new approach allowed us to efficiently rescue recombinant LCMV and Candida#1, as well as their corresponding tri-segmented versions, in human (293T) and FDA-approved (Vero) cell lines. Moreover, we document the efficient rescue of recombinant arenaviruses using plasmids that combine the use of RNA Pol-I and polymerase II (Pol-II) to direct the synthesis of antigenome L and S RNA species (via Pol-I) and viral trans-acting factors L and NP (via Pol-II). This approach reduced by half the number of plasmids required for virus rescue, thus helping to overcome difficulties posed by transfection efficiencies associated with FDA-approved cell lines (Hoffmann et al., 2000; Murakami et al., 2008; Nicolson et al., 2005).

RESULTS

Assessment of the RNA polymerase I terminator (Pol-I T) and hepatitis delta virus ribozyme (HDVR) efficiencies in the generation of plasmid-derived arenavirus antigenome RNA species

Efficient rescue of most NS RNA viruses via reverse genetics approaches has shown that plasmid-derived genome (or antigenome) RNA species should contain authentic vRNA 3’ ends to facilitate encapsidation and subsequent amplification by the intracellularly reconstituted virus polymerase complex (Flatz et al., 2006; Fodor et al., 1999; Neumann et al., 1999). Both the self-cleaving HDVR (Fodor et al., 1999) and the Pol-I T (Neumann et al., 1999) have been widely used to generate authentic vRNA 3’ ends. Recent reports have suggested that Pol-I T sequences are more efficient than HDVR sequences in producing influenza-virus-like RNAs with precise 3’ ends in a plasmid-based virus rescue system (Feng et al., 2009). To examine whether the same finding could be extended to arenaviruses, we established a human cell-based arenavirus minigenome (MG) assay (Fig. 1). We generated a MG hpPol-I plasmid that contains two reporter genes: a fusion of GFP to the puromycin (Pur) resistance gene (Pur-GFP) and the secreted Gaussia luciferase (Gluc), flanked by the
UTRs of the LCMV S segment in antigenomic orientation and separated by the corresponding intergenic region (IGR) (Emonet et al., 2009). In this construct, the Pur-GFP fusion substitutes the arenavirus NP, and Gluc substitutes the arenavirus GP. We elected to use Pur-GFP rather than GFP alone because the sequence length (1389 nt) of Pur-GFP more accurately resembles the NP sequence length (1677 nt). We generated two versions of this MG. In both of them RNA synthesis was directed by the hPol-I (Schickli et al., 2001), but termination of RNA synthesis to generate authentic viral 3′-termini was mediated by the Pol-I T (Fig. 1a, top) or the HDVR (Fig. 1a, bottom). To compare the activity of both arenavirus MGs, we co-transfected human 293T cells with each of the dual-reporter MG plasmid, together with pCAGGs (pC)-NP and pC-L LCMV plasmids, and a *Cypridina* luciferase-encoding plasmid (SV40-Cluc) (Nakajima et al., 2004) to normalize transfection efficiencies. At 48 h post-transfection (p.t.), reporter gene activities were assessed. Both GFP (Fig. 1b) and Gluc (Fig. 1c) expression levels were greater in assays using the MG plasmid containing Pol-I T than HDVR. Although we have not biochemically characterized the 3′ ends of the RNA species produced by the MG plasmids containing either Pol-I T or HDVR, these results would suggest that, as with influenza virus (Feng et al., 2009), the Pol-I T is more efficient in generating the appropriate LCMV 3′ end.

**Rescue of recombinant infectious arenaviruses in human 293T and FDA-approved Vero cell lines**

Arenavirus vaccine development would be facilitated by the ability to rescue recombinant arenaviruses using FDA-approved cell substrates (e.g. Vero). Reverse genetics approaches based on the use of the mouse RNA Pol-I promoter to direct intracellular synthesis of S and L genome, or antigenome, RNA species have been successfully implemented (Carne et al., 2011; Emonet et al., 2011b; Flatz et al., 2006). However, unlike the RNA Pol-II promoter, the transcriptional activity of the RNA Pol-I promoter exhibits stringent species specificity (Heix & Grummt, 1995). We therefore examined the use of the hPol-I promoter system, previously shown to be efficient in the generation of other recombinant NS RNA viruses from Vero cells (Billecocq et al., 2008; Fodor et al., 1999), to rescue arenaviruses.

We first confirmed the host specificity of our Pol-I based MG reporter systems (Fig. 2). For this, we co-transfected BHK-21 or 293T cells with either the human-based or mouse-based MG dual-reporter plasmid (Fig. 2a top and bottom, respectively) together with pC-NP and pC-L LCMV plasmids and SV40-Cluc plasmid. At 48 h p.t., reporter gene activities were assessed (Fig. 2b, c). Reporter gene expression confirmed the species-specific activity of our Pol-I based LCMV MG reporter system.

To establish the system for virus rescue, we incorporated the S and L segments of either LCMV or Candida1 into the hpPol-I T plasmids in antigenome orientation with respect...
to the hPol-I promoter (Fig. 3). For virus rescue, both human 293T and Vero cells were co-transfected with the pC-NP and pC-L plasmids together with the hpPol-I T plasmids containing the S and L sequences for LCMV or Candida#1 (Fig. 3a). Transfections without pC-NP plasmid were used as negative controls. Successful virus rescue was determined by infecting fresh Vero cells with tissue culture supernatants (TCSs) collected from transfected cells. At 48 h post-infection (p.i.), cells were fixed and viral antigen was detected by immunofluorescence using antibodies to NP (Fig. 3b, c). We have been able consistently to rescue recombinant LCMV and Candida#1 viruses in multiple independent attempts, indicating the robustness and high efficiency of the system. We observed similar rescue efficiencies in both 293T and Vero cell lines. To further characterize the efficiency of the rescue of infectious

Fig. 2. Specificity of the Pol-I promoter. (a) Schematic representation of plasmids expressing dual-reporter LCMV S RNA analogues (MG) under the hPol-I (top) or mPol-I (bottom) promoters: dual-reporter LCMV S RNA analogue plasmids encoding Pur-GFP and Gluc instead of the viral NP and GP, were inserted between the human RNA polymerase I promoter (hPol-I, top) or the murine RNA polymerase I promoter (mPol-I, bottom) and the polymerase terminator (Pol-I T) sequences. (b, c) Species specificity of the Pol-I promoter. Human 293T cells and rodent BHK-21 cells were co-transfected with the pC LCMV NP and L expression plasmids together with hPol-I or mPol-I Gluc/Pur-GFP reporter plasmids, together with pSV40-Cluc vector to normalize transfection efficiencies. As negative control, cells were co-transfected only with LCMV pC-L, using empty pC to keep constant the total amount of transfected DNA. At 48 h p.i., GFP expression was detected by fluorescence microscopy (b) and TCSs were analysed for the presence of secreted Gluc and Cluc (c); representative GFP expression images and Gluc fold induction over the negative controls for three independent experiments are shown. Scale bar, 100 μm.
rLCMV and rCandid#1 in 293T and Vero cells using the human Pol-I based system, we titrated the TCS of the corresponding transfections (see Fig. 6 below).

Tri-segmented arenavirus rescue for vaccine vector purposes

The generation of recombinant tri-segmented (r3) arenaviruses capable of expressing two additional foreign genes in infected cells has opened new strategies for designing an arenavirus vaccine (Emonet et al., 2009, 2011b; Popkin et al., 2011). Because rescue of r3 arenaviruses has been documented only using rodent BHK-21 cells, we assessed the feasibility of generating tri-segmented arenaviruses (r3LCMV and r3Candid#1) in Vero cells (Fig. 4). For this, we split the S segments of LCMV and Candid#1 into two hPol-I-based plasmids, one containing the Gluc ORF in lieu of GP and the other containing the GFP ORF in lieu of NP (Emonet et al., 2009, 2011b; Neumann et al., 1999) (Fig. 4a). These plasmids (hpPol-I GP/GFP and hpPol-I Gluc/NP) were co-transfected, together with pC-NP, pC-L and hpPol-I L plasmids into Vero cells. Six days after transfection, TCSs were used to infect fresh Vero cells and at 48 h p.i. the presence of virus was determined based on GFP expression [Fig. 4b(i), c(i)] and levels of Gluc activity [Fig. 4b(ii), c(ii)]. Levels of NP expression were similar in cells infected with wt of r3 viruses as determined by Western blot (WB) of total cell lysates [Fig. 4b(iii), c(iii)]. As expected, GFP and Gluc expression were detected only in lysates from cells infected with tri-segmented recombinant viruses. Since Gluc is secreted after its synthesis, only low levels of Gluc protein were detected in cell lysates (Tannous et al., 2005).

Generation of recombinant arenaviruses using a two-plasmid-based system

With the goal to facilitate the generation of future arenavirus vaccine candidates, we aimed at developing a two-plasmid-based reverse genetics to rescue LCMV and Candid#1 in the FDA-approved Vero cell line (Fig. 5). In this approach, the two plasmids contain both RNA Pol-II and -I promoters, to allow for Pol-II mediated expression of the minimal viral trans-acting factors (L and NP) required for viral replication and transcription, and Pol-I mediated initial intracellular synthesis of S and L RNA species (Fig. 5a). To avoid possible recombination that could occur by including the sequence of the same viral protein twice in the same plasmid, we cloned the NP ORF within the plasmid containing the L segment (pC-NP/hpPol-I L) and the L ORF within the plasmid containing the S segment (pC-L/hpPol-I S). We first used the MG assay described above (Fig. 1) to assess whether expression levels of NP and L supplied by pC-NP/hpPol-I L and pC-L/hpPol-I S, respectively, resulted in levels of MG expression comparable to those obtained with pC-NP and pC-L plasmids used in the four-plasmid-based arenavirus rescue system. For this, we co-transfected 293T cells with the two-plasmid system (pC-NP/hpPol-I L and pC-L/hpPol-I S) or with the plasmids used in the four-plasmid rescue approach (pC-NP and pC-L) together with the dual-reporter MG plasmid using the conditions established in Fig. 1. At 48 h p.i., we observed fewer GFP-expressing cells (Fig. 5b) and lower levels of Gluc expression (Fig. 5c) in the two-plasmid system than in the four-plasmid system. It is worth noting that in the two-plasmid-based system the Z protein would be expressed by the intracellularly reconstituted virus polymerase complex, and Z is known to inhibit arenavirus RNA replication and transcription (Flatz et al., 2006; Kranzusch & Whelan, 2011; Wang et al., 2012). Despite the observed lower values of reporter gene expressions using the two-plasmid system, we tested the feasibility of using the system to produce rLCMV and rCandid#1 in Vero cells (Fig. 6). For this we co-transfected Vero cells with pC-NP/hpPol-I L and pC-L/hpPol-I S of LCMV or Candid#1 plasmids. Six days after transfection, rescue of LCMV and Candid#1 was determined by infecting fresh Vero cells with TCS collected from transfected cells (Fig. 6a). When the TCSs, directly obtained from the transfected cells, were compared side by side, the two-plasmid-based system produced viral titres of rLCMV (Fig. 6b) or rCandid#1 (Fig. 6c) to levels similar to those obtained with the four-plasmid approach in both 293T and Vero cells.

DISCUSSION

Existing arenavirus reverse genetics systems have proven to be of great value for the investigation of arenavirus biology. However, these current systems may face significant barriers for vaccine development. Because of the species specificity of the Pol-I promoter, previously developed systems based on the use of the murine RNA Pol-I promoter would perform extremely poorly in human and non-human primate (NHP) cell lines that already have FDA approval for vaccine development. Recent approaches incorporating the use of the T7 RNA polymerase to direct the initial intracellular synthesis of S and L antigenome RNA species have proven able to efficiently rescue recombinant arenaviruses. However, this approach also relied on the use of murine cells that constitutively express the bacteriophage T7 polymerase (BSRT7/5) (Albariño et al., 2011a; Lan et al., 2009; Sánchez et al., 2005), which are not yet approved by the FDA for vaccine development (Schiff, 2005). Plasmid supplying T7 RNA polymerase could be used to adapt this system to other cell lines, including those approved by the FDA, but it would require the transfection of an additional plasmid into a single cell for production of virus progeny, which could affect the overall rescue efficiency with cell lines that have low transfection efficiencies, such as some FDA-approved cell lines (Hoffmann et al., 2000; Murakami et al., 2008; Nicolson et al., 2005). To overcome these limitations, we developed a rescue system to produce efficiently recombinant OW
(a) Diagrams showing the expression of different viral proteins under various conditions.

(b) Fluorescence images of 293T cells transfected with rLCMV or rCandid#1, showing expression in Mock and P1 conditions.

(c) Fluorescence images of Vero cells transfected with rLCMV or rCandid#1, showing expression in Mock and P1 conditions.
and NW arenaviruses in human cells, and also in the FDA-approved NHP Vero cell line.

We first established a hPol-I based MG system and showed that the Pol-I terminator is more efficient than the HDVR in generating the precise vRNA 3’ ends which are required for efficient amplification of NS RNA viral genomes (Fig. 1). This result could be explained by the fact that the cleavage efficiency of HDVR is lowest when the nucleotide located immediately after the cleavage site is a guanosine, as is the case in arenavirus genomes (Lee et al., 2000; Perrotta & Been, 1990). To our knowledge, this is the first time that a human cell-based MG system has been developed for arenaviruses (Fig. 2). Advantages of our new MG reporter system are as follows: (i) the dual-reporter nature of the assay can be easily monitored qualitatively, based on GFP expression detected by epifluorescence, and quantitatively based on Gluc activity; (ii) Gluc is efficiently secreted into the cell TCS, which allows for multiple measurements over time, as well as collection of data from cell lysates at the experiment end point; (iii) the system works in human cells, which should facilitate investigations aimed at the identification and characterization of host cellular proteins contributing to the regulation of arenavirus RNA replication and gene transcription that may be unique to humans and could play a role in arenavirus infection of humans and associated disease: in this regard it is worth noting the evidence suggesting that adaptation of the polymerase complex of highly pathogenic avian influenza viruses to interact with human rather than avian host proteins contributes to disease severity in humans (Bortz et al., 2011); (iv) the use of a dual-reporter gene system could represent an advantage in antiviral drug screens by selecting for compounds that affect expression of both reporter genes and thereby diminish the rate of false positive hits (James et al., 1996).

Our ability to efficiently rescue wt (Fig. 3) and r3 (Fig. 4) LCMV and Candida#1 in human and Vero cells should facilitate new avenues for the development of arenavirus vaccines. Particularly appealing would be the use of a Candida#1 platform, for which there is already extensive safety data in humans, to express antigens able to elicit protective immune responses against LASV to generate a single arenavirus vaccine covering the two HF arenaviruses with the highest impact in public health. We further refined our approach by developing a rescue system that eliminates the need for individual Pol-II based expression plasmids to supply the required L and NP viral trans-active factors (Figs 5 and 6), providing a more cost-effective reverse genetics technique to produce vaccine candidates in FDA-approved Vero cell lines.

Previous viral reverse genetics techniques aimed at reducing the number of plasmids required for efficient rescue of recombinant NS RNA viruses have employed vectors using bi-directional expression strategies (Hoffmann et al., 2000; Quinlivan et al., 2005). We therefore attempted to implement a similar approach with LCMV. Interestingly, despite LCMV-based bi-directional expression plasmids expressing levels of NP and L that were capable of promoting detectable levels of LCMV MG activity (data not shown), all our attempts to rescue rLCMV using this approach were unsuccessful. It is plausible that the arenavirus ambisens coding strategy poses barriers to this approach. The use of a bi-directional strategy with LCMV would involve plasmids directing synthesis of an NP mRNA containing the IGR-S and GP ORF, and an L mRNA containing the IGR-L and Z ORF. The rather large 3' UTR of these mRNAs, together with the presence of the highly structured IGR sequences and the distance between the end of the ORF and the polyadenylation (pA) signal might have affected their stability and expression, thereby preventing the rescue of rLCMV.

A recent report has described the successful generation of rCandida#1 without the requirement of protein-encoding supporting plasmids, relying solely on full-length copies of the two RNA genome segments (S and L) from T7-based transcription vectors (Albarino et al., 2009). This novel experimental approach represents an important advantage over previously reported systems, as it requires only two plasmids. However, this technology relies on the use of T7-expressing BHK-21 cells (BSRT7/5) that are not FDA-approved for vaccine development. The use of T7-expressing plasmids could potentially allow the generation of recombinant arenaviruses from different cell lines. However, recovery of recombinant NS RNA viruses based on a T7 RNA polymerase vector has been shown to be less efficient that the use of RNA Pol-I and RNA Pol-II approaches to synthesize rRNA and mRNA, respectively, from plasmids into susceptible cells (de Wit et al., 2007). In addition, it would require transfecting a third plasmid that could impact the efficiency of the system with cell lines, including those that are FDA-approved, that are not highly transfectable.

The ability to generate recombinant arenaviruses rapidly and efficiently in the FDA-approved Vero cell line should facilitate the design and production of novel live-attenuated arenavirus vaccines similar to recent successful efforts with influenza virus (Murakami et al., 2008; Nicolson et al., 2005).
**METHODS**

**Cells.** Baby hamster kidney (BHK-21) cells (ATCC CCL-10), human embryonic kidney (293T) cells (ATCC CRL-11268) and African green monkey kidney epithelial (Vero) cells (ATCC CCL-81) were grown in a 5% CO₂ humidified atmosphere at 37°C and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹).

**Plasmids.** Minigenome (MG) reporter plasmids: The dual LCMV murine reporter plasmid mpPol-I Gluc/Pur-GFP has been described

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**Fig. 4.** Rescue of recombinant tri-segmented (r3) LCMV and Candid#1 in Vero cells. (a) Schematic representation of the plasmid used for the generation of r3LCMV and r3Candid#1. (b, c) Virus rescue. Protein expression plasmids (pC) encoding NP and L were co-transfected together with the respective LCMV (b) or Candid#1 (c) hpPol-I GP/GFP, hpPol-I Gluc/NP and hpPol-I L plasmids into Vero cells. At 72 h p.t., cells were transferred into 10 cm dishes for an additional 72 h before TCSs were collected and assessed for virus rescue based on their infectivity in fresh Vero cells by GFP expression [b(i) and c(i)]. Representative images of at least three independent virus rescues are shown. Scale bar, 100 μm. TCSs from infected Vero cells were collected to assess levels of Gluc activity [b(ii) and c(ii)]. Mock-infected cells were included as negative controls. Cell lysates were prepared and analysed for protein expression by WB [b(iii) and c(iii)] using a monoclonal antibody for GFP or a polyclonal antibody for Gluc. LCMV and Candid#1 NP expressions were assessed with monoclonal antibodies. GAPDH protein expression was included as a loading control.
Briefly, the puromycin resistance (Pur) ORF fused to the N-terminal of the GFP (Pur-GFP) and the ORF of *Gaussia luciferase* (Gluc) replaced the NP and GP ORFs, respectively, in the S genome segment, which is flanked by the murine Pol-I promoter and Pol-I terminator (T) sequences to direct intracellular synthesis of recombinant S antigenome RNA species. To generate the corresponding MG reporter plasmid under the control of human (h) Pol-I promoter, the hpPol-I vector (Schickl et al., 2001) was modified to incorporate the 3' and 5' UTRs of the LCMV S segment (hpPol-I S UTRs). This plasmid contained an internal AvrII site that was used for subcloning the reporter genes and IGR from the mpPol-I Gluc/Pur-GFP where the reporter genes were in antigenome orientation with respect to the hPol-I and the hepatitis delta virus ribozyme (HDVR), and was designated hpPol-I Gluc/Pur-GFP HDVR. To produce the hpPol-I Gluc/Pur-GFP T, the HDVR found in the hpPol-I S UTRs was replaced by the terminator sequence of the RNA Pol-I to generate the hpPol-I S UTRs T plasmid. Subsequently, the reporter genes were cloned into this vector, using the AvrII site, in an antigenome orientation and under the control of hPol-I promoter and T sequences.

### Reverse genetics plasmids: LCMV and Candida plasmids were described (Emonet et al., 2011b; Lee et al., 2002). LCMV and Candida plasmids were made by digesting the LCMV (Flatz 2002) and Candida plasmids (Casas et al. 2011b) with SalI and fragments containing the viral ORFs and IGR were subcloned into the hpPol-I S UTRs T plasmid. To generate the LCMV and Candida plasmids, the hpPol-I S UTRs T was modified by site-directed mutagenesis to introduce two nucleotide changes (A9C and T11A) in the highly conserved 3' UTR to generate hpPol-I S UTRs T plasmids. In a second step, LCMV and Candida plasmids were digested with AvrII and subcloned into the hpPol-I S UTRs T plasmids. Subsequently, the reporter genes were cloned into this vector, using the AvrII site, in an antigenome orientation and under the control of hPol-I promoter and T sequences.

### Virus rescue

**Wild-type (wt) virus rescue:** For the four-plasmid rescue, Vero and 293T cells were co-transfected (six-well plate format, 10^6 cells per well, triplicates) with 0.8 μg of pC-NP, 1.0 μg of pC-L, 0.8 μg of hpPol-I S, and 1.4 μg of hpPol-I L LCMV or Candida plasmids, using 2.5 μg of LF20000 μg⁻¹ DNA. For the two-plasmid (pC/pC-hpPol-I) rescue system, cells were co-transfected with 2.0 μg of pC-NP/hpPol-I L and 1.0 μg of pC-hpPol-I S LCMV or Candida plasmids, using 2.5 μg of LF20000 μg⁻¹ DNA.

**Tri-segmented virus rescue:** For the generation of recombinant tri-segmented (3) LCMV and Candida viruses, Vero cells were co-transfected with 0.8 μg of pC-NP, 1.0 μg of pC-L, 0.8 μg of hpPol-I GP/GFP, 0.8 μg of hpPol-I Gluc/NP, and 1.4 μg of hpPol-I L, using 2.5 μg of LF20000 μg⁻¹ DNA.

At 12 h p.t., media were replaced with 2 ml of post-infection media (1:2 ratio of 10% FBS DMEM and Opti-MEM). At 72 h p.t. cells were trypsinized and passed into 10 cm dishes for another 72 h before TCSSs were collected and assessed for viral rescue by infecting fresh monolayers of Vero cells and detecting viral antigen expression by immunofluorescence microscopy (wt virus) or GFP expression (tri-segmented viruses) 48 h after infection.

### Virus titrations and immunofluorescence

Subconfluent Vero (4 × 10^6) cells in 96-well plates were infected for 90 min at 37 °C with serial dilutions of virus-containing TCSS samples. After viral adsorption, the virus inoculum was replaced with infection media. For wt viruses, titres [focus-forming units (FFU) ml⁻¹] were determined using an immunofocus centre assay (Battegay et al., 1991). For this, cells were fixed with 4% formaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min at room temperature, followed by overnight blocking at 4 °C with 2.5% BSA in 1x PBS. After overnight blocking, cells were incubated with antibodies to LCMV NP (1:1.3) (Rodrigo et al., 2011) or Candida NP (BEI Resources, SA02-BG12) for 1 h at 37 °C. Cells were then washed three times with 1x PBS followed by incubation with a secondary FITC-conjugated rabbit anti-mouse antibody (Dako, 1:140 dilution) for 30 min at 37 °C. Finally, cells were washed with 1x PBS and visualized under a Leica fluorescent microscope and luciferase activities in TCSSs, using a Biorad BioTuk fluorometer (New England Biolabs) to normalize transfection efficiencies. Transfections were done using 2.5 μg of Lipofectamine 2000 (LF2000) μg⁻¹ DNA. For the MG reporter assays with pC/hpPol-I plasmids, pC-NP and pC-L plasmids were substituted with the pC-NP/hpPol-I-L (0.3 μg) and pC-L/hpPol-I S (0.6 μg) vectors. At 48 h post-transfection (p.t.), reporter gene expression was assessed by fluorescence microscopy using a Leica fluorescent microscope and luciferase activities in TCSSs, using a Luminiecut lumiminator (Packard). Microscope images were coloured using Adobe Photoshop CS4 (v11.0) software. Representative fields of at least three independent transfections are shown. Gluc activity was determined with the Biolum *Gaussia Luciferase Assay kit* (New England Biolabs) and Gluc activity by the Biolum *Cypridina Luciferase Assay kit* (New England Biolabs). Reporter gene activation (Gluc) is indicated as fold induction over the negative controls where the transfected pC-NP plasmid was replaced by the empty pc plasmid. All MG assays were performed in triplicate. Mean value and standard deviation were calculated using Microsoft Excel software.

**Arenavirus two-plasmid-based rescue:** To establish the two-plasmid rescue system for LCMV, pC-NP and L plasmids were digested with *SalI* and the polyadenylation (pA) signal were subcloned into the hpPol-I L UTRs T plasmid. A similar strategy was followed to produce Candida plasmids with hpPol-I L and hpPol-I S vectors.

**Plasmids for rescue of tri-segmented arenaviruses:** hpPol-I GP/GFP and mpPol-I Gluc/NP (Ortiz-Riaño et al., 2012) were digested with AvrII and fragments containing the viral ORFs and IGR were subcloned into the hpPol-I S UTRs T plasmid to generate hpPol-I GP/GFP and hpPol-I Gluc/NP plasmids, respectively. Plasmids to rescue tri-segmented Candida were constructed following a similar approach. The GFP ORF was amplified by PCR with primers containing BbsI restriction sites and cloned into the Candida S segment backbone BbsI plasmid (Emonet et al., 2011b) to generate the mpPol-I GP/GFP plasmid. Similarly, *Gaussia luciferase* (Gluc) was amplified by PCR from the pC-Gluc plasmid (Capul & de la Torre, 2008) with primers containing BamHI restriction sites and cloned into the Candida S segment backbone mpPol-I BsmBI/NP plasmid (Emonet et al., 2011b) to generate the plasmid mpPol-I Gluc/NP. Both plasmids (mpPol-I GP/GFP and mpPol-I Gluc/NP) were digested with AvrII and fragments containing the ORFs and IGR subcloned into hpPol-I S UTRs plasmid to produce hpPol-I GP/GFP and hpPol-I Gluc/NP plasmids.

All plasmids were generated using standard cloning techniques. Primers for making the described plasmid constructs are available upon request. All plasmid constructs were verified by DNA sequencing.
(a) 

(b) 

(c) 

Fold induction

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4-plasmid 2-plasmid
**Fig. 5.** A two-plasmid arenavirus rescue system. (a) Schematic representation of the two-plasmid system for the generation of rLCMV and rCandid#1: Plasmids encoding either NP and the vRNA L segment (pC-NP/hpPol-I L), or the L protein and the vRNA S segment (pC-L/hpPol-I S) for the generation of recombinant arenavirus using the two-plasmid approach are indicated. (b, c) Comparative activity of pC and pC/hpPol-I-based expression plasmids in a MG assay. Human 293T cells were co-transfected with the LCMV dual-reporter MG plasmid and the pC expression plasmids encoding the viral NP and L (four-plasmid viral rescue) or the pC/hpPol-I (two-plasmid viral rescue) expression plasmids for LCMV, together with pSV40-Cluc vector to normalize transfection efficiencies. As a negative control, cells were co-transfected only with pC-L or pC-L/hpPol-I S (-NP), using empty pC to keep constant the total amount of transfected DNA. At 48 h p.t., MG expression was analysed by GFP using fluorescence microscopy (b) and TCSs were collected and analysed for Gluc and Cluc expression (c). Representative images of three independent experiments and fold induction over the negative controls are represented. Scale bar, 100 μm.

![Image of Fig. 5](image_url)

**Fig. 6.** Rescue of rLCMV and rCandid#1 in Vero cell using a two-plasmid-based approach. (a) Virus rescue. Vero cells were co-transfected with plasmids illustrated in Fig. 5(a). Six days after transfection, TCSs were assessed for rLCMV and rCandid#1 viral rescues by infecting fresh monolayers of Vero cells and detection of viral antigen by immunofluorescence. Mock-infected cells were included as controls. Representative images of at least three independent virus rescues are shown. Scale bar, 100 μm. Comparison between pC+pPol-I (four-plasmid) and pC/hpPol-I (two-plasmid) based systems for the generation of rLCMV (b) and rCandid#1 (c). Viral titres in TCSs from transfected 293T and Vero cells were determined by immunofocus centre assay (FFU ml⁻¹) in Vero cells.

![Image of Fig. 6](image_url)
microscope. For r3LCMV and r3Candid1, infected cells expressing GFP were directly detected by fluorescence microscopy. Mean value and standard deviation were calculated using Microsoft Excel software. Mock-infected cells were included as controls. Images were coloured using Adobe Photoshop CS4 (v11.0) software. Representative images of at least three independent transfections are shown.

**Gaussia luciferase (Gluc) assays.** Gluc expression from r3LCMV and r3Candid1 was determined by infecting Vero cells with the r3 viruses and at 48 h post-infection (p.i.), 100 μl of TCS was collected and analysed with the Biolux Gaussia Luciferase Assay kit. Reporter gene (Gluc) expression is indicated as fold induction over the mock-infected controls. Mean value and standard deviation from three independent infections were calculated using Microsoft Excel software.

**Protein gel electrophoresis and WB analysis.** Total cell protein content was measured with a MicroBCA kit (Promega) and 200 μg of protein per sample was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) overnight at 4 °C. After blocking for 1 h at room temperature with 10% dry milk in 1× PBS, membranes were incubated with monoclonal primary antibodies against LCMV NP (1:1,3), Candid1-NP (BEI Resources, KA03-AA01), a monoclonal antibody against GFP (Clontech, 632381), a polyclonal antibody against Gluc (New England Biolabs, E8023S), or a polyclonal antibody against GAPDH (AbCAM, AB9485) for 1 h at room temperature. Membranes were washed three times with 1× PBS containing 0.1% Tween-20, and probed with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (lg) antibodies (GE Healthcare) for 1 h at room temperature. After three washes with 1× PBS containing 0.1% Tween-20, proteins were detected using a chemiluminescence kit and autoradiography films from Denville Scientific.

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