T7 RNA polymerase-dependent and -independent systems for cDNA-based rescue of Rift Valley fever virus

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INTRODUCTION

Rift Valley fever virus (RVFV) is a serious pathogen affecting humans and livestock in sub-Saharan Africa, Egypt, Yemen and Saudi Arabia. Recurrent epidemics have killed thousands of animals, hundreds of humans, and caused significant economic losses (Balkhy & Memish, 2003). The severity of RVFV zoonosis as well as the capability to cause major epidemics have prompted authorities to list RVFV as a notifiable disease and a potential biological weapon (Borio et al., 2002).

RVFV belongs to the genus Phlebovirus, family Bunyaviridae (Elliott, 1997). Bunyaviruses are enveloped and have a tri-segmented single-stranded RNA genome of negative or ambisense polarity, replicate in the cytoplasm, and bud into the Golgi apparatus. RVFV encodes five structural proteins: the viral polymerase on the large (L) segment, two glycoproteins (Gn and Gc) and the 78 kDa protein on the medium (M) segment, and the viral nucleocapsid protein (N) on the smallest (S) segment (Struthers et al., 1984). In addition, there are two nonstructural proteins, encoded on the M segment (termed NSm) and the S segment (termed NSs). These accessory proteins are dispensable for viral multiplication in cell culture (Gerrard et al., 2007; Vialat et al., 2000; Won et al., 2006), but play important roles for pathogenesis in vivo. In particular, the NSm and 78 kDa proteins were found to enhance intrahost viral spread (Bird et al., 2007), whereas NSs is important to suppress the antiviral type I interferon (IFN) system (Billecocq et al., 2004; Bouloy et al., 2001; Le May et al., 2004, 2008).

The general features of RVFV transcription and replication are similar to those of other negative-stranded RNA viruses (Elliott, 1996). The viral genomic RNA (vRNA) segments contain untranslated regions (UTRs) on both the 5’ and the 3’ ends that serve as promoters for replication of the segment and transcription of the encoded reading frames. The vRNAs are encapsidated by the N protein and associate with the L protein both intracellularly and in the virion, and only these ribonucleoprotein particles (RNPs) are
functional templates for mRNA synthesis and RNA replication by the viral polymerase.

Recently, rescue systems to generate infectious RVFV particles from cloned cDNA plasmids have been developed for the attenuated mutant MP12 (Ikegami et al., 2006) and the virulent strain ZH501 (Gerrard et al., 2007). Similar to most other reverse genetics systems of cytoplasmic negative-strand RNA viruses (Blakqori & Weber, 2005; Bridgen & Elliott, 1996; Conzelmann, 2004; Lowen et al., 2004; Sanchez & de la Torre, 2006), RVFV rescues were based on the expression of positive-sense copies of the three viral RNA segments by the bacteriophage T7 RNA polymerase (T7 pol). A potential disadvantage is the strict dependency on cell lines which constitutively express high levels of T7 pol, e.g. BSR-T7/5 (Buchholz et al., 1999). Moreover, the 5′-triphosphorylated transcription products of T7 pol were recently shown to induce high levels of IFN due to activation of the intracellular pathogen receptor RIG-I (Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006; Plumet et al., 2007). It is thus conceivable that T7 systems may not be optimal, especially for the generation of mutant viruses lacking IFN-antagonistic functions. In line with this, experiments with a T7 minireplicon system of RVFV using 293T cells had shown that the IFN antagonist NSs strongly promoted viral gene expression (Ikegami et al., 2005). Moreover, in an Ebola virus minireplicon system (also based on 293T cells) the replacement of T7 plasmids with constructs driven by the RIG-I-neutral cellular RNA pol I and pol II promoters significantly enhanced the performance and the signal-to-noise ratio (Groseth et al., 2005). Here, we describe the establishment and comparison of T7 pol-dependent and T7 pol-independent rescue systems for the RVFV strain ZH548. We found that – different from what the published minireplicon findings and the RIG-I mechanism would imply – the T7 system (based on BSR-T7/5 cells) and the pol I/II system (based on 293T cells) are of similar efficiency, even for virus mutants lacking the IFN antagonist NSs. Further experiments revealed that in BHK-21 and in BSR-T7/5 cells the RIG-I pathway is severely compromised, thus providing a possible explanation why T7 pol transcripts are exceptionally well tolerated.

METHODS

**Chemicals, cells and viruses.** Geneticin G418 (Biochrom AG) was dissolved in H₂O to 100 mg ml⁻¹ and used at a concentration of 1 mg ml⁻¹ cell culture medium. Vero E6, A549, BHK-21, Huh-7.5 and 293T cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. BSR-T7/5 cells (Buchholz et al., 1999) were additionally provided with G418 on every second passage. RVFV strains were propagated in Vero cells under BSL-3 conditions.

**Plasmids.** All plasmids were generated using standard molecular cloning techniques and confirmed by DNA sequencing. PCR was performed as described previously for La Crosse virus (Blakqori et al., 2003). Viral genes and fragments were amplified from cDNA by PCR using specific primers (Supplementary Table S1). The constructs pL18-RVFV-L, pL18-RVFV-M, pL18-RVFV-N and pL18-RVFV-S contained the appropriate coding sequences subcloned into the eukaryotic high-level expression plasmid pL18 (kindly provided by Jim Robertson, National Institute for Biological Standards and Control, Hertfordshire, UK). For construction of pL18-RVFV-L, the L segment coding region was assembled in a stepwise manner from four overlapping cDNA fragments. These fragments were generated by PCR using primer pairs RVFV_SapI_L1_for/RVFV_L1_rev, RVFV_L2_for/RVFV_L2_rev, RVFV_L3_for/RVFV_L3_rev, and RVFV_L4_for/RVFV_L4_rev_SapI. The PCR fragments were individually TA-cloned into the pcDNA3.1-TOPO Vector (Invitrogen), giving rise to plasmids pcDNA3.1_RVFV_L1, pcDNA3.1_RVFV_L2, pcDNA3.1_RVFV_L3 and pcDNA3.1_RVFV_L4. Then, the insert of pcDNA3.1_RVFV_L2 was cut out with EcoRI and XhoI and cloned 3′ of the L1 insert into the EcoRI/XhoI-digested pcDNA3.1_RVFV_L1. The resulting plasmid was named pcDNA3.1_RVFV_L1+L2. In parallel, fragment L4 was subcloned into pl.18 using the BamHI and Xhol restriction sites. The resulting plasmid, pL18-L4, was then digested with SacI and EcoRI. The gel-purified fragment flanked by SacI/EcoRI restriction sites at the 5′ and 3′ end, respectively, was then subcloned together with the BamHI/SacI-digested L3 fragment (cut out of pcDNA3.1_RVFV_L3) into the BamHI/EcoRI-digested pL18 vector. The resulting construct, pL18_L3+L4, was then reopened with BamHI and Apal and joined with the L1+L2 fragment cut out of pcDNA3.1_RVFV_L1+L2, again using BamHI/Apal. The amino acid sequence of the full-length insert in pL18-RVFV-L corresponds to the GenBank accession number DQ357403. For construction of pL18-RVFV-M, two overlapping fragments were generated using primer pairs RVFV_SapI_m1_for/RVFV_m1_rev and RVFV_m2_for/RVFV_m2_rev_SapI. The two fragments were TA-cloned to generate plasmids pcDNA3.1_RVFV_M1 and pcDNA3.1_RVFV_M2, respectively. To obtain pL18-RVFV-M, both fragments were finally cloned into the pL18 vector in a three-way ligation reaction, where an internal DraI site served for joining M1 and M2, and KpnI and XhoI restriction sites were used for ligation into pL18. The cloning sequence of the insert corresponds to the GenBank accession number DQ380206. Two additional plasmids, pL18-RVFV-N and pL18-RVFV-S, were constructed based on the cloning sequence of the S segment (GenBank access no. DQ380151). First, overlapping fragments of the S segment were amplified from first-strand cDNA using primer pairs RVFV_SapI_m1_for/RVFV_C13_Srev_SapI and RVFV_S2_for/ RVFV_ZH_Srev_SapI. TOPO-cloning of these two fragments gave rise to plasmids pcDNA3.1_RVFV_S1 and pcDNA3.1_RVFV_S2, respectively. Subcloning of fragment S1 into the pL18 vector using KpnI/XhoI sites resulted in pl.18-RVFV-N. For construction of pL18-RVFV-S fragment S1 was digested with KpnI and PvuII, and cloned 5′ of PvuII/XhoI-digested fragment S2 into the pL18 vector. The three antigenomic T7 pol-driven rescue constructs p7triibo-RVFV-cL, p7triibo-RVFV-cM and p7triibo-RVFV-cS were obtained by cloning the full-length antigenomic sequences of all three segments into the p7triiboSM2 backbone plasmid (Fig. 1). This plasmid is an advanced version of p7triiboGB (Blakqori & Weber, 2005), lacking SapI and EspII restriction sites of the DNA backbone. First, we generated precursor plasmids p7triibo-RVFV-cLPro, p7triibo-RVFV-cMPro and p7triibo-RVFV-cSPro, containing the viral antigenic UTR of each segment separated by a linker sequence containing two flanking SapI restriction sites and one central KpnI site. The UTRs including the linker sequences were rebuilt from overlapping cloning. TA cloning was done using the pcDNA3.1/V5-His TOPO TA Expression kit (Invitrogen) according to the manufacturer’s instructions. The preparation of RVFV ZH548 first-strand cDNA was performed as described previously for La Crosse virus (Blakqori et al., 2003). Viral genes and fragments were amplified from cDNA by PCR using specific primers (Supplementary Table S1).
oligonucleotides as described previously (Blakqori & Weber, 2005). We used oligonucleotides RVFV_cLPro1for, RVFV_cLPro2rev, RVFV_cLPro3for, RVFV_cLPro4rev for the L segment, RVFV_cMPro1for, RVFV_cMPro2rev, RVFV_cMPro3for, RVFV_cMPro4rev, RVFV_cMPro5for and RVFV_cMPro6rev for the M segment, and RVFV_cSPro1for, RVFV_cSPro2rev, RVFV_cSPro3for, RVFV_cSPro4rev, RVFV_cSPro5for and RVFV_cSPro6rev for the S segment. The resulting fragments were TA-cloned for sequence analysis, and further cloned into pT7riboSM2 via the flanking Esp3I restriction sites. In a second step, the open reading frames of each segment were cut out from pl.18-RVFV-L, pl.18-RVFV-M, and pl.18-RVFV-S via SacI and cloned into the linker region of the respective precursor plasmid.

For construction of the three genomic Pol-I-driven rescue constructs (Supplementary Fig. S1, available in JGV Online), the UTR-linker sequences of all three segments were amplified from pT7ribo-RVFV-cLPro, pT7ribo-RVFV-cMPro and pT7ribo-RVFV-cSPro using primer pairs RVFV_vLProfor/RVFV_vLProrev, RVFV_vMProfor/RVFV_vMProrev and RVFV_vSProfor/RVFV_vSProrev, respectively. Each of the resulting three PCR fragments was then cloned into the backbone plasmid pH21 (Neumann et al., 1999) via primer-encoded Esp3I restriction sites, thereby giving rise to three precursor plasmids. The corresponding open reading frames of the pl.18 plasmids were finally inserted via SacI restriction sites as described above, resulting in pH21-RVFV-vL, pH21-RVFV-vM, and pH21-RVFV-vS.

Generation of pH21-RVFV-vN_TCS and pT7ribo-RVFV-cN_TCS was achieved by replacing the NSs sequence with a unique tandem cloning site (TCS). In addition to deletion of the NSs gene, the TCS allows for precise replacement of the NSs gene by foreign genes via two AarI restriction sites generating Ncol/Xhol-compatible ends (outlined in Fig. 3a). The TCS was built up from oligonucleotides at the NSs-proximal end of the intragenic region by PCR amplification with primers p1.8for, RVFV_N1TCS1rev, RVFV_N1TCS2for and RVFV_N1TCS3rev, using pl.18-RVFV-S as a template. The resulting fragment was subsequently cloned into the pl.18 vector, from which it was cut out and ligated into pH21-RVFV-vSPro or pT7ribo-RVFV-vSPro via SacI restriction sites. For generation of pH21-RVFV-vN_GFP and pT7ribo-RVFV-cN_GFP, the green fluorescent protein (GFP) open reading frame was inserted into the TCS of pH21-RVFV-vN_TCS or pT7ribo-RVFV-cN_TCS.

The firefly luciferase plasmid used to analyse activation of the ISG54 promoter (pJSI54-Luc) was kindly provided by David Levy, Department of Pathology, NYU School of Medicine, USA (Bluyssen & Levy, 1997). The control plasmid pRL-SV40 (Promega) contains the Renilla luciferase gene under control of the constitutive simian virus 40 promoter. The expression plasmid for the constitutively active RIG-I mutant (RIG-I CA) fused to the C terminus of enhanced GFP (EGFP; pEGFP-RIG-I CARD) was constructed by cloning the RIG-I CARD domain (aa 1–284) into pEGFP-C1 (Clontech) via HindIII/KpnI restriction sites. The control construct pl.18-FAAMx encodes the first 100 aa of the N terminus of MxA, fused to an N-terminal Flag tag.

**Rescue of recombinant RVFV.** To recover recombinant RVFV using the T7 pol-dependent rescue system, semiconfluent layers of BSR-T7/5 cells were grown in six-well plates. Each well was transfected with 1 µg each of pT7ribo-RVFV-cl, pT7ribo-RVFV-cM, pT7ribo-RVFV-cS (for rZH548) or pT7ribo-RVFV-cN_GFP (for pT7ribo-RVFV-cN_GFP), using 9 µl FuGENE6 transfection reagent (Roche) in 100 µl OPTIPAN (PAN-Biotech). For generation of recombinant RVFV with the pol I/II rescue system, 293T and BHK-21 cells were seeded in a 1:1 ratio in six-well plates. If not indicated otherwise, semiconfluent layers of co-cultured cells were transfected with 1 µg each of pH21-RVFV-vL, pH21-RVFV-vM, pH21-RVFV-vS (for rZH548), and pH21-RVFV-vN using 12.8 µl Nanofectin transfection reagent (PAA Laboratories). For generation of recombinant RVFV with the pol I/II rescue system, 293T and BHK-21 cells were transfected with 1 µg each of pH21-RVFV-vL, pH21-RVFV-vM, pH21-RVFV-vS (for rZH548), and 0.5 µg of pl.18-RVFV-L and pl.18-RVFV-N using 12.8 µl Nanofectin transfection reagent (PAA Laboratories). For generation of rZH548 NSs: : GFP, pH21-RVFV-vS was replaced by pH21-RVFV-vN_TCS or pT7ribo-RVFV-cN_GFP, respectively. Supernatants of transfected cells were harvested on day 3 and 5 post-transfection and the cells were provided with fresh DMEM.
order to screen for the presence of recombinant virus, Vero E6 cells grown in six-well plates were inoculated for 1 h with 500 µl supernatant at 37 °C and incubated further with 1 ml fresh DMEM. The presence of recombinant virus was then confirmed either by the appearance of a cytopathic effect, GFP expression or immunofluorescence analysis.

**Plaque assay.** Vero E6 cells grown to 90% confluency in six-well plates were inoculated with tenfold serial dilutions of supernatants from infected cells in DMEM with 2% FCS and 20 mM HEPES (pH 7.3). After 1 h incubation at 37 °C, the inoculum was removed and cells were overlaid with 3 ml DMEM containing 2% FCS, 0.02% DEAE-dextran and 0.4% Agar Noble (Difco) and further incubated for 72 h at 37 °C. Cells were fixed and stained with 1% crystal violet, 3.6% formaldehyde, 1% methanol, 20% ethanol, and titres were calculated from the plaque numbers according to the dilution.

**Diagnostic RT-PCR.** Vero E6 cells were infected with an m.o.i. of 10 for 16 h and total RNA was extracted. For reverse transcription, 1 µg DNase I-treated RNA was incubated with 200 U RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) in the presence of 100 ng random hexanucleotides, 1 mM each of dNTPs, and RNase inhibitor (Fermentas) in 20 µl of 1 x reaction buffer (Fermentas). The resulting cDNA was denatured for 5 min at 95 °C and amplified by 30 cycles of PCR, with each cycle consisting of 1 min at 95 °C, 1 min at 55 °C and 1 min at 68 °C, followed by 10 min at 68 °C. The upstream and downstream primers for amplifying M segment RNA were 5’-GGAGAGGAGCATTCCAGGTC-3’ and 5’-TCTGTCGCTTCTCTATAGACACAAAGACCGTGCACCTCA-3’.

**Transient transfection and reporter gene assays.** Activation of the hamster ISG54 promoter was monitored using the firefly luciferase reporter plasmid pISG54-Luc (Bluyssen & Levy, 1997). Briefly, subconfluent cell monolayers grown in 12-well dishes were transfected with 250 ng reporter plasmid together with 50 ng control plasmid pRL-SV40 (Promega) in 100 µl OptiPAN (PAN Biotech) containing 0.9 µl FuGENE HD (Roche). After 6 h at 37 °C, cells were either mock treated, transfected with 500 ng of RVFV particle RNA containing 5’ triphosphates (Habjan et al., 2008), transfected with 5 µg of the dsRNA analogue poly(I:C) (Blakqori et al., 2007), or infected with RVFV clone 13 at an m.o.i. of 1. Alternatively, cells were cotransfected with reporter constructs and 500 ng of pL18-FAMx or pEGFP-RIG-I CARD. Eighteen hours later, cells were harvested and lysed in 100 µl of Passive Lysis Buffer (Promega). An aliquot of 10 µl lysate was used for dual luciferase assays according to the manufacturer’s instructions (Promega).

**Test for antiviral IFN.** BHK-21 cells seeded in 12-well dishes were incubated with 500 µl conditioned medium harvested from BHK-21 or BSR-T7/5 cells which had been transfected with either pL18-FAMx (CTRL) or pEGFP-RIG-I CARD (RIG-I CA) as described above. After overnight incubation, cells were infected for 1 h with vesicular stomatitis virus at an m.o.i. of 0.01. Then, virus inoculum was removed and cells were incubated with another 500 µl conditioned medium. Supernatants were collected after overnight incubation, and virus titres were measured by plaque assays on Vero cells. Non-conditioned fresh medium and medium containing 10 µl pan-specific recombinant IFN-α A/D (BylII) (PBL Biomedical Laboratories) were used as controls.

### RESULTS AND DISCUSSION

#### T7 pol-driven rescue system for RVFV

The previously reported RVFV rescue systems were based on either the attenuated mutant MP12 (Ikegami et al., 2006) or the virulent strain ZH501 (Gerrard et al., 2007). We used the virulent strain ZH548 (Meegan et al., 1979) to construct cDNA plasmids for T7 pol-driven transcription of the viral genome segments L, M and S. The plasmid (named pT7riboSM2) in which the cDNA fragments were inserted was derived from our original rescue vector pT7riboGB (Blakqori & Weber, 2005) and had the backbone Esp3I and SapI restriction sites removed to facilitate cloning by means of these enzymes. cDNA copies of full-length ZH548 segments L, M and S were inserted in positive-sense (i.e. antigenomic polarity) into pT7riboSM2. The multistep cloning strategy is outlined for the M segment in Fig. 1. First, a short DNA cassette containing segment-specific UTRs flanking a linker of two SapI sites was inserted into pT7riboSM2, giving rise to a so-called provector. Then, the cDNAs for the coding sequences were assembled in a different vector, provided with SapI sites on the 5’ and 3’ ends, and inserted via SapI into the provector. The resulting plasmids, named pT7ribo-RVFV-cL, pT7ribo-RVFV-cM and pT7ribo-RVFV-cS were transfected into BSR-T7/5 cells seeded in six-well plates. After 5 days incubation, a cytopathic effect (CPE) was observed and supernatants were harvested and transferred to Vero E6 cells to monitor recovery of recombinant virus, again indicated by a strong CPE. Table 1 shows that transfection of the three antigenomic plasmids was sufficient to rescue recombinant ZH548 (rZH548) in more than 50% of the cases. To verify that rZH548 was derived from the transfected cDNAs, we performed a silence mutation into the M segment, using RT-PCR. Fig. 2(a) shows that rZH548 indeed contained the mutated site was amplified by RT-PCR. Moreover, as plaque formation (Fig. 2b) and growth kinetics on IFN-deficient Vero cells and IFN-competent A549 cells (Fig. 2c) were indistinguishable between ZH548 (Fig. 2c) and rZH548 (Fig. 2d), it can be concluded that the rescued virus is a faithful copy of the parental strain. Thus, transfection of the three antigenomic constructs results in efficient rescue of recombinant ZH548.

### Table 1. Rescue of rZH548 using the T7 system

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<th>Plasmids*</th>
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<tr>
<td>pT7ribo-RVFV-cL</td>
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<td>pT7ribo-RVFV-cM</td>
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<td>pT7ribo-RVFV-cS</td>
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*BSR-T7/5 cells were transfected with indicated amounts of plasmids per six-well dish.
†Number of wells with virus/number of transfected wells.
In agreement with results achieved for Bunyamwera virus, La Crosse virus and RVFV strains MP12 and ZH501 (Bird et al., 2007; Blakqori & Weber, 2005; Ikegami et al., 2006; Lowen et al., 2004), no additional support plasmids expressing N and L were needed for ZH548 rescue. This suggests that our antigenomic cDNA constructs can be translated to provide L and N proteins for initial packaging and replication of the recombinant positive-sense RNA segment copies.

pol I/II-driven rescue system for RVFV

We further developed the RVFV rescue in order to become independent of stably T7-expressing cells and to avoid potential interference of T7 transcripts with the antiviral RIG-I signalling. Rescue systems based on a combination of RNA pol I (for expression of the viral genome) and RNA pol II-driven plasmid vectors (for expression of helper proteins) are standard for influenza viruses, which replicate in the nucleus (Neumann et al., 1999). For cytoplasmic negative-strand RNA viruses, however, pol I/II rescue systems were so far only established for lymphocytic choriomeningitis virus, a member of the family Arenaviridae (Flatz et al., 2006), and the Akabane bunyavirus (Ogawa et al., 2007). To adopt this for RVFV, three rescue plasmids were constructed which express the viral segments in negative-sense (i.e. genomic polarity) under control of a human pol I promoter. These plasmids were generated by a similar two-step strategy as described above, i.e. three segment-specific provectors containing the UTRs received the corresponding full-length coding regions via two engineered SapI sites (Supplementary Fig. S1). In addition, expression constructs driven by the cellular pol II were generated to provide the viral N and L proteins for packaging and replication of the viral genomic RNAs. Human 293T cells were transfected with optimized amounts of the pol I-driven genomic plasmids and the pol II-driven support plasmids. Co-culture with BHK-21 cells (which are unable to express the viral segments from the human pol I promoter) served to amplify the recombinant virus produced by the 293T cells, as has been demonstrated for influenza A virus (Hoffmann et al., 2000).
shows that rZH548 can be generated efficiently with the pol I/II system. The identity of the recombinant virus was again verified by restriction marker analysis of the BamHI tag (data not shown). Apparently, neither the potential splicing of viral transcripts in the nucleus nor the non-viral location of template RNA synthesis poses major problems for RVFV rescue performance.

Rescue of a RVFV mutant lacking the NSs gene

To demonstrate the feasibility of the pol I/II system for rescue of IFN-sensitive virus mutants, we replaced the NSs gene, which is located in positive-sense on the ambisense S segment, with a tandem cloning site (Fig. 3a). The resulting virus, termed rZH548ΔNSs, was rescued with high efficiency and displayed the typical turbid-plaque phenotype of the NSs-less RVFV (Ikegami et al., 2006) (Fig. 3b). Moreover, growth curve analysis on IFN-deficient Vero cells and IFN-competent A549 cells (Fig. 3c and d) demonstrated that NSs of ZH548 is needed to counteract the antiviral IFN system. These results confirm previous findings obtained with the natural RVFV NSs deletion mutant clone 13 (Billecocq et al., 2004; Bouloy et al., 2001) and a recombinant MP12 deletion mutant (Ikegami et al., 2006), and show that the pol I/II system is suitable for the generation of attenuated virus mutants.

Rescue performance of the T7 and the pol I/II systems

The triphosphate group at the 5’ end of T7 transcripts is a strong activator of the pathogen recognition receptor RIG-
I which, in turn, triggers the synthesis of the antivirally active type I IFNs (Hornung et al., 2006; Kim et al., 2004; Pichlmair et al., 2006; Plumet et al., 2007). T7-mediated rescue of wild-type (wt) RVFV (see Table 1) may have been facilitated by the S segment-encoded NSs protein, which is known to suppress IFN induction (Billecocq et al., 2004) and was in fact shown to boost transcription and replication in a T7 minireplicon system of RVFV (Ikegami et al., 2005). Innate immune activation by plasmid-driven transcription should not occur in the pol I/II system, because transcript 5' ends are processed to remove the 5' triphosphate group (pol I) or masked by addition of a 7-methyl guanosine cap (pol II) (Fromont-Racine et al., 2003; Shatkin & Manley, 2000). We were interested in assessing the efficiency of the T7 and the pol I/II systems in rescuing viruses which are deficient in suppressing the IFN system. To facilitate this analysis, we inserted the GFP gene into the tandem cloning site of the respective S segment constructs. This NSs-deficient virus, termed rZH548ΔNSs::GFP, served as a model of an attenuated virus with an easily detectable marker gene. BSR-T7/5 cells and 293T/BHK-21 co-cultures were seeded in parallel dishes and transfected with the appropriate plasmid mixtures to allow virus rescue. After 3 and 5 days of incubation, transfected cells were monitored for the expression of GFP by fluorescence microscopy and supernatants were taken to inoculate fresh Vero E6 cells. In Fig. 4a (upper panel) it is shown that, after 3 days, green fluorescent cells appeared in both the T7 pol and the pol I/II rescue cells, indicating expression of the S segment-encoded GFP. The GFP gene is present in negative-sense on the antigenomic T7 transcripts, and in positive-sense on the genomic pol I transcripts. In none of the cases was a GFP signal obtained when L protein expression was omitted (data not shown), indicating negligible background activity. Thus, the strong GFP signal observed was produced by transcriptionally active, recombinant RVFV RNPs. Nonetheless, neither system produced detectable amounts of free virus on day 3, as Vero E6 cells inoculated with supernatants were not infected (Fig. 4a, lower panel). By contrast, supernatants taken on day 5 post-transfection contained sufficient amounts of virus to infect Vero cells (Fig. 4b). Overall, production of NSs-deficient virus particles using the T7 system or the pol I/II system was of similar efficiency, as was revealed by several repetitions of the experiment (Table 3).

**Impaired RIG-I pathway in BHK-21 and BSR-T7/5 cells**

Contrary to our expectations, the IFN-sensitive mutant rZH548ΔNSs::GFP was rescued from T7 constructs in BSR-T7/5 cells as efficiently as from pol I/II constructs in 293T cells. BHK-21 (baby hamster kidney) cells, the parental cell line of BSR-T7/5 (Buchholz et al., 1999; Conzelmann, 2004), are widely used for propagation of viruses (Karabatsos & Buckley, 1967). Their IFN induction capability is not entirely clear, as some studies have demonstrated IFN production (Ferran & Lucas-Lenard, 1997; Stanwick & Hallum, 1974), whereas other studies suggested non-responsiveness to virus infection (Andzhaparidze et al., 1981; MacDonald et al., 2007; Otsuki et al., 1979). Interestingly, for the BSR-T7/5 line a deficiency in the response to IFN-inducing mutants of rabies virus and bovine respiratory syncytial virus was reported (Brzozka et al., 2006; Conzelmann, 2004). As the
genome RNA of most negative-stranded RNA viruses contains the RIG-I-activating triphosphate group at the 5' end (Habjan et al., 2008; Hornung et al., 2006; Pichlmair et al., 2006), these latter findings indicate that BSR-T7/5 cells and possibly also BHK-21 cells may have a defect in the RIG-I signalling pathway. We tested this assumption by comparing the activation of the IFN system via the RIG-I pathway in 293T, BHK-21 and BSR-T7/5 cells. As a sensitive marker for IFN induction, we used a reporter construct containing the hamster ISG54 promoter (Bluyssen & Levy, 1997). As IFN inducers we initially employed either vRNA isolated from RVFV particles [a strong activator of RIG-I (Habjan et al., 2008)], the established IFN inducer poly(I:C) or the NSs-deficient RVFV mutant clone 13 (Cl 13). Fig. 5(a) shows that in 293T cells the ISG54 promoter was strongly upregulated

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<th>Table 3. Efficiency of rZH548ΔNSs::GFP rescue</th>
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*BSR-T7/5 cells were transfected with 1 μg of each antigenomic T7-driven rescue construct per six-well dish.
†Co-cultures of 293T/BHK-21 were transfected with 1 μg of each pol I-driven rescue construct and 0.5 μg of the pol II-driven helper plasmids per six-well dish.

Fig. 5. RIG-I-dependent IFN induction. (a) Human 293T cells, BHK-21 cells and BSR-T7/5 cells seeded in 12-well plates were transfected with a firefly luciferase construct under control of the hamster ISG54 promoter and a Renilla luciferase construct under control of the constitutively active SV40 promoter. At 6 h post-transfection, cells were either mock treated (M) or transfected with 5' triphosphorylated RNA (vRNA) isolated from particles of RVFV. Luciferase activities were measured after overnight incubation and normalized to the mock-induced samples. Means (given above the respective columns) and SD from three independent experiments are shown. (b) Similar experimental set-up using transfection with the dsRNA analogue poly(I:C), or (c) infection with the IFN inducer clone 13 (Cl 13). (d) Test for antiviral activity. BHK-21 or BSR-T7/5 cells were transfected either with the control construct pL18-FAMx (CTRL) or with pEGFP-RIG-I CARD, an expression construct for the constitutively active RIG-I mutant (RIG-I CA). EGFP autofluorescence of RIG-I CA showed a transfection efficiency of approximately 60% in both cell lines (data not shown). After overnight incubation, supernatants were harvested and transferred onto a monolayer of fresh BHK-21 cells. Antiviral activity contained within the supernatants was determined using the highly IFN-sensitive vesicular stomatitis virus. Means and SD from three independent experiments are shown. As further controls, BHK-21 indicator cells were mock treated (M) or incubated with 10 U IFN-α ml⁻¹.
after transfection of vRNA. BHK-21 cells, by contrast, responded only poorly to vRNA transfection and BSR-T7/5 cells did not respond at all. Similar results were obtained after transfection of poly(I:C) (Fig. 5b) or infection with Cl 13 (Fig. 5c). To corroborate the reporter assays, we directly measured the production of antiviral IFN by BHK-21 or BSR-T7/5 cells in response to T7-driven reverse transcription. Conditioned medium from cells expressing a constitutively active form of RIG-I (RIG-I CA) or a control protein was collected, transferred onto BHK-21 cells, and tested for the ability to establish an antiviral state against a challenge virus. Fig. 5(d) shows that transfection of RIG-I CA into BHK-21 cells mediated some antiviral activity, whereas in BSR-T7/5 cells RIG-I CA expression had no significant effect. Given that as little as 10 U IFN resulted in a more than tenfold stronger inhibition of the challenge virus (see Fig. 5d), we concluded that the antiviral activity mediated by RIG-I CA transfection of BHK-21 cells is minor by comparison. Together, these findings imply that BHK-21 cells are impaired and BSR-T7/5 cells are completely deficient in RIG-I-mediated IFN induction. The weak residual RIG-I response of BHK-21 cells may explain why there are conflicting reports about their IFN-inducing capability. It is conceivable that BSR-T7/5 cells have deleted all residual RIG-I signalling activity of BHK-21 cells during the course of selection for high-level expression of the T7 pol (Buchholz et al., 1999). Interestingly, a RIG-I deficiency has also been described for the human cell line Huh-7.5 which is highly permissive for hepatitis C virus replication (Blight et al., 2002; Sumpter et al., 2005; Zhong et al., 2005). In contrast to Huh-7.5 cells (Binder et al., 2007), however, the non-responsiveness of BSR-T7/5 cells could not be rescued by overexpression of the RIG-I CA mutant (see Fig. 5d and Supplementary Fig. S2, available in JGV Online). This indicates that the signalling defect of BSR-T7/5 cells is situated downstream of RIG-I.

Our findings suggest caution in using cells other than BHK-21, BSR-T7/5 or Huh-7.5 for T7-driven reverse genetics systems and interpreting their data. In line with this, it has been shown that in 293T cells the relocation of T7 pol from the cytoplasm (where RIG-I is present) into the nucleus enhances the efficiency of T7-driven influenza virus rescue (de Wit et al., 2007). In the case of BHK-21 and BSR-T7/5 cells, we cannot claim that RIG-I deficiency is the sole responsible factor promoting growth and rescue of a wide variety of viruses. The striking parallel of BSR-T7/5 cells and Huh-7.5 cells, however, underlines the importance of the RIG pathway for virus inhibition and the versatility of RIG-I-deficient cells for rescue and propagation of viruses.

In summary, we have established two different systems to recover the RVFV strain ZH548 from cloned cDNA plasmids. Rescue systems for cytoplasmic negative-strand RNA viruses are mostly based on T7 pol (Conzelmann, 2004). To our knowledge, the present study is the first side-by-side comparison of optimized T7-dependent and T7-independent systems and indicates that both are equally suitable for rescuing wt strains and attenuated mutants of this important pathogen. Moreover, our studies indicate that the well-established suitability of BHK-21-derived cells for virus rescue and propagation is based on a deficiency in RIG-I signalling.

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