Rescue of Akabane virus (family \textit{Bunyaviridae}) entirely from cloned cDNAs by using RNA polymerase I

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Reverse-genetic systems are often used to study different aspects of the viral life cycle. To date, three rescue systems have been developed for the family \textit{Bunyaviridae}. These systems use T7 RNA polymerase, which is generally used in rescue systems for \textit{Mononegavirales}. In the present study, we describe a rescue system for Akabane virus (family \textit{Bunyaviridae}) that uses cDNAs and RNA polymerase I instead of T7 RNA polymerase. The utility of this system was demonstrated by the generation of a mutant with a deletion of the non-structural protein (NSs) on the S RNA segment. These results offer a new option for bunyavirus rescue.

INTRODUCTION

Reverse-genetic systems are often used to study different aspects of the viral life cycle. In the family \textit{Bunyaviridae}, three rescue systems have been developed to date, for Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bunyamwera virus (BUNV), La Crosse virus and Rift Valley fever virus (Bridgen \& Elliott, 1996; Blakqori \& Bun...
studies have so far been limited. It is essential for research into these areas to establish a reverse-genetic system. In the present report, we describe a rescue system for AKAV that uses RNA polymerase I instead of T7 RNA polymerase.

**METHODS**

**Viruses and cells.** The plaque-cloned OBE-1 strain of AKAV (Akashi et al., 1997a) was used throughout this study. Hamster lung cells (HmLu-1) and African green monkey kidney cells (Vero) were cultivated in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 5% fetal bovine serum (FBS) at 37 °C. Baby hamster kidney cells (BHK-21) were maintained in Eagle's minimum essential medium containing 5% FBS. Medium without serum was used for viral propagation.

**Plasmid construction.** The pcDNA/L and pcDNA/S plasmids express the L and N of AKAV, respectively, under the control of the CMV promoter (Ogawa et al., 2007b). The pcDNA/M plasmid, which expresses the viral glycoproteins, was generated using a method similar to that employed for pcDNA/L. Briefly, the full-length M RNA segment was amplified by PCR using KOD Plus (Toyobo) and the phosphorylated primers RF42-T-MF (5'-aattcgacctggagagtgtgcaaactcagcggagagaga-3') and RF42-P-MR (5'-aattcgacctggagagtgtgcaattatataataataatg-3') (the restriction sites are underlined, the AKAV sequences are capitalized and italicized). The PCR product was cloned into the mammalian expression vector pcDNA3.1/myc-His version A (Invitrogen).

The pRF42/L, pRF42/M, and pRF42/S plasmids exhibit RNA polymerase I-driven transcription of the respective genomic RNAs. To generate pRF42/L and pRF42/M, pcDNA/L and pcDNA/M were digested with EspI and cloned between the murine polymerase I promoter and the terminator of the pRF42 vector (Flick & Petterson, 2001). The S RNA segment was generated using the primers RF42-T_SF (5'-aattcgacctggagagtgtggaaacctcagcggagagaga-3') and RF42-P_SMR (5'-aattcgacctggagagtgtgcccaataaatcacaatcaga-3') (the restriction sites, the ATG start codon (nt 59–61 of the S cRNA) was changed to ACG and the primer-encoded alterations in overlapping regions were generated using the primer pairs RF42_T_SF plus SdelNSs-R (5'-aatcgtctctgggggaggtagtgtaacccctaaaatatcaatatcaga-3') and RF42_P_MMR (5'-aattcgacctggagagtgtgtaacccctaaaatatcaatatcaga-3') and the full-length N-coding region, which was then cloned into the EspI and BpiI sites of pRF42, thereby generating pRF42/SANs.

**Rescue of recombinant AKAV.** A subconfluent monolayer of HmLu-1 cells was transfected with 0.5 µg pRF42/S or pRF42/SANs, 1.5 µg pRF42/M, 2 µg pRF42/L, 0.1 µg pcDNA/SANs and 0.4 µg pcDNA/L, with or without 0.15 µg pcDNA/M, using TransIT-LT1 (2 µg µl−1 DNA; Mirus) in 100 µl serum-free medium (OPTI-MEM; Invitrogen). If no cytopathic effect (CPE) was observed, supernatants were harvested. The p.f.u. were determined in HmLu-1 cells. The experiments were performed in triplicate.

**RT-PCR analysis.** Viral RNA was extracted from the supernatant of infected cell cultures using the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNA was synthesized using the primer AKAccon (5'-ggggtctggtaagtagtgg-3'). The S RNA segment was amplified by PCR using Ex Taq (Takara Bio) with the primers SF (5'-agtagtggaagttctccactatt-aactgac-3') and NSs_C_XhoI (5'-ggggtctggagtaagtagtgc-3'). The amplicons were digested with HpyCH4IV.

The whole genomes of the rescued viruses were confirmed by direct sequencing of the PCR products. The three segments of the recombinant viruses were amplified by RT-PCR using the primer set: RF42_T_SF plus RF42_P_SR for the S RNA segment, RF42_T_MF plus RF42_P_MR for the M RNA segment, and RF42_T_LF (5'-aattcgacctggggagagtgtgtaacccctaaaatatcaatatcaga-3') plus RF42_P_3R (5'-aattcgacctggagagtgtgtaacccctaaaatatcacaatcaga-3') for the L RNA segment. The sequencing reaction was resolved on an ABI Prism 3100 automated sequencer (Applied Biosystems).

**Plaque morphology and size.** To examine differences in plaque morphology between the wild-type and recombinant viruses, HmLu-1 or Vero cells were seeded into six-well plates. After virus adsorption to HmLu-1 or Vero cells at 37 °C for 1 h, the inocula were removed and the infected cells were washed with PBS and overlaid with DMEM that contained 0.8% agar and 2.5% FBS. Three days later, the cells were stained with neutral red. Fifty plaques were randomly selected and sized using the NIH Image software. Statistical significance was assessed by the Student’s t-test.

**Experimental infections.** Three groups of 12–14 one-day-old BALB/c mice were inoculated intracerebrally with 0.02 ml wild-type or recombinant viruses (TCID50, 101.25 ± 0.1 ml−1) and observed for mortality over a period of 21 days. Mice that died within 1 day of inoculation were not included in the results of these experiments. The surviving mice were sacrificed 21 days after inoculation and tested for the presence of antibodies against AKAV, to confirm that the animals were infected with viruses. The animal experiments were conducted according to the guidelines of the Animal Care Committee of the University of Tokyo.

**RESULTS**

**Rescue of AKAV from cDNAs using RNA polymerase I**

Although RNA polymerase I localizes to the cell nucleus, transcripts generated by this enzyme are exported from the nucleus to the cytoplasm, which is the site of bunyavirus replication (Flick & Petterson, 2001). Therefore, we postulated that a rescue system for bunyaviruses could be devised using RNA polymerase I. Essentially, we followed the strategies described previously by Flick & Petterson (2001) for constructing a bunyavirus minigenome system, and that described previously by Neumann et al. (1999) for a rescue system for influenza virus. To rescue AKAV from cDNAs, plasmids that encode AKAV proteins (pcDNA/L, pcDNA/SANs and pcDNA/M) and negative-sense viral RNAs (pRF42/L, pRF42/M and pRF42/S) were constructed (see Methods). Since the NSs of AKAV inhibits minirePLIC.
activity, we used pcDNA/S\textsuperscript{NSs}, which expresses only N (instead of pcDNA/S, which expresses both N and NSs; Ogawa et al., 2007b). To recover infectious viruses, HmLu-1 cells were transfected with pRF42/L, pRF42/M, pRF42/S, pcDNA/L and pcDNA/S\textsuperscript{NSs}, with or without pcDNA/M. At 4–5 days post-transfection, CPE was observed regardless of the existence of the M protein-expressing plasmid. This resulted in the recovery of infectious AKAV (rAKAV) entirely from the cDNAs.

To characterize the rescue of AKAV, we measured the development of virus titres (Fig. 1a–c). Although the titre of rAKAV was slightly lower than that of the wild-type virus at 12 h post-infection in HmLu-1 cells, the growth kinetics of these viruses were similar (Fig. 1a). Similar growth curves for these viruses were observed in BHK-21 (Fig. 1b) and Vero cells (Fig. 1c), although the virus titres were generally lower in Vero cells. Furthermore, the nucleotide sequence of the rAKAV genome was determined by direct sequencing of PCR products. No mutations were found in the rescued virus.

Rescue of AKAV with a deleted NSs gene

To test whether our rescue system could be used for the introduction of functionally important mutations into the AKAV genome, we generated a mutant virus with an inactivated NSs gene. Since the S RNA segment encodes N and NSs on overlapping reading frames, complete deletion of the NSs gene without affecting the N gene is impossible. Therefore, we applied a strategy that has been used for the abrogation of Bunyavirus NSs gene expression (Bridgen et al., 2001) and changed the ATG start codon of NSs to ACG (Fig. 2a). The resulting genomic AKAV S construct, pRF42/NSs, expresses an unaltered N but no NSs.

For virus rescue, HmLu-1 cells were transfected with the plasmids pcDNA/L, pcDNA/NSs, pRF42/L, pRF42/M and the mutant construct pRF42/NSs. The supernatants were collected 5 days post-transfection and aliquots were transferred onto fresh HmLu-1 cells. CPE was observed 4 days post-infection. Analysis of the growth kinetics of the mutated recombinant virus (rAKAV\textsuperscript{NSs}) in HmLu-1, BHK-21 and Vero cells revealed slightly poorer multiplication compared with the wild-type and rAKAV viruses (Fig. 1a–c).

To establish that rAKAV\textsuperscript{NSs} is derived from cDNA plasmids, we distinguished the wild-type and rAKAV\textsuperscript{NSs} viruses by Hpy\textsuperscript{CH4IV} restriction analysis of their S RNA segments, as mutagenesis of the NSs start site created a unique recognition site for this restriction enzyme (Fig. 2a). RT-PCR amplification of the recovered viral RNA and subsequent digestion of the PCR products with Hpy\textsuperscript{CH4IV} showed that the recovered recombinant virus carried the introduced Hpy\textsuperscript{CH4IV} site in the S RNA segment (Fig. 2b). No PCR products were obtained from control reactions in which the reverse-transcription step was omitted, indicating that no contaminating plasmid DNA was present in the RNA preparations. This resulted in the recovery of rAKAV\textsuperscript{NSs} entirely from cDNAs. Additionally, this mutation was confirmed by direct sequencing of PCR product of rAKAV\textsuperscript{NSs}. No other mutations were found in the whole genome of rAKAV\textsuperscript{NSs}.

Plaque morphology and size

To characterize the recombinants, plaque assays were performed. Although there was a great variation in the plaque size of the wild-type virus on HmLu-1 cells, the plaque size for rAKAV was strikingly smaller than that for the wild-type virus (Fig. 1d). Similarly, the plaques for rAKAV\textsuperscript{NSs} appeared to be significantly smaller than those for rAKAV (P<0.01). Furthermore, the average sizes of the plaques for the three viruses after five successive passages were approximately similar to those before passage (data not shown). In contrast, rAKAV\textsuperscript{NSs} formed slightly smaller plaques than rAKAV on Vero cells but the size difference was not significant (data not shown).

Pathogenesis

Wild-type or recombinant viruses were inoculated intracerebrally into BALB/cAJcl suckling mice, and the inoculated animals were observed over a period of 21 days. All of the mice inoculated with wild-type or rAKAV viruses died, but there was a clear difference in disease progression (Fig. 3). The wild-type virus killed all the mice by 10 days post-inoculation, whereas it took 14 days post-inoculation for the rAKAV to kill all the mice. In contrast, the rAKAV\textsuperscript{NSs}-infected mice showed a survival rate >78% during the 21 day observation period. These results suggest that rAKAV\textsuperscript{NSs} is attenuated. All of the surviving mice carried antibodies against each strain, with neutralizing antibody titres greater than 1:2, which suggests that the animals were infected with the recombinant virus (data not shown).

DISCUSSION

Current reverse-genetic systems for negative-strand RNA viruses rely on the production of viral transcripts from transfected plasmids by either bacteriophage T7 RNA polymerase or cellular RNA polymerase I (reviewed by Kawaoaka, 2004). For T7 RNA polymerase-driven systems, the viral transcripts are produced in the cytoplasm, whereas RNA polymerase I is a cellular protein that synthesizes unmodified RNA species with defined terminal sequences in the nuclei of transfected cells. With regard to members of the family Bunyaviridae, both RNA polymerases have been utilized to develop minigenome systems for representatives of each of the four genera of the family Bunyaviridae that infect animals (Dunn et al., 1995; Lopez et al., 1995; Flick & Pettersson, 2001; Flick et al., 2003a, b), although to date, only T7 RNA polymerase-driven systems have been established for the rescue of infectious viruses from cDNA (Bridgen & Elliott, 1996; Bridgen et al., 2001; Lowen et al., 2004; Blakqori & Weber,
However, for influenza virus and arenavirus, rescue systems that use both polymerases have been established (Kawaoka, 2004; Flatz et al., 2006; Sanchez & de la Torre, 2006).

As the 3’ ends of the transcripts formed by T7 RNA polymerase are correctly trimmed by the hepatitis delta virus ribozyme, the T7 RNA polymerase-driven system appears to be necessary for both antigenomic and supporting plasmids. However, in the case of the family Bunyaviridae, small amounts of the antigenomic RNAs that act as mRNA can be translated into viral proteins without the supporting plasmids (Lowen et al., 2004; Blakqori & Weber, 2005; Ikegami et al., 2006). Thus, in these systems,

Fig. 1. Growth kinetics and plaque size of recombinant viruses. Monolayers of HmLu-1 (a), BHK-21 (b) and Vero (c) cells were infected with each AKAV strain at an m.o.i. of 0.01. Supernatants were recovered at the time points shown in each part, and the p.f.u. were determined. The results are shown as mean ± SD (error bars) of three independent experiments. (d) Plaque size of the recombinant viruses. The results are shown as mean ± SEM of the relative sizes (%) of 50 randomly selected plaques for each virus. The average plaque size of wt virus was arbitrarily set at 100. Asterisks indicate a significant difference (P<0.01) compared with the mean plaque size of the examined viruses.
it is not clear whether the M polyprotein-expressing plasmid is necessary for bunyavirus rescue. Our results suggest that the rescue of bunyavirus, as well as influenza and arenavirus, is independent of the envelope-glycoprotein-expressing plasmid. The genomic plasmids and minimum supporting plasmids (N and L) that form the ribonucleoprotein complex are necessary for this rescue system.

Although the growth kinetics of the wild-type and rAKAV viruses were very similar in the three different cell lines used in this study (Fig. 1a–c), their plaque sizes and the mortality patterns of the virus-infected mice were slightly different (Figs 1d and 3). Mutation frequencies of RNA viruses are considered to be 1 mutation in 10^3–10^5 nt per replication, while DNA viruses have rates of less than 1 in 10^8 (Leider et al., 1988; Steinhauer et al., 1992). This difference is attributed to a lack of proofreading ability in RNA replication enzymes. Although plaque-cloning of the wild-type virus was performed, it seems likely that the wild-type virus still contains a quasispecies virus population. The plaque size of the wild-type virus varied widely after five successive plaque pickings (data not shown), and that of rAKAV was within the range of the wild-type virus plaque size. Furthermore, when the whole genomes of recombinant viruses were compared to that of the original virus, no mutations were found in rAKAV. We have also reported previously that the genomes of the same AKAV strains with different passage histories were slightly different (Ogawa et al., 2007c). Thus, despite the slight differences in biological characteristics between the wild-type and rAKAV viruses, we conclude that rAKAV has a wild-type phenotype.

It has been reported that the NSs protein of BUNV acts as a virulence factor by suppressing the production of IFN-α/β (Weber et al., 2002). In the genus Orthobunyavirus, NSs deletion mutants have been generated by a reverse-genetic system (Bridgen et al., 2001; Blakqori & Weber, 2005). The NSs deletion mutant of BUNV (BUNdelNSs) formed smaller plaques in BHK cells than the wild-type virus. When inoculated intracerebrally, BUNdelNSs killed BALB/c mice more slowly and exhibited reduced cell-to-cell spread, and the titres of virus in the brain were lower compared with infection with the wild-type virus (Bridgen et al., 2001). The attenuation of BUNdelNSs in infected mice suggests that NSs acts as a virulence factor. Similarly, rAKAVΔNSs formed smaller plaques than the wild-type virus (Fig. 1b). Furthermore, rAKAVΔNSs-infected mice had a survival rate >78% (Fig. 3). Our results suggest that rAKAVΔNSs is also attenuated, and that the NSs of AKAV plays the role of a virulence factor.

The present study describes for the first time, to our knowledge, the establishment of an RNA polymerase I-driven system for the rescue of infectious viruses from the cDNAs of the family Bunyaviridae. It is possible now to choose between the T7 RNA polymerase-driven and RNA polymerase I-driven systems. Since T7 RNA polymerase is a foreign protein, this enzyme must be introduced by using
plasmids, stable cell lines or helper viruses, such as the recombinant vaccinia virus, that express T7 RNA polymerase. In contrast, RNA polymerase I is a cellular protein and all eukaryotes possess this enzyme, although it is species-specific. Both T7 RNA polymerase- and RNA polymerase I-driven systems of arenaviruses efficiently produced infectious viruses (Flatz et al., 2006; Sanchez & de la Torre, 2006). In contrast, the efficiency of our RNA polymerase I-driven system was still lower than that of the T7 RNA polymerase-driven system for other bunyaviruses. This might be due to the number of essential plasmids for the arenavirus systems and bunyavirus T7 RNA polymerase-driven system. Although further development and optimization of this system are still needed, it can facilitate studies of the biology and pathogenesis of bunyaviruses. This technology provides an important basis for investigating viral virulence determinants and molecular virology, and for producing a live-attenuated vaccine candidate.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid from the Japan Livestock Technology Association and the Ministry of Education, Culture, Sports and Technology of Japan. We thank Ramon Flick of the University of Texas Medical Branch and Ralf F. Pettersson of the Karolinska Institute, Stockholm, Sweden, for providing pRF42, and Hitotoshi Ikeda of the National Institute of Animal Health, Tsukuba, Japan, for providing BALB/cAJcl mice.

REFERENCES


