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Efficient Reverse Genetics Generation of Infectious Junin Viruses Differing in Glycoprotein Processing▽†

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The New World arenaviruses, Junin, Machupo, Guanarito, Sabia, and Chapare, are associated with rapidly progressing severe hemorrhagic fever with a high rate of case fatality in various regions of South America. The threat of natural or deliberate outbreaks associated with these viruses makes the development of preventive or therapeutic measures important. Here we describe a Junin virus functional minigenome system and a reverse genetics system for production of infectious Junin virus. This robust, highly efficient system involves transfection of cells with only two plasmids which transcribe the virus S and L antigenomic RNAs. The utility of the system is demonstrated by generating Junin viruses which encode a glycoprotein precursor (GPC) containing the following: (i) the wild-type (SKI-1/S1P peptidase) cleavage site, (ii) no cleavage site, or (iii) a cleavage site where the SKI-1/S1P motif (RSLK) is replaced by a furin cleavage site (RRKR). In contrast to the wild-type virus, Junin virus lacking a GPC cleavage site replicated within successfully transfected cells but failed to yield infectious virus particles. This confirms observations with other arenaviruses suggesting that GPC cleavage is essential for arenavirus infectivity. In contrast, infectious Junin virus which encoded GPC cleaved by furin-like proteases was easily generated. The two-plasmid, high efficiency aspects of this Junin virus reverse genetics system show great promise for addressing important questions regarding arenavirus hemorrhagic fever disease and for development of precisely attenuated live arenavirus vaccines.

The family *Arenaviridae* contains several viruses associated with severe human disease (9). Of particular note among the Old World group of arenaviruses is Lassa virus, which is associated with hemorrhagic fever (HF), and lymphocytic choriomeningitis virus (LCMV), which is associated with severe neurologic disease in immunocompromised individuals and fetal abnormalities. The pathogenic New World group of arenaviruses includes Junin, Machupo, Guanarito, Sabia, and Chapare, each associated with severe HFs in various parts of South America. Junin virus (JUNV) has been known to be associated with the Argentine HF outbreaks since the 1950s (31). Arenaviruses exist in rodent reservoirs, and the main natural host of JUNV is *Calomys musculinus*, although the virus has also been found in other diverse rodent species (15, 25, 28, 29, 35). Virus transmission to humans is usually linked to contact with the blood, urine, and feces of infected rodents (34, 41). The associated aerosol infectivity, rapid onset of severe disease, and limited vaccine and antiviral options have resulted in these HF viruses being placed on the select agent list of potential bioterrorism threat agents. No FDA-approved vaccine exists for any of the HF-associated arenaviruses, although a live attenuated Junin vaccine (Candid 1) has been in use in Argentina for some years. In addition, while ribavirin has been shown to be of some value for treatment when used early in the course of arenavirus infections, it does have associated toxicity. The need for better tools to combat these diseases and more knowledge of arenavirus-associated pathogenesis remains critical.

The arenaviruses are enveloped viruses with negative-strand bisegmented genomes which encode four viral proteins in an ambisense orientation. The L segment (~7,200 nucleotides [nt]) encodes the matrix protein (Z) and the viral polymerase (L), while the S segment (~3,400 nt) encodes the glycoprotein precursor (GPC) and the nucleoprotein (N). As is commonly the case with many other negative-stranded RNA viruses, arenavirus GPC proteins are cleaved at specific amino acid signatures by a proprotein convertase (PC) to generate the mature G1 and G2 surface glycoproteins (9). Unlike most negative-stranded RNA viruses which utilize basic amino acid-specific PCs commonly referred to as furin-like proteases (PC5, PACE4, furin, and PC7) for this cleavage event (38), arenavirus G1 and G2 proteins are cleaved by SKI-1/S1P (6, 22, 33). SKI-1/S1P is localized mainly in the Golgi cisternae, which is where the virus GPC is most likely cleaved (22). The only other negative-stranded RNA virus known to share this unusual glycoprotein cleavage feature is Crimean-Congo HF (CCHF) virus, genus *Nairovirus*, family *Bunyaviridae* (40). It is unclear why these evolutionarily distinct HF-associated viruses use SKI-1/S1P rather than the more common furin-like PCs, but one can hypothesize that these viral glycoproteins may have structural or functional constraints that prevent utilization of furin-like PCs for this important glycoprotein maturation step.

Over the last two decades, reverse genetics systems have been successfully developed for several negative-stranded RNA viruses. This powerful technology has been used to pre-

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cisely address numerous questions regarding viral pathogenesis and replication and to develop prototypic live attenuated vaccines (5, 8, 12, 16). Reverse genetics systems for arenaviruses have been developed utilizing virus minigenomes for the study of LCMV, Lassa virus, and Tacaribe virus transcription and replication mechanisms. With these systems, transcription of the virus minigenome version of the S RNA segment is produced either in the cytoplasm by the T7 RNA polymerase or by the polymerase I in the nucleus, and L and N expression initiates transcription and replication from the primary transcripts (18, 21, 23, 32).

More recently, reverse genetics have been developed to allow the rescue of infectious recombinant LCMV, an Old World arenavirus. In these systems, primary transcription of the LCMV genome was driven by either the polymerase I or T7 polymerase in the presence of support plasmids expressing L RNA polymerase and N (11, 13, 20, 37). Given the substantial genetic and biologic differences between the LCMV and the New World arenaviruses associated with HF disease, we initiated a project to attempt to design an efficient reverse genetics system for the rescue of infectious JUNV variants. Such a system would be highly valuable to address fundamental questions regarding the biology and pathogenesis of New World arenaviruses and allow development of prototypic live attenuated vaccines with precisely engineered disruptions of pathogenic properties.

We report here the successful development of a highly efficient two-plasmid reverse genetics system for the generation of JUNV variants. The robust nature of the system likely reflects the simplicity of the two-plasmid procedure. The utility of the system was validated by generation of JUNV variants with altered GPC cleavage properties. These experiments demonstrated that JUNV GPC cleavage is essential for the release of infectious virus particles but that furin-like PCs can effectively be substituted for SKI-1/S1P in the cleavage process and generation of infectious virus.

MATERIALS AND METHODS

Cell culture, virus propagation, and biosafety. BSR-T7/5 cells constitutively expressing T7 polymerase were a generous gift of K. Conzelmann (Max-von-Pettenkofer-Institut, Munich, Germany). BSR-T7/5 and Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin at the standard concentration suggested by the manufacturers. BSR-T7/5 cells were propagated in the presence of 1 mg/ml of the selective agent G418 (Geneticin; Invitrogen) once every other passage. SRD12B cells (SKI-1/S1P deficient) were propagated as described previously (4). Manipulations of wild-type (wt) JUNV or recombinant JUNV (rJUNV) were performed within a biosafety level 4 (BSL-4) containment laboratory.

Virus propagation was routinely done by infecting confluent monolayers of Vero E6 cells in 25-cm^2 flasks. Infected cells and/or viral supernatants were harvested at 3 to 5 days postinfection (p.i.), depending on the experiment. Virus supernatants were harvested for RNA extraction using TriPure (Roche) at a ratio of 5:1; tubes containing the resulting noninfectious lysates were surface decontaminated and then transferred into a BSL-2 laboratory. RNA was extracted using either RNAid (Qbiogene) or RNeasy (Qiagen) following the manufacturer's recommendations.

Determination of terminal sequences. The terminal sequences of JUNV strain XJ13 were determined by 3′ rapid amplification of cDNA ends (RACE) and RNA ligation as described previously (1). Briefly, viral RNAs were extracted from supernatants or from Vero E6-infected cells as described above. All primers were designed according the sequences available in GenBank for JUNV XJ13 (L, NC_005080; S, NC_005081). To detect JUNV genomes and antigenomes, we used primers corresponding to the following respective positions: S-fwd (nt 3207 to 3226) and S-rev (nt 250 to 270) for S RNA and L-fwd (nt 6636 to 6656) and L-rev (nt 352 to 368) for L RNA.

For 3′ RACE, RNAs were polyadenylated using the poly(A) polymerase tailing kit (Epicentre) by following standard protocols and then purified using the RNeasy kit (Qiagen). Ten microliters of in vitro polyadenylated RNA was used as a template in reverse transcription-PCR (RT-PCR) analyses by using the SuperScript III one-step RT-PCR system with Platinum *Taq* High Fidelity (Invitrogen), following the manufacturer's protocol. Parallel RT-PCR analyses were done using an oligo(dT)-containing primer in combination with primers specific for S and L RNAs.

For RNA ligation, viral RNAs were treated with tobacco acid pyrophosphatase (Epicenter), following the manufacturer's protocol, purified using the RNeasy kit (Qiagen), and then ligated with T4 RNA ligase (NEB) using standard conditions. Ligated RNAs were further purified and used as templates in RT-PCR analyses. The resulting RT-PCR products were separated by electrophoresis in agarose gels, further purified by using the QIAquick gel extraction kit (Qiagen), and sequenced using standard protocols (ABI). Additional technical details may be supplied upon request.

Plasmid construction. Cloning of the JUNV S and L followed the standard T7-based strategy we had successfully applied to Rift Valley fever RNA segments previously (1, 7). Cloning of full-length JUNV S RNA (3,411 nt) was a relatively straightforward process. Basically, the S RNA was amplified by RT-PCR, digested with BsmBI, and ligated into a modified version of the V0.0 transcription plasmid, a generous gift from L. A. Ball at the University of Alabama at Birmingham. Although we obtained several clones of full-length S, none of them presented a sequence identical to the viral RNA. For this reason, we randomly chose one of them, sequenced it to completion, and corrected several nucleotide changes in consecutive steps while keeping some minor mutations as genetic markers.

As expected, due to the total length of JUNV L RNA (7,114 nt), the cloning process was more complicated and took several additional steps. From the beginning, the amplification of full-length L could not be easily achieved, possibly due to the strong RNA secondary structure that somehow was an impediment to the cDNA synthesis and/or the amplification. Eventually, we managed to amplify a full-length L copy, but the yield was small, and the cloning process proved unsuccessful. After these initial attempts, we amplified JUNV L RNA in two halves, digested with appropriated restriction enzymes and ligated into the transcription plasmid. An additional problem emerged in the cloning process due to the unstable nature of the resulting clones containing JUNV L sequences. Indeed, most of the clones that were well tolerated in the competent bacteria contained severe mutations such as stop codons and frameshift mutations in the L open reading frame (ORF). Based on this finding, we took an approach similar to that used to obtain functional S clones; i.e., one of the L clones that was well tolerated in the bacteria was chosen and sequenced to completion, and then several nucleotide changes corrected in consecutive steps while keeping some minor mutations as genetic markers.

The final plasmid constructions, pJunS and pJunL, were designed to transcribe the full-length antigenomic copies of JUNV S and L RNAs and contained two nonviral nucleotides, 5′ G and 3′ C, required for the efficient function of the T7 promoter and the hepatitis delta virus ribozyme. Moreover, we replaced the GPC ORF in pJunS by the enhanced green fluorescent protein (GFP) ORF by conventional cloning techniques. The resulting clone, pJunS-ΔGPC:GFP, carries the N gene in positive sense and GFP in negative sense. Variants of the full-length L and S clones were constructed by classic PCR mutagenesis which would encode altered virus protein motif changes. The SDD polymerase motif in pJunL was changed to AAA (pJunL-ΔSDD); the GPC cleavage site (RSLK) in pJunS was changed to AAAA (pJunS-ΔSKI and RRKR [pJunS-Furin]). Additional technical details may be supplied upon request.

Transfection of plasmid DNA and viral rescue. The plasmids described above were transfected in monolayers of BSR-T7/5 growing in 6-well, 12-well, or 24-well plates, using LT1 transfection reagent (Mirus) in a ratio of 3:1 or 5:1 reagent to DNA. Transfection medium was replaced 6 to 14 h later with regular DMEM containing 5% FBS. To check replication of GFP minigenomes, the supernatant was removed and cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS), washed with PBS, treated with Triton 1%, mounted with 4′,6-diamidino-2-phenylindole (DAPI), and photographed with an inverted microscope. Alternatively, we checked expression of JUNV proteins in transfected cells by immunofluorescence assay (IFA) using a rabbit serum raised against JUNV or monoclonal antibodies (MAb) against N that were described previously (36). The following commercially available secondary antibodies were routinely used: goat anti-rabbit, goat anti-mouse, and chicken anti-rabbit conjugated with Alexa Fluor 488, 546, and 594 (Molecular Probes; Invitrogen). IFAs were done with standard protocols and photographed with an Axio-Imager

(Zeiss) direct microscope or a Leica DM6000B confocal microscope. All transfection reactions with the potential to generate infectious virus were carried out in the BSL-4 laboratory.

For viral rescue, 0.2 to 0.5 ml of the 3 ml of supernatant was removed from the well of the six-well plate of transfected BSR-T7/5 cells 3 to 5 days posttransfection. This was diluted to 1 ml of medium and used to infect a Vero E6 cell monolayer in a 25-cm^2 flask (first passage), followed by the addition of 5 ml of tissue culture fluid. Infected cells were analyzed 3 to 5 days later by IFA as described above, or successive passages were done by transferring 1 ml of the 5 ml of supernatant from the 25-cm^2 flask, diluting it into 5 ml of medium, and infecting fresh monolayers of Vero E6 cells in 75-cm^2 flasks.

Virus growth curve. Vero E6 cells growing in 25-cm^2 flasks were infected with the wt JUNV, rJUNV, or RRKR mutant viruses (rJUNV-Furin) at a multiplicity of infection of 0.01. After 1 h of adsorption, the monolayers were washed three times with PBS and supplied with DMEM containing 2% FBS. The first sample was taken at 1 h p.i. ($t = 0$), and subsequent samples were collected at 1, 3, 5, and 7 days p.i. and stored at −70°C. These samples were diluted in DMEM and titrated, and plaques were detected by immunochemistry and by staining with crystal violet using previously described protocols (39).

Western blot analysis. Infected cells were lysed in mammalian protein extraction reagent (Pierce) with Halt protease inhibitor cocktail (Pierce). Cell lysates were γ-irradiated with 2×10^6 rad before aliquots of 50 μl were denatured for 15 min at 65°C with denaturation buffer, followed by digestion with PNGase F (500 U) or recombinant EndoH (1,000 U) for 1 h at 37°C (New England Biolabs). Proteins were separated by electrophoresis on a NuPAGE 4 to 12% gradient Bis-Tris gel in morpholineethanesulfonic acid (MES) buffer (Invitrogen) under reducing conditions. Resolved proteins were transferred onto nitrocellulose membranes by using dry electrotransfer with an iBlot apparatus (Invitrogen) before being incubated with JUNV G2 MAb (QD04-AF03) (36), actin MAb (GenScript), and anti-mouse horseradish peroxidase-coupled secondary antibody (Pierce). Bands were revealed with SuperSignal West Dura chemiluminescence substrate (Pierce) and detected with the FluorChem HD2 imaging system (Alpha Innotech). Densitometry of the bands was analyzed with AlphaEaseFC software version 6.0.2 (Alpha Innotech).

RESULTS

Determination of terminal sequences of JUNV genome RNAs. The 3′ RNA termini of arenavirus genomic and antigenomic RNAs contain critical regulatory regions important for polymerase binding and encapsidation processes involved in virus transcription and replication. Before starting the cloning process, it was important to experimentally determine these terminal sequences for JUNV S and L RNA segments as we could find no evidence in GenBank entries or in the literature that this had been done previously. The JUNV entries in GenBank (L, NC_005080; S, NC_005081) include segment terminus sequences, but these are identified as primer binding sites, i.e., the sequences were not determined but merely represent the sequences of the primers used to amplify the PCR products. Using 3′ RACE and RNA ligation approaches, the JUNV strain XJ13 RNA terminus sequences were directly determined and compared to those primer bind sites reported in the JUNV entries in GenBank (L, NC_005080; S, NC_005081). Six nucleotide differences were found (Fig. 1A). The S RNA sequence differed in two positions (7AxC and 9TxG) that correspond to the 5′ end; while the L RNA sequences differed in four positions that correspond to both the 5′ end (7AxC and 9TxG) and the 3′ end (7107AxT and 7109TxG). As previously observed (13, 14), about 25 to 50% of the S RNA molecules had an additional 5′ G relative to the complementary 3′ end, while no additional 5′ G was found at the end of the L RNA molecules. Interestingly, the predicted panhandle structures for the terminal 19 nt reveals perfect complementarity for the L RNA segment termini but not for the S RNA segment (Fig. 1B).

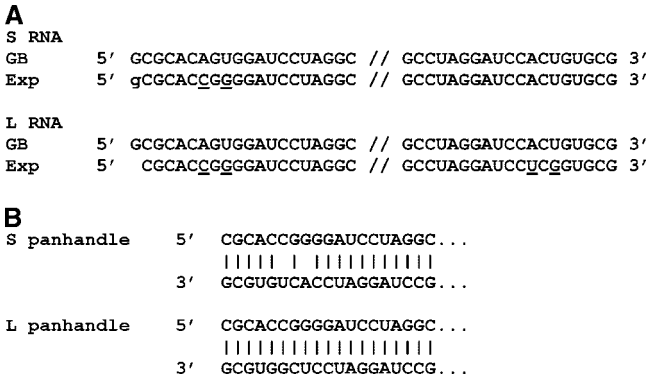


FIG. 1. JUNV RNA genome termini. (A) Virus RNA terminal sequences were experimentally determined by 3′ RACE and RNA ligation (Exp) and are shown in comparison with available sequences from GenBank (GB). Nucleotide differences are shown underlined, and g signifies an extra G found at the 5′ end of approximately 25 to 50% of the population of JUNV S RNAs analyzed. (B) Predicted panhandle structures for both S and L genomic RNAs.

Replication of JUNV minigenomes and rescue of recombinant virus. In order to generate recombinant viruses, we cloned full-length antigenomic copies of JUNV S and L RNAs in a T7-based system described previously (1, 7). The final constructions, pJunL and pJunS, have the viral insert flanked by the T7 promoter and the hepatitis delta virus ribozyme, followed by the T7 terminator. Transfection of these plasmids into T7-expressing BSR-T7/5 cells was expected to generate primary transcripts that correspond to JUNV complete S and L anti-genome RNAs that may be translated to produce sufficient N and L proteins to initiate viral RNA replication.

Following construction of the initial virus L clones, we made two additional constructions in order to test their functionality. First, a fluorescent reporter gene was inserted into the S template by replacing the GPC ORF by enhanced GFP (pJunS-ΔGPC:GFP). The orientation of the resulting plasmid is such that GFP would be produced only when RNA replication occurred (Fig. 2A). This construction allowed us to test the functionality of about 60 L clones and choose the active clones before sequencing them to completion. Four out of the 60 L clones demonstrated polymerase activity. The defects in the nonfunctional L clones frequently included single-base deletions which altered the reading frame or mutations creating stop codons within the ORF. The functional L plasmid clone selected for subsequent use encoded an L polymerase which differed from the authentic virus L polymerase by only three amino acid positions (see Table S1 in the supplemental material).

In order to generate an appropriate negative control plasmid for the reverse genetics experiments, one of the active L clones (i.e., one that supported GFP production in cells transfected with pJunS-ΔGPC:GFP) was modified to inactivate polymerase activity by replacing the core active motif, SDD, with AAA (Fig. 2A), generating plasmid pJUNL-ΔSDD. As shown in Fig. 2B, transfection of the pJunS-ΔGPC:GFP mini-replicon along with the active pJUNL clone resulted in GFP-expressing cells, while the inactive pJUNL-ΔSDD did not show any sign of activity (Fig. 2) and was used as a control in all subsequent experiments.

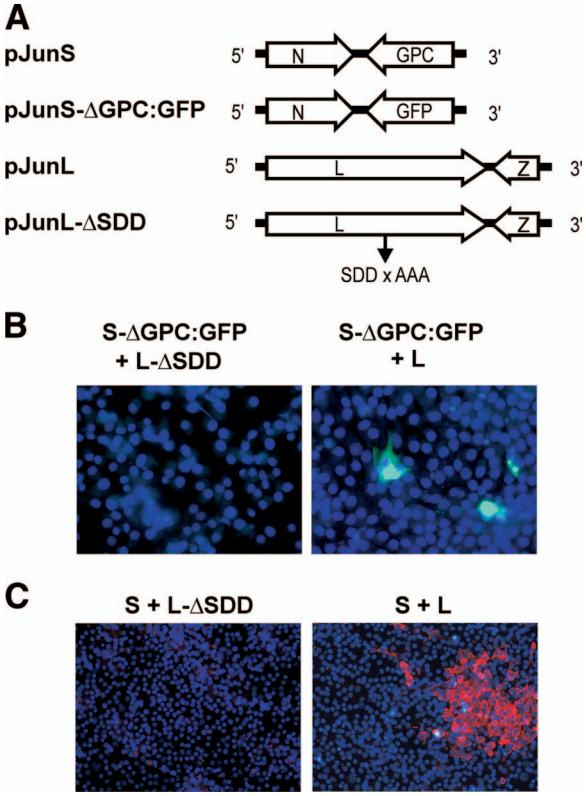


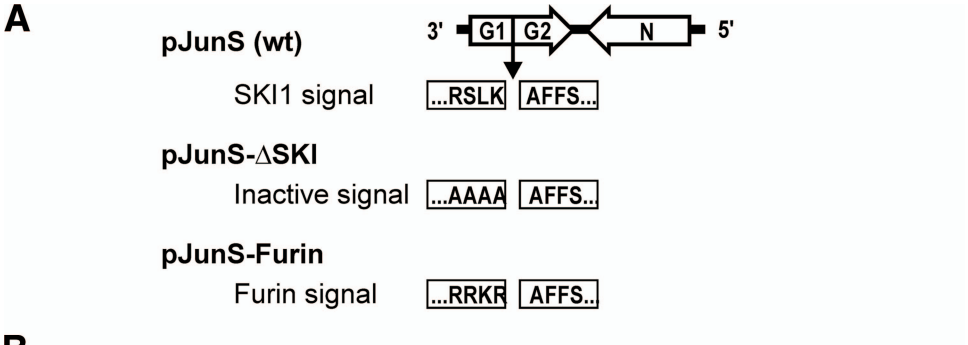
FIG. 2. (A) Schematic of plasmids indicating resulting primary transcripts. (B) Functional L protein required for minigenome replication. BSR-T7/5 cells growing in 24-well plates were transfected with pJunS-ΔGPC:GFP and pJunL (right) or pJunL-ΔSDD (left). Transfected cells were fixed 3 dpt, counterstained with DAPI and photographed with UV light in the GFP range (green cells). (C) Functional L protein required for virus replication and spread. BSR-T7/5 cells were transfected with pJunS and pJunL (right) or pJunL-ΔSDD (left). JUNV proteins were detected 5 dpt with anti-Junin rabbit serum and anti-rabbit Alexa Fluor 594 (red stain). Cells were counterstained with DAPI and photographed with UV light in a regular upright microscope.

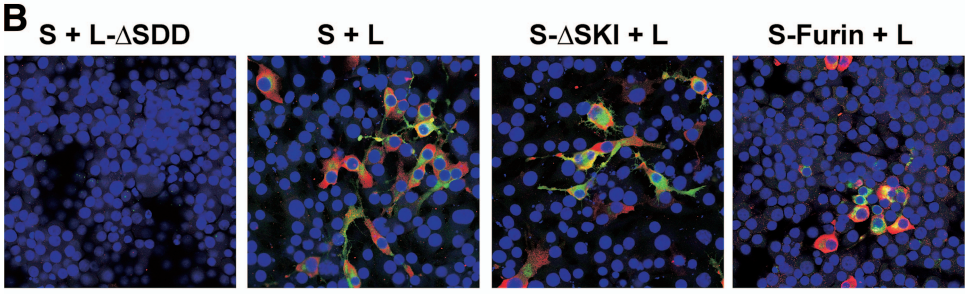
With these basic tools successfully developed, attempts were made to generate infectious JUNV entirely from plasmid DNA. For this purpose, BSR-T7/5 cells were transfected with the full-length plasmids, pJunS and pJunL or pJUNL-ΔSDD, as a negative control. Six days after transfection, cells were analyzed for evidence of virus production by staining them with JUNV reactive antibody (Fig. 2C). Approximately two to five large foci of positively stained cells were seen in each well of the six-well plate, indicating successful rJUNV replication and spread. These were not observed in the cell monolayers transfected with the negative control, inactive L polymerase (pJUNL-ΔSDD). Rescue of infectious virus was demonstrated by infection of fresh monolayers of Vero E6 cells with supernatant off the positively transfected cell monolayers. The majority of cells were demonstrated by IFA to be infected, recombinant virus master stocks were prepared, and the virus genome was sequenced to verify that the recombinant virus S and L segment sequences were identical to those of the template plasmids, including the marker mutations included in the plasmid construction process (GenBank entries FJ805379 to FJ805380). Following the initial successful rescue of infectious virus, the procedure has been repeated multiple times with 100% success, indicating the robust nature of the technique and the high level of efficiency of a system requiring the transfection of cells with only two plasmids.

Proof of system utility by generation of virus GPC cleavage mutants. The arenaviruses and CCHF virus (genus *Nairovirus*, family *Bunyaviridae*) share the unusual feature of having the major cleavage step which generates the virus mature surface glycoproteins mediated by the SKI-1/S1P protease rather than the furin-like proteases (PC5, PACE4, furin, and PC7) utilized by other negative-stranded RNA viruses (6, 22, 33, 38). Why these evolutionarily unrelated HF-associated viruses utilize SKI-1/S1P and not furin-like PCs is an enigma. Perhaps there is some structural or functional constraint on these glycoproteins which prevents furin utilization for this cleavage event. In addition, data from experiments utilizing SKI-1/S1P cell lines deficient in SKI-1/S1P have strongly suggested that GPC cleavage by SKI-1/S1P is strictly required for Lassa virus and CCHF virus infectivity (4, 22). However, some other role for SKI-1/S1P in the virus life cycle could not be ruled out in these studies. The newly developed JUNV reverse genetics system provided the opportunity to precisely examine these issues in the context of authentic JUNV.

A version of the pJunS plasmid was designed where the virus GPC SKI-1/S1P cleavage signal (RSLK) was abolished (pJUNS-ΔSKI) (Fig. 3). If this cleavage event was strictly required for generation of infectious virus particles, then it was anticipated that transfection of cells with this plasmid (in conjunction with pJunL) would result in virus replication but not in the release of infectious virus. This is exactly the result obtained. Cells supporting active virus replication were observed in the monolayers of cells transfected with pJUNS-ΔSKI plus pJunL but not in cells transfected with pJUNS-ΔSKI plus the negative control pJunL-ΔSDD (Fig. 3B). However, supernatant from the pJUNS-ΔSKI plus pJunL-transfected cell monolayers was used to initiate two blind passages on new Vero E6 monolayers with no evidence of virus growth (Fig. 3C). These results clearly demonstrated that in the context of authentic JUNV, SKI-1/S1P cleavage appears to be strictly required for the release of infectious virus particles.

To determine if there was a functional or structural constraint preventing arenavirus GPC cleavage by furin-like PCs, another version of pJunS was constructed where the SKI-1/S1P cleavage signal, RSLK, was replaced by a canonical furin-like cleavage site, RRKR (pJUNS-Furin). Transfection of cells with pJUNS-Furin plus pJunL did successfully yield infectious virus (Fig. 3C), indicating that furin-like PCs could effectively replace SKI-1/S1P in the GPC cleavage process despite the strict conservation of the SKI-1/S1P motif among the GPCs of all arenaviruses. However, the furin-cleaved mutant virus (rJUNV-Furin) appeared to grow less efficiently, as evidenced by immunofluorescence staining for JUNV following passages in Vero E6 cells. The number of positively staining cells was significantly less than that observed with the wt (rJUNV) rescued in parallel experiments (Fig. 3C). In addition, the rJUNV-Furin virus still grew to a lower titer by the second Vero E6 cell passage (Fig. 3C). The rJUNV and rJUNV-Furin genome sequences were verified and shown to be identical except for the engineered cleavage site mutation, confirming





C

	Foci formation		Titer (pfu/ml)
	1st pass	2nd pass	2nd pass
rJUNV (wt)	+5	+5	$1x10^6$
rJUNV-ΔSKI	0	0	NA
rJUNV-Furin	+2	+3	$1x10^4$

FIG. 3. (A) Schematic of plasmids encoding virus GPC cleavage variants. Plasmid primary transcript is shown 3′ to 5′, and the encoded GPC cleavage site is indicated for each plasmid. (B) Replication of wt and cleavage variant JUNV in transfected cells. BSR-T7/5 cells were transfected with pJunS, pJunS-ΔSKI or pJunS-Furin with pJunL or pJunL-ΔSDD. N protein was detected 3 dpt with anti-Junin rabbit serum and an anti-rabbit Alexa Fluor 594 (red stain). GPC was detected with a MAb anti-Junin GPC and anti-mouse Alexa Fluor 488 (green stain). Cells were counterstained with DAPI and photographed with UV light in a confocal microscope. (C) Virus GPC cleavage by SKI-1/S1P or furin-like PCs is required for rescue of infectious rJUNV. The efficiency of viral rescue was examined by focus formation in Vero E6 cells (1st and 2nd passages) and by plaque assay of virus stocks (2nd passage). +2 to + 5 indicates an average of 2 to 5 large (>50 cell) foci of infected cells/well on a six well plate.

that the growth defect was directly related to the cleavage site alteration (see Table S1 in the supplemental material).

rJUNV-Furin virus growth is attenuated in cell culture. Since we recovered rJUNV-Furin virus with a lower efficiency than rJUNV, we compared the growth in cell culture of the mutant virus with that of the authentic wt JUNV and the reverse genetics-derived wt virus (rJUNV). Vero E6 cells were infected with a multiplicity of infection of 0.01, supernatant samples were collected at 0, 1, 3, 5, and 7 days p.i., and virus titers were obtained by using a conventional plaque assay (Fig. 4A). The authentic JUNV and rJUNV viruses exhibited similar growth curves, where maximum virus titers of approximately 10^5 PFU/ml were obtained about 5 to 6 days p.i. In contrast, growth of rJUNV-Furin appeared to be slower, and the maximum titers reached were approximately ~50- to 100-fold less than those for rJUNV and JUNV.

In order to determine whether the defect in rJUNV-Furin resided in GPC processing, infected cell lysates were analyzed by Western blot analysis using a G2-specific MAb (Fig. 4B). Examination of the glycoproteins following removal of all N-glycans with the endoglycosidase F (EndoF) (Fig. 4B, lanes 3 and 6) showed that the total amounts of GPC and G2 proteins synthesized by both rJUNV and rJUNV-Furin were approximately equal. In addition, GPC cleavage was equally efficient (~65%) based on similar ratios of uncleaved GPC to G2 bands observed for the two viruses (Fig. 4B, lanes 3 and 6). However, a comparison of the various glycosylated forms of the glycoproteins synthesized by rJUNV and rJUNV-Furin revealed clear differences between these viruses with regard to the modifications of their N-glycans (Fig. 4B, lanes 1 to 2 and 4 to 5). For instance, examination of nontreated lysates (lanes 1 and 4) showed that rJUNV-Furin synthesized less of the ~38-kDa form of G2 than rJUNV. Treatment with endoglycosidase H (EndoH) to remove high levels of mannose sugars on the wt rJUNV lysate (Fig. 4B, lane 2) resulted in a mobility shift of the ~64-kDa form of GPC to an ~49-kDa form, and the ~38-kDa form of G2 shifted to an ~35-kDa form. Comparison of the EndoH-treated lysates of rJUNV-Furin relative to those of rJUNV (Fig. 4B, lane 5 versus lane 2) revealed an increase in the amount of the ~44-kDa form of G2 and a large reduction in the amount of the ~35-kDa form of G2 relative to that of the ~49-kDa form of GPC. Together, these data indicate

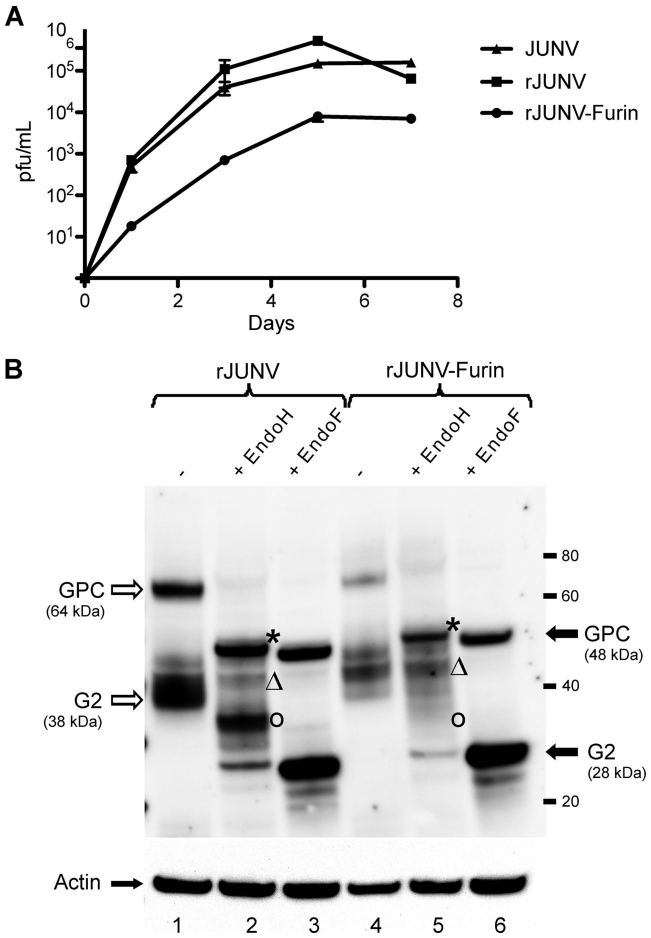


FIG. 4. rJUNV-Furin exhibits altered virus growth and glycoprotein maturation. (A) Vero E6 cells were infected with wt JUNV (triangle), rJUNV (square) and rJUNV-Furin viruses (circle). Duplicate samples were collected and titers determined at 0, 1, 3, 5, and 7 days p.i. (B) Protein lysates from Vero infected cells were examined by Western blot using anti-JUNV GPC MAbs. Glycosylated forms of GPC and G2 forms are indicated with open arrows and unglycosylated forms with black arrows. EndoH resistant forms of GPC (asterisk), and G2 (triangle and circle) are also indicated. Blot was reprobed with anti-actin MAb to control the amount of loaded proteins in the gel.

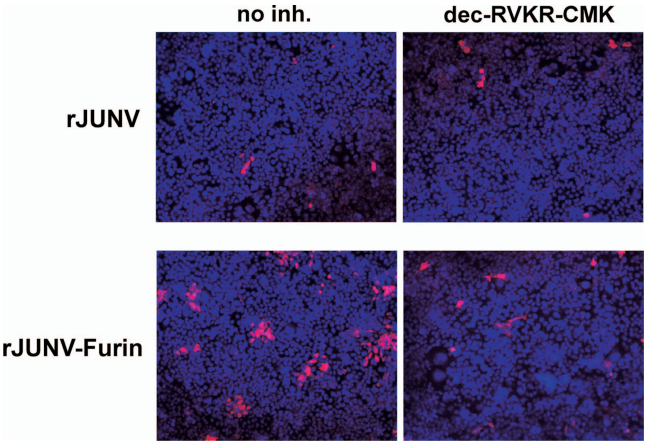


FIG. 5. rJUNV-Furin grows in SKI-1/S1P deficient cells. SRD12B cells deficient in SKI-1/S1P were infected with rJUNV or rJUNV-Furin in the absence or presence of 30 μM the furin inhibitor dec-RVKR-CMK. Thirty-six hours p.i. supernatants were replaced with fresh media with or without inhibitor. JUNV proteins were detected 3 dpi using an anti-Junin rabbit serum and an anti-rabbit Alexa Fluor 546 (red stain). Cells were counterstained with DAPI and photographed with UV light in a regular upright microscope.

that SKI-1/S1P cleavage can be substituted by a furin-like motif without affecting GPC production and cleavage but that this substitution leads to reduced growth of the rJUNV-Furin and differences in the N-glycan maturation of the virus glycoproteins.

SKI-1/S1P-independent growth of rJUNV-Furin virus. The absence of infectious virus released from cells successfully transfected with pJUNS-ΔSKI and pJunL plasmids is consistent with earlier data (using SKI-1/S1P-deficient cells), indicating a strict requirement for cleavage of Lassa virus or JUNV GPC for generation of infectious virus particles (6, 19, 22, 33). What remains unclear is whether SKI-1/S1P is required for other aspects of the arenavirus replication cycle in addition to GPC cleavage. To investigate this possibility, SKI-1/S1P-deficient cells (SRD12B cells) were infected with rJUNV or rJUNV-Furin viruses. If the arenavirus requirement for SKI-1/S1P is to cleave only GPC, then the rJUNV-Furin should grow in SKI-1/S1P-deficient cells due to cleavage of its GPC by furin-like PCs. The rJUNV-Furin virus infection clearly spread in cell monolayers in the absence of active SKI-1/S1P (Fig. 5, bottom left). Approximately seven or eight large infected cell foci (more than five cells) were observed per field by 3 days p.i. As expected, infected cell foci were not seen in any of the negative control monolayers. The wt rJUNV could infect SKI-1/S1P-deficient cells, as evidenced by virus-specific antibody staining of initial individual infected cells, but no spread of virus or infected cell foci was observed (Fig. 5, top left). In addition, spread of the rJUNV-Furin virus infection in SKI-1/S1P-deficient cells was shown to be prevented by a nontoxic dose of a commonly used furin-like protease inhibitor, dec-RVKR-CMK (Fig. 5, bottom right). These data indicate that the strict requirement of SKI-1/S1P for generation of infectious arenavirus particles is directly related to the need for virus GPC cleavage.

DISCUSSION

The development of JUNV reverse genetics systems for minigenome replication and generation of infectious virus represent an important breakthrough in efforts to understand and combat infections associated with the HF-associated New World arenaviruses. This success was achieved by first determining the correct sequence of the virus RNA genome termini which contain critical promoter and encapsidation signals, followed by development of a JUNV minigenome system to allow screening of functional JUNV L polymerase-encoding clones. These elements, coupled with the construction of an appropriate negative control L polymerase (lacking the SDD core motif), laid the necessary foundation for the infectious virus rescue attempts. The distillation of the procedure down to a simple two-plasmid system (pJUNS and pJUNL) resulted in generation of a highly efficient and robust virus rescue system.

With regard to the JUNV RNA terminal sequences derived

here, it is now clear that all the known arenavirus RNA termini share the feature whereby the termini of the L RNA segment have the potential to form a perfect panhandle structure, but the termini of the S RNA segment contain mismatches that would result in an imperfect structure (30). The strict conservation of this feature suggests it has a likely role in balancing the relative transcription, replication, and packaging efficiency of the S and L RNA segments. We now have the required tools on hand to initiate study of the impact of sequence and predicted structure variation of the RNA termini relative to these processes.

Lowen and colleagues had earlier pioneered a reverse genetic system for Bunyamwera virus (genus *Orthobunyavirus*, family *Bunyaviridae*) that generated infectious virus from a system, avoiding the requirement of additional protein-encoding support plasmids by relying solely on full-length plus-strand copies of the three RNA genome segments (S, M, and L) to generate sufficient virus proteins to initiate virus replication (24). We had successfully applied the same concept for the generation of a high-efficiency reverse genetics system for the rescue of Rift Valley fever viruses (genus *Phlebovirus*, family *Bunyaviridae*) (7). Based on these experiences, we decided to attempt to apply the same concept to the JUNV ambisense RNA genome. We based our reverse genetic rescue of infectious virus solely on synthesis of the full-length antigenomic S and L RNAs in T7-based transcription vectors. This approach also relies on sufficient translation of L and N proteins from the T7-generated antigenomic transcripts to initiate virus genome replication. This novel experimental approach for arenavirus reverse genetics presents an important advantage over previously reported LCMV systems (11, 13, 20, 37), as only two plasmids are required to rescue an rJUNV, without the need for additional expression vectors to express the nucleoprotein, viral polymerase, glycoprotein, or T7 RNA polymerase. The system has proven to be remarkably robust, as infectious rJUNV and rJUNV-Furin virus have now been consistently generated in many replicas and variations of the original protocol. These data suggest that this simplified approach should be equally applicable for the development of efficient reverse genetics systems for other arenaviruses.

The JUNV reverse genetics system described above represents a powerful tool to precisely address questions regarding arenavirus biology and pathogenicity, particularly with regard to the New World HF-associated arenaviruses. The utility of the system was illustrated here by using it to examine elements of virus glycoprotein processing. Arenavirus G1/G2 heterodimers associate in three noncovalently associated subunits, which are incorporated in budding virions at the cell surface. Previous findings strongly suggested that arenavirus GPC processing by the protease SKI-1/S1P was absolutely required for arenavirus growth in cell culture (6, 19, 22, 33). These experiments clearly identified SKI-1/S1P as a critical cellular factor for production of infectious virus, but it remained unclear whether glycoprotein cleavage was solely involved or whether other steps in infectious virus production required functional SKI-1/S1P. Here we demonstrated (Fig. 3C) in the context of actual JUNV that the protease cleavage-resistant mutant pJUNS-ΔSKI replicated successfully in transfected cells but did not yield infectious progeny virus, despite these cells producing active SKI-1/S1P. These data confirmed that JUNV GPC processing is strictly required for production of infectious virus particles. The JUNV reverse genetics system allowed this point to be made in a more unequivocal manner, avoiding the use of SKI-1/S1P inhibitors or deficient cell lines or pseudotype systems.

The inability of the cleavage-defective pJUNS-ΔSKI mutant to yield infectious virus is consistent with earlier studies which suggested that while uncleaved GPC is expressed at the cell surface, it is not incorporated into virus particles (6, 19, 22, 33). In addition, it had previously been shown that overexpression of a cleavage-resistant form of the JUNV GPC failed to induce cell-cell fusion (42), suggesting that GPC cleavage is also critical to generate the fusion-competent glycoprotein form.

Generation of the mature virion glycoproteins by SKI-1/S1P cleavage is a feature apparently shared by arenaviruses and CCHF virus, an evolutionarily unrelated virus of the family *Bunyaviridae* (4). We hypothesized that there must be some functional or structural feature of these glycoproteins which prevents cleavage by furin-like PCs which is the more common cleavage mechanism among the negative-strand RNA viruses. This hypothesis was proved to be incorrect by the successful generation of the infectious rJUNV-Furin where the SKI-1/S1P cleavage site (RSLK) had been replaced by a furin cleavage site (RRKR) (Fig. 3B). In addition, the growth of rJUNV-Furin in SKI-1/S1P-deficient cells (Fig. 5) confirmed that the GPC of this virus was not cleaved by SKI-1/S1P but by furin-like PCs. This result also demonstrated that there was no additional SKI-1/S1P-dependent step (beyond GPC cleavage) essential for production of infectious virus.

It was noted, however, that the rJUNV-Furin virus was recovered less efficiently and grew to lower titers in Vero E6 cells than the wt rJUNV, suggesting that SKI-1/S1P GPC-cleaved virus has a fitness advantage over furin GPC-cleaved virus. At first the basis for this was unclear as biochemical analysis of the virus glycoproteins revealed that the level of GPC processing was not significantly different between rJUNV-Furin and rJUNV, confirming that the RRKR motif was cleaved with very similar efficiency (Fig. 4B). However, significantly less GPC and G2 proteins with a large mannose content (EndoH sensitive) and more EndoH-resistant GPC and G2 were observed for the rJUNV-Furin. Why the switch in cleavage from SKI-1/S1P to furin should increase the GPC and G2 complex oligosaccharide chain content may be a matter of timing. As SKI-1/S1P is located predominantly in the early Golgi, whereas furin is predominantly in the *trans*-Golgi network, it is possible that GPC cleavage in a different compartment as it transits the Golgi results in subtle differences in the carbohydrate chain addition during trafficking from the endoplasmic reticulum to cell surface. Such alterations could correlate with the growth differences observed.

The development of safe and efficacious live attenuated vaccines for the New World HF-associated arenaviruses is an important long-term goal of our reverse genetics efforts with these viruses. The observation that the rJUNV-Furin virus had reduced growth properties in tissue culture led us to examine whether switching the virus GPC cleavage from SKI-1/S1P to furin-like PCs had produced a virus with attenuated pathogenicity. To test this, a pilot experiment was done in which 9-day-old suckling mice (two groups of 10) were inoculated intracerebrally with either a lethal dose (400 PFU) of wt rJUNV or an

equivalent dose of rJUNV-Furin. Both rJUNV and rJUNV-Furin killed all the mice within 2 weeks (data not shown). The lack of attenuation suggests that this specific protease cleavage site switch would not be a good candidate for incorporation into a vaccine design. However, other engineered protease variants can be envisaged that might have potential, whereby virus production in the presence of a specific protease leads to high production yields but the absence of the protease in human target cells could contribute to virus vaccine attenuation.

Considering the significant genetic and pathogenic differences between LCMV and the HF-associated New World arenaviruses, the development of an efficient two-plasmid reverse genetics system for the rescue of infectious JUNV variants represents a significant advance which will allow fundamental questions regarding the biology and pathogenesis of these viruses to be addressed. In addition, this system has the potential to be utilized for the development of prototypic live attenuated vaccines with precisely engineered disruptions of pathogenic properties. A live attenuated JUNV, Candid 1, was proven effective in challenge studies in rhesus macaques and has been in use in the at-risk population in Argentina (3, 26, 27). While the consensus sequence of this vaccine virus has been determined (2, 17), the basis for attenuation and potential risk of reversion to a virulent phenotype remains unclear (10), and no JUNV vaccine is currently licensed for use in the U.S. The reverse genetics system can be used to specifically identify the attenuation features of Candid 1, and precisely engineer a prototypic live attenuated vaccine incorporating these and other attenuating features, thereby improving on the vaccine identity, efficacy, and safety.

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