Rescue from Cloned cDNAs and In Vivo Characterization of Recombinant Pathogenic Romero and Live-Attenuated Candid #1 Strains of Junin Virus, the Causative Agent of Argentine Hemorrhagic Fever Disease

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The New World arenavirus Junin virus (JUNV) is the causative agent of Argentine hemorrhagic fever (AHF), which is associated with high morbidity and significant mortality. Several pathogenic strains of JUNV have been documented, and a highly attenuated vaccine strain (Candid #1) was generated and used to vaccinate the human population at risk. The identification and functional characterization of viral genetic determinants associated with AHF and Candid #1 attenuation would contribute to the elucidation of the mechanisms contributing to AHF and the development of better vaccines and therapeutics. To this end, we used reverse genetics to rescue the pathogenic Romero and the attenuated Candid #1 strains of JUNV from cloned cDNAs. Both recombinant Candid #1 (rCandid #1) and Romero (rRomero) had the same growth properties and phenotypic features in cultured cells and in vivo as their corresponding parental viruses. Infection with rRomero caused 100% lethality in guinea pigs, whereas rCandid #1 infection was asymptomatic and provided protection against a lethal challenge with Romero. Notably, Romero and Candid #1 trans-acting proteins, L and NP, required for virus RNA replication and gene expression were exchangeable in a minigenome rescue assay. These findings support the feasibility of studies aimed at determining the contribution of each viral gene to JUNV pathogenesis and attenuation. In addition, we rescued Candid #1 viruses with three segments that efficiently expressed foreign genes introduced into their genomes. This finding opens the way for the development of a safe multivalent arenavirus vaccine.

Arenaviruses are enveloped viruses with a bisegmented negative-strand (NS) RNA genome. Each genomic RNA segment, L (ca. 7.3 kb) or S (ca. 3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientations and separated by a noncoding intergenic region (IGR) that acts as a transcription termination signal for the virus polymerase (21, 29). The S RNA encodes the viral glycoprotein precursor (GPC) and the nucleoprotein (NP). The GPC is posttranslationally cleaved by the cellular site 1 protease to yield the two glycoproteins GP1 and GP2, which, embedded in the lipid bilayer, form the viral spikes in the mature virion that are crucial for receptor recognition and cell entry. The L RNA encodes the viral RNA-dependent RNA polymerase (or L polymerase) and the small (ca. 11-kDa) RING finger protein Z that is the arenavirus counterpart of the M protein found in many other NS RNA viruses (24, 28, 30).

Arenaviruses cause chronic infections in rodents with a worldwide distribution (5). Infection of humans usually occurs through mucosal exposure to aerosols or by direct contact of abraded skin with infectious materials and may result in severe disease. Thus, the Old World Lassa virus (LASV) and several New World (NW) arenaviruses cause hemorrhagic fevers (HF), posing a serious public health problem in the regions where they are endemic (4, 19, 25).

The NW arenavirus Junin virus (JUNV) causes Argentine HF (AHF), a disease mostly endemic to the Pampas region of Argentina. AHF is a severe illness with hemorrhagic and neurological manifestations and a case fatality rate of 15 to 30% (13, 25, 31). In addition to its impact on public health, JUNV possesses features that make it a potential biological weapon. JUNV is very stable, is highly infectious by aerosol, and produces high morbidity and significant mortality at low doses. Accordingly, the development of antiviral strategies against JUNV is one of the top priorities within the Implementation Plan of the HHS Public Health Emergency Medical Countermeasures Enterprise.

Immune plasma therapy can ameliorate AHF symptoms and reduce mortality if administered during the prodromal phase. However, 10% of patients still present with late neurologic syndrome due to unknown mechanisms (9, 25). Further limitations of this treatment are dictated by a short supply of plasma and the risk of transmission of blood-borne pathogens.
In vitro and in vivo studies have documented the prophylactic and therapeutic value of the nucleoside analogue ribavirin (Rib) against several arenaviruses, including JUNV (3, 7). However, Rib is only partially effective and causes significant side effects, including anemia and congenital disorders, which, together with the need for its intravenous administration for optimal efficacy, underscores the need for the development of novel antiviral drugs. The JUNV live-attenuated Candid #1 strain, derived from the 44th mouse brain passage of the prototype XJ strain of JUNV, was found to be attenuated in guinea pigs, and preclinical studies at USAMRIID supported the safety, immunogenicity, and protective efficacy of Candid #1 in both guinea pigs and rhesus macaques (20). Moreover, a TC83 replicon vectored vaccine expressing GPC of Candid #1 has been shown to be sufficient to induce a protective immune response against virulent JUNV in guinea pigs (27). More importantly, clinically significant studies involving agricultural workers in the area where JUNV is endemic have shown Candid #1 to be an effective and safe vaccine in humans (17). This vaccine was licensed in 2006 for use exclusively in Argentina, whereas in the United States, Candid #1 remains only an investigational new drug and studies addressing long-term immunity and safety have not been conducted. The current availability within the United States of a Candid #1 master virus seed (MVS) is uncertain, and reimportation of Candid #1 vaccine from Argentina is likely to meet unsolvable obstacles due to foot-and-mouth-disease virus activity in several geographic regions of Argentina and the potential lack of FDA-compliant documentation. In addition, a detailed genetic composition and the bases for the attenuated phenotype of Candid #1 have not been documented. Therefore, the development of a vaccine against AHF for licensure in the United States will be facilitated by the generation of a genetically well-characterized MVS from a source that has not been in Argentina and the identification of the viral genetic determinants and virus-host interactions underlying JUNV pathogenesis. To this end, we have used reverse genetics to rescue from cloned cDNAs infectious, live-attenuated Candid #1 and pathogenic Romero strains of JUNV with well-defined genetic compositions. Here we describe the rescue of recombinant Candid #1 (rCandid #1) and Romero (rRomero) strains that were genetically identical to their parent viruses and grew to high titers in cultured cells without a need for cell culture adaptation. Moreover, we have, for the first time, characterized the recombinant JUNV strains (rCandid #1 and rRomero) in vivo. Guinea pigs infected with rRomero or the parental Romero field isolate succumbed with the same kinetics and symptoms. Likewise, guinea pigs infected with rCandid #1 did not exhibit clinical symptoms and were protected against a subsequent challenge with the pathogenic Romero strain.

The rescue of the XJ13 strain of JUNV from cloned cDNAs using a T7-based system has been recently documented (1). XJ13 was derived by carrying out two passages in guinea pigs and 13 passages in mouse brain of the XJ strain originally isolated from a case of AHF in Junin City (Buenos Aires, Argentina) (23). During these passages, XJ acquired several mutations, and therefore its derivative XJ13 may not accurately reflect the pathogenicity of JUNV in humans. In addition, the ability of the rescued XJ13 virus to induce AHF-like disease in a guinea pig has not been demonstrated (1). In contrast, the Romero strain, also isolated from a patient with AHF, has not been subjected to multiple passages in cultured cells or animals in the laboratory and therefore it is expected to represent a truly human pathogenic JUNV. In addition, over the years, we have accumulated a large body of data regarding the parameters associated with Romero-induced AHF-like disease in guinea pigs (32), which would facilitate the use of rRomero for the investigation of viral determinants of JUNV pathogenesis.

The development of a reverse genetics system for both live-attenuated Candid #1 and pathogenic Romero strains of JUNV will facilitate the investigation of the genetic determinants responsible for JUNV pathogenesis and attenuation, as well as the role of cis-acting sequences and trans-acting factors that control JUNV replication and gene expression, as well as assembly and budding. This knowledge will permit the merging of selected genetic information from different genomes of Candid #1-related viruses to generate a novel Candid #1 MVS with optimal properties for vaccine development, including immunogenicity, safety, and efficacy, as well as genetic stability, while retaining optimal growth in appropriate cell substrates used in vaccine production. In addition, we have generated a genetically marked, fully virulent Romero strain that should help with future studies aimed at testing vaccines and antivirals by different research groups using the same challenge virus to facilitate the comparison of data.

MATERIALS AND METHODS

Cells, viruses, and biosafety. Baby hamster kidney (BHK-21) and Vero cells (American Tissue Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and l-glutamine. The wild-type Romero strain of JUNV (GenBank accession no. AY619640 and AY619641) was obtained from Thomas G. Ksiazek (University of Texas Medical Branch [UTMB]). Viral stocks of the Romero and Candid #1 strains were prepared by infecting Vero cells (multiplicity of infection [MOI] = 0.01) and collecting virus-containing tissue culture supernatants (TCS) at 72 h postinfection (p.i.), followed by elimination of cell debris by centrifugation (10,000 × g for 10 min at 4°C). Work with virulent strains of JUNV and animal experiments were performed in the UTMB biosafety level 4 (BSL-4) facility in accordance with institutional health and safety guidelines.

Sequencing of full-length S and L RNA genome segments from Romero and Candid #1. (i) Sequencing of Candid #1 full-length genome. RNA (0.5 to 1.0 μg) isolated at 72 h p.i. from Candid #1-infected Vero cells was used in two reverse transcription (RT) reactions. One RT reaction was primed with a primer reverse complementary to an internal region of the Candid #1 polymerase gene, and the corresponding cDNA was used for amplification of the 5′ half of the L segment. The entire S and L segments were amplified in three and four, respectively, PCRs. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and directly sequenced to obtain the corresponding master sequences for the Candid #1 S and L genome RNA species, and its cDNA was used to amplify the complete S segment and the 3′ half of the L segment. The other RT was primed with a primer reverse complementary to an internal region of the Candid #1 polymerase gene, and the corresponding cDNA was used for amplification of the 5′ half of the L segment. The entire S and L segments were amplified in three and four, respectively, PCRs. PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and directly sequenced to obtain the corresponding master sequences for the Candid #1 S and L genome RNA species. Sequencing data were analyzed using the program Sequencher 4.9 (Gene Codes).

(ii) Sequencing of Romero full-length genome. The complete L and S genome RNA species of Romero were amplified using three and two, respectively, overlapping PCR fragments and procedures similar to those described for Candid #1 and directly sequenced to obtain their corresponding master sequences.

Determination of 5′ and 3′ termini of S and L genome RNAs from Romero and Candid #1. To determine the 5′ and 3′ termini of the S and L RNA segments of Candid #1, total RNA extracted from infected Vero cells was treated with an RNAse-free DNase I (Epicentre), purified using the QIAacell and the RNasey Minikit procedure, and self-ligated using T4 RNA ligase I. The product of the ligation was purified using the RNasey Minikit procedure and reverse transcribed using primers JUNGPrEx5′-5′-TCTAAACGTCTGAGAAATG AAGCA-3′ and JUNZSpo5′-ACGGTGTGTGTTGGTGCAGTT
GG-3') for the S and L segments, respectively. The cDNAs were used in PCRs to get products containing the 5' and 3' ends of the S and L segments. The primers for the S segment were JUNPRrev and JUNNPFP1 (5'-CTCCTAATCTGTTGAGATCTCT-3'), and those for the L segment were JUNZSop and XJLR2 (5'-TGGGACATGATGCGATTCGTA-3'). Amplified products were cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen). Multiple individual clones derived from L and S PCR products were sequenced to obtain their corresponding terminal sequences. The 5' and 3' termini of the S and L RNA segments of Romero were determined by 5' and 3' rapid amplification of cDNA ends using the FirstChoice RLM-RACE kit (Ambion) and following the protocol provided by the manufacturer.

**Plasmid constructs.** (i) Generation of Candid #1 plasmids. The Candid #1 plasmid was prepared by amplifying using primers located at the 5' and 3' ends of the open reading frame (ORF) and containing BamHI restriction sites, gel purified (QIAquick gel extraction kit;Qiagen), and cloned into pCR2.1 (TA cloning kit; Invitrogen). After sequencing, a clone with the correct insert was digested with BsmBI and the NP gene was introduced into the pCAGGS vector to generate the Candid #1 pC-NP plasmid. Candid #1 pC-L was generated by using a similar strategy but involving a two-step cloning approach in which (i) the first and last thirds of the Candid #1 L polymerase gene were amplified and ligated together via an inserted NotI site and introduced into pCAGGS via BsmBI restriction to generate the intermediate pCAGGS-Candid #1 polymerase vector and (ii) the central part of the LR ORF was amplified and inserted into the intermediate pCAGGS-Candid #1 polymerase vector via BstXI restriction sites to create plasmid Candid #1 pC-L. To create a pCAGGS vector expressing the L polymerase of JUNV strain XJ13, we introduced six mutations into the Candid #1 L polymerase vector via mutational PCR to recombine the amino acid differences previously reported between the strains XJ13 and Candid #1 (Y74A, A103V, L111P, P247S, N293L, K1156R, and V883I) (11). The NP amino acid sequences of JUNV strains XJ13 and Candid #1 are identical. To generate plasmids containing the full-length S and L genome sequences flanked by the mouse RNA polymerase I (mPol-I) promoter and terminator sequences, the backbones of the Candid #1 S and L segments (5' and 3' untranslated regions, IGR, and cloning sites) were synthesized (Integrated DNA Technologies, San Diego, CA) based on the sequence published for strain Romero. The overlapping medium minus strand (mPol-I) templates were later modified by mutational PCR to reflect either published reading frame (ORF) and containing BsmBI restriction sites, gel purified (QIAquick gel extraction kit; Qiagen), and cloned into pCR2.1 (TA cloning kit; Invitrogen). The plasmid mixture included 1.2 μg of pC-NP and 1.5 μg of pC-L, together with plasmids mPol-I-Lag (2.1 μg) and mPol-I-Sag (1.2 μg). At 3 days posttransfection, cells in each transfected M6 well were trypsinized and passed into a 12-well plate. After 72 h, virus-containing TCS were collected. For the rescue of rCandid virus containing 1L and 2S genome RNAs, each Pol-I-S (1.2 μg) expressing the modified S segment was incorporated into the transfection mixture. Trisegmented rCandid #1 viruses were referred to as rCandid XY, where X and Y represent the reporter genes in lieu of GPC and NP, respectively.

**Identification of genetic tags incorporated into the genomes of rRomero and rCandid #1.** Total RNA isolated from infected Vero cells or brain tissue from inoculated guinea pigs was reverse transcribed, and cDNAs were subjected to PCR using specific primers to amplify fragments of 822 bp (within the L polymerase gene of Romero and rRomero) or 664 bp (within the NP gene of Candid #1 and rRomero #1). The genetic tag in the Romero genome consisted of a silent change from C to U at nucleotide position 976 within the S segment that was confirmed by sequencing of the 822-bp PCR fragment. The genetic tag in the rCandid #1 genome consisted of two silent mutations within the NP gene (U1236C and C1262U) that created a second NcoI restriction site within the 664-bp PCR fragment of the virus NP. To confirm this genetic tag, the 664-bp PCR product was digested with NcoI and analyzed by agarose gel electrophoresis to reveal one (Candid #1), bands of 308 and 356 bp or two (rCandid #1), bands of 308, 227, and 129 bp NcoI restriction sites. The sequences of the detailed PCR protocols are available upon request.

**Viruses, propagation, and titration.** Virus propagation and growth kinetics in cultured cells were done by infecting (MOI = 0.01) Vero cells and collecting TCS at the indicated times. Virus titers in TCS and infected tissues were determined by either plaque assay (Romero and rRomero) or determination of 50% tissue culture infective doses (TCID50, Candid #1 and rCandid #1). For the plaque assay 10-fold dilutions of TCS specimens were added to Vero cell monolayers in six-well culture plates for 1 h at 37°C in an atmosphere of 5% CO2, after which overlaid with medium minus mPol-I and 5% fetal bovine serum, 1% penicillin-streptomycin (P/E) solution, and 0.5% agarose. Plates were then incubated for 7 days at 37°C, and plaques were revealed by fixation of cell monolayers with formaldehyde, followed by crystal violet staining. Infected tissues were dissected at necropsy and homogenized in MEM containing 1% P/E. Homogenates were clarified by centrifugation, and the supernatants were used for plaque assays as described for TCS specimens. Virus TCID50 were determined using the endpoint dilution assay and the Reed-Muench calculation method. Briefly, 10-fold virus dilutions were used to infect Vero cell monolayers in quadruplicate in a 96-well plate. At 3 days p.i., cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in a 0.3% Triton X-100–3% bovine serum albumin-PBS solution. The cells were then stained using a mouse monoclonal antibody to NP (IC06-BE10) and an Alexa Fluor 488-labeled anti-mouse secondary antibody (Molecular Probes). Similar procedures were used to detect viral antigen in infected cells using a mouse monoclonal antibody to either JUNV G1 (GB03-080) or NP (IC06-BE10). The following reagents were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: monoclonal anti-JUNV antibody (clone IC06-BE10); immunoglobulin G, mouse) and monoclonal anti-JUNV antibody (clone GB03-080; immunoglobulin G, mouse).

**Animal experiments.** Ten-week-old female Hartley guinea pigs were purchased from The Charles River Laboratory and housed for at least 7 days in a specific-pathogen-free environment before being used in any experimental procedure. All virus inoculations, including vaccination with rCandid #1, were conducted under BSL-4 conditions in the Robert E. Shope BSL-4 Laboratory, UTMB. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the UTMB and were carried out according to the National Institutes of Health guidelines. Guinea pigs were anesthetized using an isoflurane precision variable-bypass vaporizer prior to virus inoculation by the intraperitoneal route with 104 PFU. Standardized recording of death and disease symptoms was performed using the following definitions: encephalitis; development of coordination, ataxia, or transient seizures with retention of the ability to drink and feed; paralysis; and hind limb (hemiplegic) or quadriplegic paralysis with the inability to reach the feeder or water bottle. The experiment was stopped at 37 days when 25% of the animals that survived infection were humanely euthanized. Telemetric monitoring of body temperature and measurement of body weight were performed during the course of study. For telemetry, animals were anesthetized and implanted subcutaneously with BMDS IPT-300 transponders (chips) obtained from Bio Med Data Systems, Inc. (Seaford, DE).
using a trocar needle assembly. Animals were monitored for signs of infection or transponder migration for 2 days prior to transfer to the BSL-4 facility. Chips were scanned using a DAS-6007 transponder reader (Bio Medic Data Systems, Inc.). Downloading of digital temperature data was performed in accordance with the manufacturer’s protocol.

Hematologic and clinical chemical analyses. Blood was collected from guinea pigs into tubes containing EDTA, and a standard hematologic analysis was performed using the HEMAVET 1700 (Drew Scientific, Inc.) on whole-blood specimens to determine platelet and differential counts, in accordance with the manufacturer’s recommendation. Clinical chemical analysis was performed using the ACE Alera Clinical Chemistry System (Alfa Wassermann) according to the manufacturer’s instructions.

Histopathological and immunohistochemical analysis. Tissue samples were fixed in 4% buffered formalin for a minimum of 7 days and stored in 70% ethanol for 12 h. The samples were then embedded in paraffin. For histopathology, sections (4 µm) were mounted on slides and subjected to standard hematoxylin and eosin (H&E) staining. For immunohistochemical analysis, tissue sections were deparaffinized and rehydrated through xylene and graded ethanol solutions. To block endogenous peroxidase activity, slides were then treated with a solution of Tris-buffered saline containing 0.1% Tween 20, 3% hydrogen peroxide, and 0.05% sodium azide for 15 min, followed by heat antigen retrieval in a water bath at 95°C for 40 min in Dako Target Retrieval Solution, pH 6.1 (Dako Corporation). To block endogenous biotin reactivity, sequential 15-min incubations with avidin D and biotin solutions (Vector Laboratories) were performed. Subsequently, to prevent nonspecific protein binding, sections were incubated in blocking solution according to the manufacturer's instructions (Histomouse-SP kit; Zymed). For detection of viral antigen in tissue sections, we used a LASV group hyperimmune ascitic fluid (HIAF) prepared in adult ICR mice, provided by Robert Tesh, World Reference Center of Emerging Viruses and Arboviruses at UTMB. This HIAF contained cross-reacting antibodies to JUNV and recognized viral antigens in sections from paraffin-embedded tissue. Tissue sections were incubated for 60 min with HIAF at a 1:500 dilution in antibody diluent solution (BD PharMingen). Tissue sections from uninfected guinea pigs were used as a negative control for immunostaining. As an additional negative control, tissue sections from Junin-infected guinea pigs were incubated with diluent alone. To detect HIAF bound to Junin antigen in guinea pig tissue, the Histomouse-SP kit (Zymed) biotinylated secondary antibody was used, followed by streptavidin-peroxidase. Color development was achieved by using the chromogenic substrate according to the manufacturer’s instructions. Slides were counterstained with Mayer’s modified hematoxylin for microscopy.

RESULTS

Identification of viral cis-acting sequences and trans-acting factors required for efficient RNA replication and gene transcription of live-attenuated Candid #1 and pathogenic Romero strains of JUNV. As a first step to rescue infectious Candid #1 and Romero from cloned cDNAs, we developed an MG rescue system for JUNV to identify functional clones for the minimal viral trans-acting factors, NP and L, required for RNA replication and transcription by the JUNV polymerase complex. For this, we generated a polymerase I-based plasmid that directed the intracellular synthesis of an S-based RNA MG containing the 5’ and 3’ noncoding regions and IGR corresponding to published Candid #1 sequences and where the ORF of the CAT or Gaussia luciferase (Gluc) reporter gene substituted for the ORF of the viral NP. We noticed that, intriguingly, the highly conserved 19 nt characteristically observed at the 5’ end of all other arenaviruses so far examined appeared to contain significant differences in the S genome

these studies clearly indicated differences from previously published 5’- and 3’-end sequences of the Candid #1 and Romero genome RNA species (Fig. 1A). We therefore investigated whether previously published sequences (i) reflected genetic differences within the S and L termini of Candid #1 and Romero potentially associated with phenotypic differences or (ii) were artifacts caused by the experimental procedures associated with the sequencing strategy used. To this end, we generated three mPol-I-S vectors expressing CAT instead of NP that differed only in their 5’ and 3’ termini. One vector had 5’ and 3’ termini from the published Candid #1 sequence (accession number AY746353), another had 5’ and 3’ termini from previously published XJ13 and Romero sequences (accession numbers NC005081 and AY619641, respectively), and the last one had 5’ and 3’ termini from the newly determined Candid #1 and Romero sequences.

To provide the minimal viral trans-acting factors required for RNA replication and expression of the virus MG, we generated full-length clones for the NP and L gene products of Candid #1 and Romero and cloned them into the Pol-II-based expression vector (pCAGGS). Based on our previous experience with the rescue of infectious lymphocytic choriomeningitis virus (LCMV) from cloned cDNAs, we were aware that single amino acid differences, including those highly conserved with respect to the virus master sequence present in L and NP expression clones, can significantly affect the activity of a virus polymerase complex. We therefore confirmed that the sequences of the L and NP ORFs cloned into pCAGGS were identical to the corresponding master sequences we determined for Candid #1 and Romero.

Results from the MG experiment clearly demonstrated that only the newly determined 5’/3’-terminal sequences were fully functional, whereas 5’/3’ terminal from the published Candid #1 sequence completely abolished MG expression (Fig.1Bi). We next compared the relative efficiencies with which different combinations of L and NP derived from Romero and Candid #1 promoted RNA replication and expression of the Candid #1 S-based MG (Fig.1Bii). We also included in these studies an L polymerase clone derived from the JUNV XJ13 strain, the parental strain of Candid #1. We assigned a value of 100% to the MG activity obtained with the NP and L of Candid #1. Our results indicated that the Romero L-NP combination was significantly more active than the Candid #1 or XJ13 L-NP combination in the MG rescue assay. These results confirmed that we had obtained functional L and NP clones from both Romero and Candid #1 that are required for virus rescue. In addition, that combinations of L and NP derived from Romero and Candid #1 exhibited a good level of activity in this assay indicated the feasibility, as predicted based on their genetic proximity, of generating chimeric viruses based on Candid #1 and Romero.

Rescue of live-attenuated Candid #1 and pathogenic Romero strains of JUNV from cloned cDNAs. To rescue infectious rRomero and rCandid #1 from cloned cDNAs, we transfected BHK-21 cells with mPol-I-Sag and mPol-I-Lag for either Romero or Candid #1, together with Pol-II expression plasmids for the minimal corresponding viral trans-acting factors, L and NP, derived from each one of the two viral strains. For rRomero, TCS collected at 96 h posttransfection consistently had titers in the range of 10⁴ to 10⁵ PFU/ml. For
Candid #1 at 72 h posttransfection, cells (M6) were transferred to a T75 flask, and 72 later, TCS were collected and used to infect a fresh monolayer of Vero cells. The rationale for this was to demonstrate that the rescued Candid #1 vaccine strain could be grown to high titers in Vero cells, an approved cell substrate for vaccine production, without requiring adaptation. Vero cell TCS were collected at 72 h p.i., and they consistently had titers in the range of $10^6$ to $10^7$ TCID$_{50}$/ml. Both rRomero and rCandid #1 were unequivocally identified based on genetic tags introduced into the recombinant L and S segments, respectively, as described in Materials and Methods.

We next assessed whether rescued rRomero and rCandid #1 exhibited growth properties in cultured cells similar to those of their natural existing counterpart Candid #1 and Romero viruses. For this, we infected Vero cells (MOI = 0.01) with each of the parental or recombinant viruses and at the indicated times after infection determined infectious virus titers in TCS. rRomero (Fig. 2A) and rCandid #1 (Fig. 2B) displayed growth properties similar to those of Romero and Candid #1, respectively, the parental isolates. In addition, rRomero and rCandid #1 exhibited plaque-forming efficiencies, as well as plaque sizes and morphologies, similar to those of their respective counterparts, Romero and Candid #1.

### Table

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![Fig. 1](http://jvi.asm.org/)
In vivo biological properties of rRomero and rCandid #1. To confirm that the experimental procedures used for the rescue of rRomero and rCandid #1 did not result in unexpected changes in their phenotypic properties, we compared rRomero and rCandid #1 to Romero and Candid #1 with respect to their abilities to induce an AHF-like disease in guinea pigs. As with strain Romero (27, 32), infection with strain rRomero was 100% lethal in Hartley guinea pigs by 17 days p.i. (Fig. 3A). We observed a steady decrease in body weight after 7 days p.i. (Fig. 3B), and all infected guinea pigs became febrile (body temperature, ≥39.5) at 10 days p.i., which was followed by a rapid decline in body temperature between 12 and 14 days p.i. (Fig. 3C). Clinical signs of infection were first observed at 12 to 13 days p.i., with 50% (2/4) of the guinea pigs developing clinical encephalitis and 25% (1/4) developing paralysis at 17 days p.i. (data not shown). Infected guinea pigs developed high levels of viremia (in the range of 10⁶ PFU/ml) and had high titers of infectious virus in the spleen, liver, kidneys, and brain (Fig. 3D). More importantly, the infection with rRomero caused thrombocytopenia, as previously reported from studies with Romero. In addition, increased levels of liver enzymes and reduced production of albumin correlate with the histopathology reported for Romero infection and indicate mild liver damage (Table 1).

We next studied the histopathology associated with rRomero infection of guinea pigs. Representative H&E-stained sections of brains, including the cerebrum, cerebellum, and hippocampus, from rRomero-infected and mock-infected control guinea pigs did not reveal significant inflammation or necrosis in the brains of rRomero-infected guinea pigs (Fig. 4A). However, we occasionally observed vascular cuffs of mononuclear cells in the cortices of rRomero-infected animals. This finding correlates with the detection of viral antigen in neurons in small foci located mostly in the cortex (Fig. 4B). These results indicated that, as reported for Romero, the rRomero virus was also able to penetrate the central nervous system and cause mild inflammatory changes in the brain. We also consistently observed pathological changes in the spleens and livers of rRomero-infected guinea pigs similar to those previously reported for Romero-infected guinea pigs (32) at 12 days p.i. (Fig. 4A). Compared to those of mock-infected controls, the spleens from rRomero-infected guinea pigs showed a “moth-eaten” appearance of the white pulp with apparently numerous macrophages. The red pulp contained clusters of neutrophils, particularly in the marginal zones. Severely affected animals showed generalized cellular depletion within the red pulp.

Liver pathology was characterized by the presence of diffuse microvesicular steatosis, mild portal inflammation, and mild lobular inflammation. Additionally, steatosis was more pronounced, predominantly macrovesicular. Scattered foci of nuclear debris were present in portal triads and in lobular foci, suggesting leukocyte degeneration.

In contrast to our findings with rRomero, guinea pigs infected with rCandid #1 did not exhibit noticeable clinical symptoms throughout an observation period of 21 days. During this time, rCandid #1 and Candid #1 induced similar neutralizing antibody titers (Fig. 5). At day 21 p.i., we challenged rCandid #1-infected guinea pigs with a lethal dose of rRomero and monitored them for the development of clinical symptoms to determine whether infection with rCandid #1 had endowed the guinea pigs with protective immunity against a pathogenic JUNV (Fig. 6). All (n = 3) mock-immunized guinea pigs showed a steady decrease in body weight starting at day 8 postchallenge, and all of them succumbed by day 17 postchallenge. In contrast, immunization with Candid #1 or rCandid #1 provided protection against the development of disease symptoms and death following a challenge with a lethal dose of rRomero. One guinea pig immunized with rCandid 1# did not recover from an anesthesia procedure done to collect blood on day 8 postchallenge with rRomero, which resulted in 66% instead of the expected 100% survival in the group immunized with rCandid #1 and challenged with rRomero.

Generation and characterization of r3Candid expressing GFP. rCandid #1 and the parental Candid #1 isolate showed the same phenotype both in vitro and in vivo. We therefore reasoned that, as we have shown for LCMV (8), we should be able to generate an r3Candid virus able to express two additional genes of interest. We were able to readily rescue two different r3Candid viruses (Fig. 7). Both r3Candid GFP/CAT and r3Candid CAT/GFP grew to high titers in cultured cells and stably expressed both reporter genes. Consistent with our previous findings with r3LCMV, levels of reporter gene, GFP
and CAT, expression where higher when the reporter gene was placed in the NP locus within the S genome. These results indicated that it would be feasible to combine the safety of a live-attenuated vaccine already extensively tested in humans with the ability to express additional immunogens to induce protective immunity against pathogens other than JUNV, including LASV.

**DISCUSSION**

Here we have documented the rescue of the pathogenic Romero and attenuated Candid #1 strains of JUNV from cloned cDNAs. We employed a rescue system based on the use of Pol-I vectors to drive intracellular synthesis of the virus S and L genome RNA species and Pol-II expression plasmids to initially provide the minimal viral trans-acting factors, L and NP, required for RNA replication and gene transcription directed by the virus polymerase complex. The characterization of rRomero and rCandid #1, both in cultured cells and in a guinea pig model of JUNV infection, demonstrated that rRomero and rCandid #1 were genetically and phenotypically undistinguishable from the corresponding parental Romero and Candid #1 viruses.

A first and necessary step to accomplish this goal was to accurately determine the complete master genome sequences for Romero and Candid #1, including the precise L and S 5'3' termini that have been shown to play critical roles in the control of arenavirus RNA synthesis. We then used this information to generate Pol-II expression plasmids for the corresponding L and NP ORFs, whose functionality was assessed in an MG rescue assay based on the S segment of Candid #1. Our sequencing data revealed several differences within the 5'3' termini between previously published Romero and Candid #1 sequences and the ones we determined (Fig. 1A). More intriguingly, we obtained a sequence for the 5' end of the Candid #1 S segment that was identical to that of LCMV, whereas the sequence published for the 5' end of the S segment of Candid #1 appeared to be extended by 3 nt and contained five nucleotide differences within the 5'-end 19 nt highly conserved among arenaviruses. Because the role played by the 5'3' termini in the regulation of viral RNA synthesis, the unique sequence features of the 5' end of the Candid #1 S segment might have a role in attenuation. Results from MG rescue assays clearly indicated that the previously published 5'-end sequence of the Candid #1 S segment was not compatible with virus RNA replication and gene expression (Fig. 1B).

Romero and rRomero exhibited similar growth kinetics and peak titers in cultured cells (Fig. 2A). More importantly, guinea pigs infected with rRomero or Romero were similar in temporal development and the magnitude of their clinical symptoms, as well as in mortality. All animals that were inoculated with Romero and rRomero developed a systemic febrile illness that led to characteristic hematologic and neurologic manifestations associated with typical histopathological changes in organs such as the liver, spleen, and brain. In this particular study, animals infected with rRomero succumbed to the diseases insignificantly faster but within the normal time range (32).

As predicted, guinea pigs infected with either Candid #1 or rCandid #1 did not develop noticeable clinical symptoms, and the two viruses induced similar levels of neutralizing antibodies against JUNV (Fig. 5). Consistent with these results, two of the three rCandid #1-infected guinea pigs were totally resistant to a lethal challenge with Romero (Fig. 6). One rCandid #1-infected guinea pig, however, exhibited a delayed onset of clinical symptoms and associated histopathological changes, which resolved spontaneously within 3 days after inoculation.

**FIG. 3.** Induction of lethal disease and viral loads in guinea pigs infected with rRomero. (A) Female Hartley guinea pigs were inoculated i.p. with 10^3 PFU of either Romero (n = 3) or rRomero (n = 3) and monitored for 21 days for survival. (B and C) Body temperatures and weight changes were recorded throughout the course of the study. Shown are average values and standard deviations. (D) Virus titers in organs of rRomero-infected guinea pigs. Necropsies were performed on three rRomero-infected guinea pigs euthanized at 12 days p.i. Organs were homogenized, and virus titers in the organ samples and sera were determined by plaque assay.
immunized guinea pig developed some fever and lost some weight upon a lethal challenge with Romero. This animal did not recover from anesthesia during a blood collection procedure, and therefore it is unknown whether it would have survived the challenge as the other animals in the same group did. Different reasons could account for this unexpected finding. First, most protocols allow 6 to 8 weeks between Candid #1 immunization and a lethal challenge with pathogenic JUNV. In contrast, in our protocol, rCandid #1-immunized guinea pigs were subjected to a lethal challenge with Romero only 21 days after immunization. This significantly shorter time between immunization and challenge may result in some guinea pigs having suboptimal neutralizing antibody titers at the time of the challenge. In addition, we use outbred Hartley guinea pigs instead of inbred guinea pig strain 13, which is commonly used for vaccination studies with Candid #1. Therefore, a higher degree of genetic heterogeneity within outbred Hartley guinea pigs may have contributed to the higher levels of variation in the immune responses seen following immunization with rCandid #1. Likewise, a very large challenge dose (1,000 50% lethal doses) and potential genetic differences could influence guinea pig susceptibility to Romero, and therefore, even restricted Romero multiplication due to vaccination could result in the development of clinical symptoms. Although more detailed studies are required to conclusively establish the safety and efficacy of rCandid #1 in guinea pigs and nonhuman primates, our results have documented, for the first time, the generation of a genetically defined molecular clone of strain Candid #1 able to induce protective immunity against a pathogenic strain of JUNV in guinea pigs, which are a well-established model of JUNV infection and pathogenesis. A recent paper published after the original submission of this work reported the rescue of Candid #1, but it did not examine whether guinea pigs infected with this virus remained free of

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Blood was collected preinfection (day −6) and postinfection (day +11), and hematology and blood chemistry parameters were analyzed.

Three guinea pigs were euthanized 12 days after inoculation with rRomero.

![FIG. 4. Histopathology and tissue viral antigen distribution in rRomero-infected guinea pigs. (A) Histopathologic analysis. Tissue sections from guinea pigs infected with rRomero or mock infected were subjected to standard H&E staining. Magnification, ×20. (B) Dissemination of rRomero in the brains and livers of infected guinea pigs. Tissue sections were probed with an LASV group HIAF and a biotinylated secondary antibody. Color development was performed by using streptavidin-peroxidase, followed by the addition of a chromogenic substrate (brown-red).](http://jvi.asm.org/)
clinical symptoms and were protected against a lethal challenge with a pathogenic strain of JUNV (2).

Previous studies, including the comparison of LCMV Docile and Aggressive strains (6) or PICV nonpathogenic P2 and pathogenic P18 strains (15), have examined the relationship between genetic changes within the arenavirus genome and virus pathogenic potential. However, the implementation of these studies for JUNV would, for the first time, involve an arenavirus highly significant to human health. Romero was isolated from a severe, nonfatal human infection, whereas Candid #1 has been used in Argentina since 1991 to vaccinate individuals at high risk of infection with JUNV (9). Moreover, Candid #1 was derived from the prototype XJ strain originally isolated from a human with a fatal case of AHF (26). Therefore, some of the genetic differences between Candid #1 and Romero are likely to be related to the acquisition of attenuation in humans, nonhuman primates, and guinea pigs. In contrast, genetic differences between the nonpathogenic P2 and pathogenic P18 strains of PICV are likely related to the acquisition of virulence factors in guinea pigs whose relevance to arenavirus-induced disease in humans remains to be determined.

We showed that the NP and L proteins of Romero and Candid #1 were exchangeable in an MG rescue system. This finding supports the idea that it would be feasible to rescue rCandid #1 and rRomero with an exchanged gene or RNA segment(s), which would facilitate the identification of viral genes associated with pathogenicity. In this regard, we already found that the combination of rCandid #1 NP and L is less efficient than the combination of rRomero NP and L in an MG rescue assay, suggesting that Romero may replicate faster than Candid #1. Enhanced viral growth was previously associated with the ability of arenaviruses to escape or abrogate the host immune response (18, 22), and the level of viremia is a clear predictor of the clinical outcome in patients with Lassa fever (14).

The precise genetic characterization of Candid #1 vaccine remains uncertain. Several Candid #1 sequences have been deposited in GenBank, and their comparison shows several mutations among them. For example, a recent report (12) documented five amino acid differences in NP with respect to previously reported Candid #1 (AY746353) and XJ13 (NC005081) NP sequences. This lack of a precise genetic identity for Candid #1 not only complicates the identification of amino acid changes potentially responsible for the virus’s attenuation but also raises some questions about the safety of the vaccine. The use of reverse genetics to generate a Candid #1 strain with a well-defined genotype should help to develop a well-characterized Candid #1 MVS for the development of a vaccine against JUNV that is able to meet FDA requirements for licensure in the United States. Likewise, the ability to
manipulate the genome of a genetically and phenotypically well-characterized MVS of rCandid #1 raises the possibility of using Candid #1 as a platform to express antigens from LASV to generate a vaccine that is able to protect against LASV and JUNV, the two most relevant HF arenaviruses. As a first step in this direction, we explored whether, as we reported for LCMV (8), Candid #1 could also be converted into a trisegmented virus. For this, we rescued r3Candid GFP/CAT and r3Candid CAT/GFP by using a strategy we recently developed for LCMV (8). As with r3LCM viruses, r3Candid expressed high levels of the additional foreign genes and grew to high titers in cultured cells (Fig. 7). We have documented that, compared to wild-type LCMV, r3LCMV is attenuated in mice, suggesting that in vivo, r3Candid viruses would be, compared to Candid #1, further attenuated and therefore safer, but this r3Candid virus should retain levels of replication in vivo sufficient to induce a protective immune response. Preexisting immunity to the vector used to vaccinate can interfere with the ability of this vector to stimulate a protective immune response against the antigens expressed by the vector (16). In this regard, the highly restricted area where JUNV is endemic makes it highly unlikely that preexisting immunity to JUNV would interfere with the use of Candid #1 as a vaccine platform.

The ability to manipulate the genomes of two genetically well-defined and closely related strains of JUNV, one pathogenic (rRomero) and the other attenuated (Candid #1), in a well-established guinea pig model of JUNV infection should facilitate studies aimed at identifying viral genetic determinants associated with virulent and attenuated phenotypes, as well as the mechanisms by which different viral genes contribute to virus-host interactions underlying the development of HF arenaviral disease.

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