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 neurologic 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 suitable as 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 antiarenavirus 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, clinical 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 guinea pigs 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 the conserved 3′-terminal 19 nucleotides (nt) of the S and L genome RNA species, and its cDNA was used to amplify the complete S segment and the 5′ 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 genome RNA segments of Candid #1, total RNA extracted from infected Vero cells was treated with an RNase A/S1 polynucleotase (Epicentre), purified using the QiAquick and the RNeasy Minikit procedure, and self-ligated using T4 RNA ligase I. The product of the ligation was purified using the RNeasy Minikit procedure and reverse transcribed using primers JUNGPrevS (5′-TATCAACAGCTATCGTACTGAAATG AAGCA-3′) and JUNZSop (5′-AGCTCTTCGTCATGTGGTGTTGCTTT

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GG-3') for the S and L segments, respectively. The cDNAs were used in PCRs to get products containing the 5′ untranslated regions, IGR, and cloning sites) were cloned instead of GPC and/or NP into Candid #1 mPol I-Sag as described previously (8). Transfections were done using FuGene HD transfection reagent (Roche) by following the standard protocol. The next day, transfected cells were trypsinized and transferred into a T75 flask, and 72 h later, 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 X,Y, 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 rCandid #1). The genetic tag in the rRomero genome consisted of a silent change from C to U at nucleotide position 715 within the L 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 (U1263C and C1266U) that created a second Ncol 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 Ncol 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) Ncol restriction sites. The sequences of the primers and detailed PCR protocols are available upon request.

**Establishment of an MG rescue assay for JUNV.** 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 guidelines of the National Institutes of Health. Guinea pigs were anesthetized using an isoflurane precision variable-bypass vaporizer prior to virus inoculation by the intraperitoneal route with 103 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 experimenital endpoint was set at 21 days when evident signs of disease were observed. Animals exhibiting severe encephalitis and/or hind-limb paralysis 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 IPTT-300 transponders (chips) obtained from Bio Med Data Systems, Inc. (Seaford, DE).

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**Plasmid constructs.** (i) Generation of Candid #1 plasmids. The Candid #1 NP was PCR amplified using primers located at the 5′ and 3′ ends of the open reading frame (ORF) and containing BsmBI 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 pCNP plasmid. Candidate #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 endonuclease to generate the intermediate pCAGGS-Candid #1 polymerase vector and (ii) the central part of the L ORF was amplified and inserted into the intermediate pCAGGS-Candid #1 polymerase vector by 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 pC-L vector by mutational PCR to recreate the amino acid differences previously reported between the Candid #1 (Joy/00/3) and XJ13 (YYA05, H11187, P935L, K1156R, and Y8833) (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 of the full-length genome RNA sequences of Romero (GenBank accession number MG222174 and MG222178) after extermities were later modified by mutational PCR to reflect either published terminal sequences from XJ13/Romero (NC005801 and Y616946, respectively) or the sequences we determined in this work. Backbone sequences were introduced into the mPol-I vector pRF2 in antigenic orientation. Each Candid #1 gene was then introduced after enzymatic restriction to produce the Candid #1 mPol-I-Sag and mPol-I-Lag vectors. For the generation of mPol-I-Sag vectors used for the rescue of rCandid viruses, genes of interest (the enhanced green fluorescent protein and chloramphenicol acetyltransferase [CAT] genes) were cloned instead of GPC and/or NP into Candid #1 mPol-I-Sag as described previously (8).

(ii) Generation of Romero plasmids. To generate the Romero mPol-I-Sag and mPol-I-Lag plasmids, the complete S and L segments were amplified in two and three fragments, respectively. Subsequently, PCR products were assembled, using appropriate restriction enzymes, into plasmid pBS-L containing the complete sequence of the L segment that corresponded to the sequence with GenBank accession number AY619640. We used a similar approach to ligate the two S fragments to generate plasmid pBS-S containing the complete sequence of the S segment that corresponded to the sequence with GenBank accession number AY619641. For generating plasmids containing the full-length S and L genome sequences of Romero flanked, in antigenic orientation, by the mPol-I promoter and terminator sequences, fragments containing full-length L and S segments were cut out of pBS-L and pBS-S, respectively, and inserted into pRF2 to generate Romero mPol-I-Sag and mPol-I-Lag. Plasmids expressing the NP and L polymerase of the Romero strain of JUNV were generated similarly to the corresponding plasmids of the Candid #1 strain.

**Establishment of an MG rescue assay for JUNV.** BHK-21 cells (seeded at 3 × 104/well of an 12-well plate) were transfected with 0.4 μg of pC-NP, 0.6 μg of pC-L, and 0.5 μg of the indicated Candid #1 plasmid pMol-I-S to direct intracellular synthesis of the minigenome (MG) RNA of interest. Three days later, we assayed for the rescue of rCandid #1, subconfluent monolayers of BHK-21 cells (2 × 105/well of an M6 plate) were transfected for 5 h by using 2.5 μL of Lipofectamine 2000 (Invitrogen)/μg of plasmid DNA. The plasmid mixture included 1.2 μg of pC-NP and 1.5 μg of pC-L, together with plasmids pMol-I-Lag (2.1 μg) and pMol-I-Sag (1.2 μg). At 3 days posttransfection, cells in each transfected M6 well were trypsinized and passed into a T75 flask, and 72 h later, 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. Transsegmented rCandid #1 viruses were referred to as rCandid XY, where X and Y represent the reporter genes in lieu of GPC and NP, respectively.

**Virus propagation, growth kinetics in cultured cells.** 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 infectious 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 and then overlaid with minimum essential medium (MEM) with 1% fetal bovine serum, 1% penicillin-streptomycin (PE) 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 568-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-BE08) 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-BE08; immunoglobulin G, mouse).

Infections were confirmed using a mouse monoclonal antibody to IC06-BE10 and an Alexa Fluor 568-labeled anti-mouse secondary antibody (Molecular Probes). Similar procedures were used to detect viral antigen in infected cells using a monoclonal antibody to either JUNV G1 (GB03-BE08) 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-BE08; immunoglobulin G, mouse).
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 RNA of Candid #1 deposited in GenBank (accession number AY746353), including the presence of an extra 3 nt (Fig. 1A), which led us to revisit the 5’/H11032/3’/H11032/ termini of both the S and L RNA genome species of Candid #1, as well as the Romero strain. For this, we conducted a comprehensive analysis of the Candid #1 and Romero 5’- and 3’-end genomic sequences by using viral genome RNA isolated at 72 h p.i. Results from 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 sequences from the published Candid #1 sequence completely abolished MG expression (Fig.1B). 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.1Bi). 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 10^4 to 10^5 PFU/ml. For...
rCandid #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 counterparts 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.
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. Severe 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 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-
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

<table>
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<tr>
<th>Animal</th>
<th>Platelet count (10^3/ml)</th>
<th>Mean platelet volume (fL)</th>
<th>Albumin (g/dl)</th>
<th>Alkaline phosphatase (U/liter)</th>
<th>Alanine aminotransferase (U/liter)</th>
<th>Amylase (U/liter)</th>
<th>Calcium (mM)</th>
<th>Globulin (g/dl)</th>
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<tr>
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<td>3.03/10.5</td>
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<td>548/217</td>
<td>5.8/3.2</td>
<td>4.30/2.00</td>
<td>112/683</td>
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<td>1,541/1,353</td>
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<td>1.7/2.7</td>
</tr>
<tr>
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<td>5.5/2.9</td>
<td>4.30/2.10</td>
<td>102/688</td>
<td>24/136</td>
<td>1,292/721</td>
<td>11.6/8.9</td>
<td>1.6/3.1</td>
</tr>
</tbody>
</table>

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.
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

FIG. 5. Immunogenicity of rCandid #1 in guinea pigs. Serum samples were collected at the indicated time points from guinea pigs immunized with either Candid #1 or rCandid #1, and the titers of Candid #1-specific neutralizing antibodies were determined by 50% plaque reduction neutralization test (PRNT). The detection range of the assay is flanked by dashed lines. Asterisks indicate the serum virus titers of the guinea pig that did not recover from anesthesia after blood collection performed on day 11 after a challenge with rRomero.

FIG. 6. Protection of rCandid #1-infected guinea pigs against a lethal challenge with rRomero. (A) Comparison of guinea pig survival after immunization with Candid #1 or rCandid #1 and a challenge with rRomero. Female Hartley guinea pigs (three per group) were inoculated i.p. with 103 PFU of either Candid #1 or rCandid #1 or mock immunized. At 21 days after immunization, all of the guinea pigs were challenged by the i.p. route with 103 PFU of rRomero and monitored for survival. (B and C) Body weight (B) and temperature (C) changes were recorded at the indicated time points pre- and postchallenge. Average values and standard deviations are shown.
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 r3LCMV 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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