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Development and Application of a Reverse Genetics System for Zika Virus

Jordan C. Frank
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DEVELOPMENT AND APPLICATION OF A REVERSE GENETICS SYSTEM

FOR ZIKA VIRUS

by

Jordan C. Frank

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal Health and Disease

Approved:

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UTAH STATE UNIVERSITY
Logan, Utah

2018
ABSTRACT

Development and Application of a Reverse Genetics System for Zika Virus

by

Jordan C. Frank, Master of Science
Utah State University, 2018

Major Professor: Young-Min Lee, Ph.D.
Department: Animal, Dairy and Veterinary Sciences

Zika virus (ZIKV) causes no-to-mild symptoms to severe neurological disorders. To investigate the importance of viral and host genetic variations in determining ZIKV infection outcomes, three full-length infectious cDNAs were cloned as bacterial artificial chromosomes for each of three spatiotemporally distinct and genetically divergent ZIKVs: MR-766 (Uganda, 1947), P6-740 (Malaysia, 1966), and PRVABC-59 (Puerto Rico, 2015). Using the three molecularly cloned ZIKVs, together with 13 ZIKV region-specific polyclonal antibodies covering nearly the entire viral protein-coding region, three conceptual advances were made: (1) a comprehensive genome-wide portrait of ZIKV gene products and their related species, with several previously undescribed gene products identified in the case of all three molecularly cloned ZIKVs. (2) The data
demonstrated that ZIKV has a broad cell tropism in vitro, being capable of establishing productive infection in 16 of 17 animal cell lines from 12 different species, although its growth kinetics varied depending on both the specific virus strain and host cell line. More importantly, one ZIKV-nonsusceptible bovine cell line that has a block in viral entry but fully supports the subsequent post-entry steps was identified. (3) In mice, the three molecularly cloned ZIKVs differed in their neuropathogenicity, depending on the particular combination of viral and host genetic backgrounds, as well as in the presence or absence of type I/II interferon signaling. Overall, the findings demonstrate the impact of viral and host genetic variations on the replicability and neuropathogenicity of ZIKV and provide multiple avenues for developing and testing medical countermeasures against ZIKV.
Development and Application of a Reverse Genetics System for Zika Virus

Jordan C. Frank

Zika virus (ZIKV) has emerged in many regions of the world, with infection outcomes spanning from no apparent illness to crippling nervous system disease. ZIKV and its close relatives, West Nile virus, Japanese encephalitis virus, dengue virus, and yellow fever virus are primarily transmitted by mosquitoes. Three ZIKVs were selected: MR-766 (Uganda, 1947), P6-740 (Malaysia, 1966), and PRVABC-59 (Puerto Rico, 2015), whose place of origin and time of isolation differ substantially. Stable, complementary DNA (cDNA) copies of the three ZIKV RNA genomes were cloned to examine the significance of viral and host genetic variations in directing ZIKV infection outcomes. Using a new toolbox for ZIKV genome engineering and protein analysis, combined with various cell culture and mouse infection model systems, the following were determined: (1) Genome-wide landscape of viral gene products and their related species, with several immuno-reactive gene products identified in the case of all three cloned ZIKVs. (2) Viral replicability in cultured cells, varied significantly depending on the virus strain and host cell type, with one cow cell line being resistant to ZIKV infection. (3) Virus induced neurological disease in mice, differed dramatically depending on the virus dose and strain, mouse age and strain, route of infection, and presence or absence of immune system components. Overall, the findings demonstrate the impact of
the viral and host genetic backgrounds on the ability of ZIKV to replicate and cause disease. The ZIKV strain-specific characterizations and molecular instruments described will provide multiple avenues for developing and testing medical countermeasures.
ACKNOWLEDGMENTS

This work has been supported by endless efforts from Dr. Young-Min Lee. He has spent innumerable hours providing feedback and guidance. I appreciate his willingness to work with me. Drs. Sang-Im Yun, Byung-Hak Song, and Jin-Kyoung Kim have also been essential in teaching protocol, and use of laboratory tools. Their efforts and availability have been vital to the progress and goals of this research. I thank them for their examples of persistence and patience. Entrusted teaching and learning opportunities to and from lab group members have been very valuable, and I am grateful for those experiences. I enjoyed collaborating with fellow researchers, Michael Woolley and Joseph Goldhardt, and appreciate their friendship. I express my thanks to undergraduates who helped with this project: Emily Robb, Jackeline Wilkinson, Michael Berentzen, and Jae-Min Eun. Aid coupled with the expert advice and experience of Drs. Chris Davies and Justin Julander, greatly contributed to the advancements of this project. I express my gratitude for their assistance, and continued interest as members of my supervisory committee. I appreciate Dr. Aaron Olsen, Lisa DeSoi and LARC staff for their time, training and unparalleled service.

Assistance, encouragement, and the example of my parents and many family members are undeniably bonded to my educational endeavors and innate interest in lifelong learning. I thank them for their undying and selfless support.

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1. INTRODUCTION

Discovered in Uganda in 1947 in a febrile, sentinel rhesus macaque [1], ZIKV is a medically important flavivirus [2] related to Japanese encephalitis (JEV), West Nile (WNV), dengue, and yellow fever viruses [3]. Originally, it was found only within an equatorial belt running from Africa to Asia, with only about a dozen cases of human illness reported [4]. In 2007, however, it caused a major outbreak of mild illness characterized by fever, rash, arthralgia, and conjunctivitis on the western Pacific Island of Yap [5, 6]. Since then, it has spread eastward across the Pacific Ocean, invading French Polynesia and other Pacific Islands in 2013-2014 [7], reaching the Americas and Caribbean in 2015-2016 [8, 9], and exhibits characteristics which may encourage virus dissemination to many regions of the world. [10, 11]. ZIKV is spread to humans mainly through the bite of an infected Aedes species mosquito, e.g., A. aegypti or A. albopictus [12], but it can also be transmitted from a mother to her child during pregnancy [13, 14] or through sexual contact [15, 16]. Serious concerns have been raised over links to congenital neurological malformations (e.g., microcephaly) and severe neurological complications (e.g., Guillain-Barré syndrome) [17, 18]. Despite its continuous rapid spread and high pandemic potential, no vaccine or drug is available to prevent or treat ZIKV infection.

ZIKV is an enveloped RNA virus with a nucleocapsid core comprising an ~11-kb plus-strand RNA genome and multiple copies of the capsid (C) protein; this core is surrounded by a lipid bilayer bearing the anchored membrane (M) and envelope (E)
proteins [19, 20]. To date, little information is available about the molecular events that occur during ZIKV infection, but our current understanding of the molecular biology of closely related flaviviruses offers a promising starting point for ZIKV research [21]. As the first step in flavivirus replication, the virion binds nonspecifically to the surface of a host cell and is then internalized via clathrin-mediated endocytosis in a viral glycoprotein E-dependent manner [22, 23]. Within endosomes, the E glycoprotein undergoes low pH-induced conformational changes, followed by fusion of the viral and host cell membranes [24-26]. In the cytoplasm, the viral genomic RNA (vgRNA) functions initially as an mRNA for the translation of a single long open reading frame (ORF) flanked by 5’ and 3’ non-coding regions (NCRs) [27, 28]; the resulting polyprotein is cleaved by viral and cellular proteases to generate at least 10 mature proteins [29, 30]: three structural (C, premembrane (prM), and E) and seven nonstructural (NS1, 2A, 2B, 3, 4A, 4B, and 5). In JEV and WNV, ribosomal frameshifting is also used for the expression of NS1’, a C-terminally extended form of NS1 [31-33]. A complex of the seven nonstructural proteins directs vgRNA replication on the distinct virus-induced membranous compartments derived from endoplasmic reticulum (ER) [34, 35]. This replication process is catalyzed by two main viral components: (1) NS3, with serine protease (and its cofactor, NS2B) and RNA helicase/NTPase/RTPase activity, and (2) NS5, with methyltransferase/guanylyltransferase and RNA-dependent RNA polymerase activity [36]. Virus assembly begins with budding of the C proteins, complexed with a newly made vgRNA, into the ER lumen, and acquisition of the viral prM and E proteins. The prM-containing immature virions travel through the secretory pathway; in the trans-Golgi
network, a cellular furin-like protease cleaves prM to yield the mature M protein, converting the immature particle to a mature virion [37].

The clinical presentation of ZIKV infection is highly variable, ranging from no apparent symptoms or mild self-limiting illness to severe neurological disorders such as microcephaly and Guillain-Barré syndrome [10, 17]. Fundamentally, the varied outcomes after infection with a pathogen depend on the specific combination of pathogen and host genotypes [38]. On the virus side, a limited but significant number of ZIKVs have been isolated from Africa, Asia, and the Americas during the past 70 years. Recent phylogenetic analyses based on complete or near-complete viral genome sequences have revealed that the spatiotemporally distinct ZIKV strains are grouped into two major genetic lineages, African and Asian, with the 2015-2016 American epidemic strains originating from a common ancestor of the Asian lineage [39-41]. Despite the continuous expansion of its genetic diversity, little is known about the effect of viral genetic variation on the pathogenicity of ZIKV between the two lineages or between different strains within a particular lineage. On the host side, much progress has recently been made in developing murine models for ZIKV infection, including mice genetically engineered to lack one or more components of the innate and adaptive immune systems that affect the development, severity, and progression of ZIKV-induced disease [42]. However, the influence of host genetic variation on susceptibility to ZIKV infection is largely unknown.

To experimentally assess the impact of viral and host genetic variations on the outcome of ZIKV infection, a unique set of three functional bacterial artificial
chromosomes (BACs), each containing a full-length infectious cDNA for one of three genetically divergent ZIKV strains, and an exclusive collection of 13 rabbit antisera capable of detecting almost all of the ZIKV gene products and their related species were generated. Using these functional genomics and immunologic tools, together with various cell culture and mouse infection model systems, the three molecularly defined cDNA-derived ZIKVs have a similar viral protein expression profile but display dramatic differences in \textit{in vitro} growth properties and \textit{in vivo} neuropathogenic potential that depend on both viral and host genetic traits. This study not only provides a powerful system for the functional study of viral and host genetics in ZIKV replication and pathogenesis but also offers a valuable platform for the rational design of vaccines and therapeutics against ZIKV.
2. MATERIALS AND METHODS

2.1. Cells and Viruses

Details of the 17 cell lines used in this study, including their growth medium and culture conditions, are presented in Table 2-1. ZIKV MR-766 and P6-740 were obtained from the World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch (Galveston, TX), and ZIKV PRVABC-59 was provided by the Centers for Disease Control and Prevention (Fort Collins, CO). In all three ZIKVs, viral stocks were amplified once in a ZIKV-susceptible African green monkey kidney (Vero) cell line at a multiplicity of infection (MOI) of 1.
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2.2. Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignments were performed via ClustalX, and the phylogenetic tree was constructed using MEGA and visualized via TreeView, as described [43]. Sequence identities between aligned nucleotide and amino acid sequences were calculated using ClustalX.

2.3. Functional cDNA Cloning

Standard molecular cloning techniques were used to create three full-length ZIKV cDNAs, each for MR-766, P6-740, and PRVABC-59 in the BAC plasmid pBeloBAC11 [44], designated pBac/MR-766, pBac/P6-740, and pBac/PRVABC-59. The cloned cDNAs were checked by restriction enzyme mapping and sequencing. The salient features of the three full-length ZIKV BACs are the SP6 promoter sequence positioned immediately upstream of the viral 5’-end and an artificial run-off site (PsrI for MR-766, BarI for P6-740 and PRVABC-59) placed just downstream of the viral 3’-end.

The oligonucleotides used in this study are listed in Table 2-2. The same cloning strategy was used to construct three full-length ZIKV cDNAs, one each for the MR-766, P6-740, and PRVABC-59 strains (Figure 3-2).
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<tr>
<td>85</td>
<td>CDS</td>
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<td>3'UTR</td>
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<tr>
<td>87</td>
<td>3'UTR</td>
<td>3'-UTR</td>
</tr>
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</table>

Table 2.2: Oligonucleotides used in this study.
pBac/MR-766: The genomic RNA of ZIKV MR-766 (GenBank accession no. KX377335) was used as a template for the synthesis of three overlapping cDNA fragments by RT-PCR with the following primer sets: Frag-A<sup>MR-766</sup> (4552 bp), Z1RT and Z1F+Z1R; Frag-B<sup>MR-766</sup> (5070 bp), Z2RT and Z2F+Z2R; and Frag-C<sup>MR-766</sup> (5008 bp), Z3RT and Z3F+Z3R. Each of the three cDNA amplicons was subcloned into pBAC<sup>SP6</sup>/JVFLx/XbaI [45], a derivative of the pBeloBAC11 plasmid, by ligating the 8381-bp Pmel-MluI fragment of pBAC<sup>SP6</sup>/JVFLx/XbaI with the 4538-, 5056-, and 4994-bp Pmel-Ascl fragments of the Frag-A<sup>MR-766</sup>, Frag-B<sup>MR-766</sup>, and Frag-C<sup>MR-766</sup> amplicons, respectively. This generated pBac/Frag-A<sup>MR-766</sup> to -C<sup>MR-766</sup>. To introduce an SP6 promoter immediately upstream of the first adenine residue of the viral genome, two cDNA fragments were first amplified individually by (1) PCR of pBAC<sup>SP6</sup>/JVFLx/XbaI with a pair of primers, S123-5sp1F+S123-5sp1R (S123-5sp1R contains the antisense sequence of the SP6 promoter) and (2) PCR of pRs/5’NCR<sup>MR-766</sup> [46] with another pair of primers, S1-5sp2F+S1-5sp2R. Subsequently, these two fragments were fused by a second round of PCR with the outer forward and reverse primers S123-5sp1F+S1-5sp2R. The 1025-bp BamHI-SacII fragment of the fused PCR amplicons was ligated with the 2718-bp BamHI-SacII fragment of pRs2, creating pRs/5’SP<sup>MR-766</sup>. To engineer a unique Psrl run-off site just downstream of the last thymine residue of the viral genome, one cDNA fragment was amplified by PCR of pRs/3’NCR<sup>MR-766</sup> [46] with primers S1-3roF+S1-3roR (S1-3roR contains the antisense sequence of the Psrl and NotI recognition sites in a row). The 649-bp SacII-NotI fragment of the resulting amplicons was ligated with the 2667-bp SacII-NotI fragment of pRs2, creating pRs/3’RO<sup>MR-766</sup>. The full-length MR-766 cDNA clone
pBac/MR-766 was then assembled by sequentially joining the 7456-bp PacI-NotI fragment of pBACSP6/JVFLx/XbaI with the following five DNA fragments: (1) the 1004-bp PacI-Xmal fragment of pRs/5'SPThrMR-766, (2) the 3160-bp Xmal-XhoI fragment of pBac/Frag-AThrMR-766, (3) the 3144-bp XhoI-NsiI fragment of pBac/Frag-BThrMR-766, (4) the 3041-bp NsiI-BamHI fragment of pBac/Frag-CThrMR-766, and (5) the 619-bp BamHI-NotI fragment of pRs/3'ROThrMR-766.

pBac/P6-740: The genomic RNA of ZIKV P6-740 (GenBank accession no. KX377336) was used as a template for the synthesis of three overlapping cDNA fragments by RT-PCR with the following primer sets: Frag-AThrP6-740 (4553 bp), Z1RT and Z1F+Z1R; Frag-BThrP6-740 (5070 bp), Z2RT and Z2F+Z2R; and Frag-CThrP6-740 (5008 bp), Z3RT and Z3F+Z3R. Each of the three cDNA amplicons was subcloned into pBACSP6/JVFLx/XbaI, by ligating the 8381-bp PmeI-MluI fragment of pBACSP6/JVFLx/XbaI with the 4539-, 5056-, and 4994-bp PmeI-AscI fragments of the Frag-AThrP6-740, Frag-BThrP6-740, and Frag-CThrP6-740 amplicons, respectively. This generated pBac/Frag-AThrP6-740 to -CThrP6-740. To introduce an SP6 promoter immediately upstream of the first adenine residue of the viral genome, two cDNA fragments were first amplified individually by (1) PCR of pBACSP6/JVFLx/XbaI with a pair of primers, S123-5sp1F+S123-5sp1R (S123-5sp1R contains the antisense sequence of the SP6 promoter) and (2) PCR of pRs/5’NCRThrP6-740 [46] with another pair of primers, S23-5sp2F+S23-5sp2R. Subsequently, these two fragments were fused by a second round of PCR with the outer forward and reverse primers S123-5sp1F+S23-5sp2R. The 1025-bp BamHI-SacII fragment of the fused PCR amplicons was ligated with the 2718-bp BamHI-SacII
fragment of pRs2, creating pRs/5’SP<sup>P6-740</sup>. To engineer a unique BarI run-off site just downstream of the last thymine residue of the viral genome, one cDNA fragment was amplified by PCR of pRs/3’NCR<sup>P6-740</sup> [46] with primers S23-3roF+S23-3roR (S23-3roR contains the antisense sequence of the BarI and NotI recognition sites in a row). The 649-bp SacII-NotI fragment of the resulting amplicons was ligated with the 2667-bp SacII-NotI fragment of pRs2, creating pRs/3’RO<sup>P6-740</sup>. The full-length P6-740 cDNA clone pBac/P6-740 was then assembled by sequentially joining the 7456-bp PacI-NotI fragment of pBAC<sup>SP6/JVFLx/XbaI</sup> with the following five DNA fragments: (1) the 187-bp PacI-Nhel fragment of pRs/5’SP<sup>P6-740</sup>, (2) the 2930-bp Nhel-SpI fragment of pBac/Frag-A<sup>P6-740</sup>, (3) the 3359-bp SpI-NgoMIV fragment of pBac/Frag-B<sup>P6-740</sup>, (4) the 4059-bp NgoMIV-StuI fragment of pBac/Frag-C<sup>P6-740</sup>, and (5) the 433-bp StuI-NotI fragment of pRs/3’RO<sup>P6-740</sup>.

pBac/PRVABC-59: The genomic RNA of ZIKV PRV ABC-59 (GenBank accession no. KX377337) was used as a template for the synthesis of three overlapping cDNA fragments by RT-PCR with the following primer sets: Frag-A<sub>PRVABC-59</sub> (4553 bp), Z1RT and Z1F+Z1R; Frag-B<sub>PRVABC-59</sub> (5070 bp), Z2RT and Z2F+Z2R; and Frag-C<sub>PRVABC-59</sub> (5008 bp), Z3RT and Z3F+Z3R. Each of the three cDNA amplicons was subcloned into pBAC<sup>SP6/JVFLx/XbaI</sup>, by ligating the 8381-bp Pmel-MluI fragment of pBAC<sup>SP6/JVFLx/XbaI</sup> with the 4539-, 5056-, and 4994-bp Pmel-AscI fragments of the Frag-A<sub>PRVABC-59</sub>, Frag-B<sub>PRVABC-59</sub>, and Frag-C<sub>PRVABC-59</sub> amplicons, respectively. This generated pBac/Frag-A<sub>PRVABC-59</sub> to -C<sub>PRVABC-59</sub>. To introduce an SP6 promoter immediately upstream of the first adenine residue of the viral genome, two cDNA
fragments were first amplified individually by (1) PCR of pBACSP6/JVFLx/XbaI with a pair of primers, S123-5sp1F+S123-5sp1R (S123-5sp1R contains the antisense sequence of the SP6 promoter) and (2) PCR of pRs/5’NCRPRVABC-59 [46] with another pair of primers, S23-5sp2F+S23-5sp2R. Subsequently, these two fragments were fused by a second round of PCR with the outer forward and reverse primers S123-5sp1F+S23-5sp2R. The 1025-bp BamHI-SacII fragment of the fused PCR amplicons was ligated with the 2718-bp BamHI-SacII fragment of pRs2, creating pRs/5’SPPRVABC-59. To engineer a unique BarI run-off site just downstream of the last thymine residue of the viral genome, one cDNA fragment was amplified by PCR of pRs/3’NCRPRVABC-59 [46] with primers S23-3roF+S23-3roR (S23-3roR contains the antisense sequence of the BarI and NotI recognition sites in a row). The 649-bp SacII-NotI fragment of the resulting amplicons was ligated with the 2667-bp SacII-NotI fragment of pRs2, creating pRs/3’ROPRVABC-59.

The full-length PRVABC-59 cDNA clone pBac/PRVABC-59 was then assembled by sequentially joining the 7456-bp PacI-NotI fragment of pBACSP6/JVFLx/XbaI with the following five DNA fragments: (1) the 187-bp PacI-Nhel fragment of pRs/5’SPPRVABC-59, (2) the 4426-bp Nhel-EcoNI fragment of pBac/Frag-APRVABC-59, (3) the 2114-bp EcoNI-SacII fragment of pBac/Frag-BPRVABC-59, (4) the 3808-bp SacII-Stul fragment of pBac/Frag-CPRVABC-59, and (5) the 433-bp Stul-NotI fragment of pRs/3’ROPRVABC-59.

A total of five bacterial expression plasmids were constructed, each of which was used to express a 32-to 51-amino acid (aa) non-hydrophobic region of the ZIKV polyprotein as a glutathione-S-transferase (GST) fusion protein. In all cases, a defined region (region-specific) of the ZIKV ORF was amplified by PCR using pBac/PRVABC-
59 as a template and the appropriate pair of primers listed in Table 2-2: (1) Frag-zC (147 199 bp), ZikaC-F+ZikaC-R; (2) Frag-zM (120 bp), ZikaM-F+ZikaM-R; (3) Frag-zE (147 bp), ZikaE- F+ZikaE-R; (4) Frag-zNS4A (177 bp), ZikaNS4A-F+ZikaNS4A-R; and (5) Frag-zNS4B (177 bp), ZikaNS4B-F+ZikaNS4B-R. Each of the resulting amplicons was cloned into pGex-4T-1 (GE Healthcare) by ligating the 4954-bp EcoRI-XhoI fragment of the pGex-4T-1 vector with 135-, 108-, 135-203 , 165-, and 165-bp EcoRI-XhoI fragments of the Frag-zC, -zM, -zE, -zNS4A, and -zNS4B amplicons, respectively. This created pGex-zC, -zM, -zE, -zNS4A, and -zNS4B.

2.4. Transcription and Transfection

Infectious transcripts were synthesized from 3 µg of Psrl/Barl-linearized BAC plasmid DNA with SP6 RNA polymerase [45] in 25-µl reaction mixtures containing 0.6 mM m7GpppA (New England Biolabs) for 1 hour (h) and 20 minutes (min) at 37°C. RNA integrity was examined by agarose gel electrophoresis using a 2-µl aliquot on a 0.6 % gel stained with ethidium bromide. The remaining transcription reaction was immediately stored at -80°C until future use in transfection experiments. RNA was transfected into Vero cells by electroporation using the BTX ECM 830 electroporator with a 2-mm-gap cuvette under optimized conditions (980 V, 99-µs pulse length, and 3 pulses). In brief, sub-confluent Vero cells were trypsinized, washed three times with cold RNase-free phosphate-buffered saline (PBS), and re-suspended at a density of 2×10⁷ cells/ml in PBS. Cell suspensions of 400 µl were then transfected with 2 µg of RNA transcripts by electroporation. RNA infectivity was quantified by infectious center assay. In summary,
Electroporated cells were serially diluted 10-fold and plated on monolayers of untransfected cells (3×10^5 cells/ml) in a six-well plate. Cells were provided 6 h for plate attachment prior to being overlaid with a 0.5% SeaKem LE agarose containing MEM and 10% fetal bovine serum (FBS). Plates were incubated for 4-5 days at 37°C with 5% CO_2 [47, 48]. 5 days after transfection, the agarose overlay was removed and cells were fixed with 7% formaldehyde in PBS followed by three PBS washes. Then cells were permeabilized in 0.25% Triton X-100 in PBS, and infectious centers of plaques were subsequently visualized either nonspecifically by counterstaining of uninfected cells with crystal violet [47] or specifically by immunostaining of ZIKV-infected cells with rabbit anti-NS1 antiserum specific to the Zika virus NS1 protein (α-ZNS1 antiserum) and horseradish peroxidase (HRP)-conjugated goat α-rabbit IgG (Jackson ImmunoResearch), followed by developing with 3,3’-diaminobenzidine [48].

2.5. Growth Kinetics and Cytopathogenicity

Viral growth kinetics and cytopathogenicity were analyzed in 17 animal cell lines from 12 different species. In each case, naïve cells were seeded into 35-mm culture dishes at a density of 3×10^5 cells/dish for 12 hours and then mock-infected or infected with cDNA derived viruses at an MOI of 1 for 1 h at 37°C. Following incubation, cell monolayers were washed and incubated with complete medium. At 6, 12, 18, 24, 36, 48, 60, 72, and 96 hours post-infection (hpi), ZIKV-infected cells were examined morphologically under a light-inverted microscope (Primo Vert, Carl Zeiss) to assess the degree of ZIKV-induced cytopathic effect (CPE) as compared to mock-infected cells, and
culture supernatants were collected to evaluate the levels of virus production by plaque assays on Vero cells [49]. The infectious centers of plaques were visualized at 5 days after infection by counterstaining of uninfected cells with crystal violet [47].

2.6. Real-Time RT-PCR

Total RNAs were extracted by direct lysis of cell monolayers with TRIzol reagent (Invitrogen). ZIKV vgRNA levels in infected Vero cells were quantified [49] by real-time RT-PCR with the primer pairs and fluorogenic TaqMan probes listed in Table 2-2: the ZikaF+ZikaR and ZikaProbe specific for the ZIKV NS3-coding region that has the identical sequences in all three ZIKVs, and the VeroF+VeroR and VeroProbe specific for the Vero β-actin-coding region. Each ZIKV vgRNA level was normalized to the corresponding β-actin mRNA level as an internal control. cDNAs were produced at 50°C for 30 min with Superscript III reverse transcriptase (Invitrogen), followed by enzyme inactivation at 70°C for 15 min. cDNAs were then amplified and detected using the TaqMan gene expression master mix (Applied Biosystems) with the 7500 Fast real-time PCR system (Applied Biosystems). Thermal cycling reactions were carried out under the following conditions: 10 min at 95°C, followed by 45 cycles of 15 seconds (s) at 95°C and 1 min at 60 °C. Relative changes in ZIKV RNA levels were quantified using the $2^{-\Delta\Delta Ct}$ method [50]. Samples were run in duplicate multiple times with the averages represented in the results.
2.7. Immunoblotting, Confocal Microscopy, and Flow Cytometry

Individual ZIKV proteins were identified by immunoblotting [30] using each of the 15 previously characterized JEV region-specific rabbit antisera that have the potential to cross-react with their ZIKV counterparts, or seven newly generated ZIKV rabbit antisera raised with a defined antigen (region-specific) of ZIKV. ZIKV infected cells were lysed, boiled for 5 min, separated by the Glycine- and/or Tricine-SDS-PAGE system, and transferred to a polyvinylidene difluoride membrane using a Trans-Blot SD electrophoretic transfer cell (Bio-Rad). Then the membrane was blocked for 1 h at room temperature (RT), followed by several washes and probed for 2 h at RT using one of the JEV or ZIKV region-specific rabbit antisera. The rabbit antibody was detected using alkaline phosphatase (AP)-conjugated goat α-rabbit IgG (Jackson ImmunoResearch), and the AP enzyme was visualized using colorimetric detection with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma). ZIKV E proteins were visualized using an LSM-710 confocal microscope (Carl Zeiss) [49] with rabbit anti-envelope antiserum specific to the Zika virus E protein (α-ZE antiserum), followed by secondary labeling with fluorescein isothiocyanate (FITC)-conjugated goat α-rabbit IgG (Jackson ImmunoResearch). ZIKV NS4A proteins were detected by flow cytometry [51] with rabbit α-ZNS4A antiserum, followed by secondary labeling with Alexa 488-conjugated goat α-rabbit IgG (Invitrogen). Samples were analyzed on a FACSIAriaIII cell sorter (BD Biosciences).
2.8. *Mouse Studies*

ZIKV neuropathogenicity was examined in male and female mice of four strains: CD-1 (1, 2, and 4 weeks, Charles River), C57BL/6J (4 weeks, the Jackson Laboratory), A129 (4 weeks, bred in-house), and AG129 (4 weeks, bred in-house). Groups of mice were inoculated by intramuscular (IM) (50 µl) or intracerebral (IC) (20 µl) routes, with 10-fold serial dilutions of virus stock in α-minimal essential medium and monitored for any ZIKV-induced clinical signs, weight loss, or death daily for 20 days. The IM and IC LD$_{50}$ values for each virus were calculated by the Reed-Muench method [52] from the respective dose-dependent survival curves of the infected mice. Survival curves were created by the Kaplan-Meier method [53].

All mouse studies were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, United States of America. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Utah State University (approved IACUC protocol #2505). Discomfort, distress, pain and injury were minimized as much as possible through limited handling and euthanization of mice when they were moribund.
3. RESULTS

3.1. Characterization of Three ZIKV Strains

As an initial step in examining the genetic diversity of ZIKV and its biological significance for viral replication and pathogenesis, three historically important strains of distinct geographical and temporal origins were selected: (1) MR-766, the first ZIKV identified from the blood of a rhesus macaque monkey in Uganda in 1947 [1]; (2) P6-740, the first non-African strain, isolated from a pool of *A. aegypti* mosquitoes in Malaysia in 1966 [54]; and (3) PRVABC-59, the recent American strain recovered from the blood of a human patient in Puerto Rico in 2015 [55]. To compare the genome sequence and composition of these three ZIKVs, the consensus nucleotide sequence for each of their full-length vgRNAs was determined [46]. In all three ZIKVs, the vgRNA is 10,807 nt long, with a single ORF of 10,272 nt flanked by a 106- or 107-nt 5’NCR and a 428- or 429-nt 3’NCR (Figure 3-1A). Also, the three vgRNAs all begin with the dinucleotide 5’-AG and end with the dinucleotide CU-3’, both of which are conserved among all mosquito- and tick-borne flaviviruses. However, pairwise sequence comparisons of the three complete genomes showed a considerable degree of genetic diversity, with a range in sequence identity of 89.1-95.6% at the nucleotide level and 96.8-98.8% at the amino acid level over the 3,423-aa polyprotein encoded by the single ORF of the vgRNA (Figure 3-1B).

To examine the genetic relationship between the three spatiotemporally distinct ZIKVs and their associations with other strains, a multiple sequence alignment for
phylogenetic analysis using the nucleotide sequence of all 29 ZIKV genomes (15 complete, 14 near-complete) in GenBank at the time of analysis (June 2016), including the complete nucleotide sequence of the genomes of MR-766, P6-740, and PRVABC-59 was performed. Construction of a genome-based rooted phylogenetic tree using JEV K87P39 as an outgroup revealed two distinct phylogenetic groups (Figure 3-1C), in agreement with previous ORF-based phylogenetic studies that classified 10-40 ZIKV isolates into two major genetic lineages, African and Asian [39-41]. The African lineage branches into two clusters, one including four different versions of the Ugandan MR-766 strain (1947) that are not identical in genome sequence, and the other including the three Senegalese isolates 41671-DAK, 41525-DAK, and 41662-DAK, all isolated in 1984. On the other hand, the Asian lineage contains a single cluster of the Malaysian P6-740 (1966), Cambodian FSS13025 (2010), Philippine CPC-0740 (2012), and Thai SV0127-14 (2014) strains, as well as 18 other isolates collected during the 2015-2016 American epidemic, including the Puerto Rican PRVABC-59 strain (2015). Notably, the four pre-epidemic Asian strains (P6-740, FSS13025, CPC-0740, and SV0127-14) are closely related to the 2015-2016 American epidemic strains, but each forms a single minor branch. Overall, the data indicate that MR-766 belongs to the African lineage, whereas both P6-740 and PRVABC-59 belong to the Asian lineage, with PRVABC-59 being derived from an ancestor of the Asian lineage.
Figure 3-1. A spectrum of ZIKV genetic diversity is represented by three historically important and spatiotemporally distinct strains: MR-766, P6-740, and PRVABC-59. The consensus nucleotide sequence for each of their full-length vgRNAs was determined by sequencing three overlapping uncloned cDNA amplicons collectively representing the entire vgRNA except for the 5’ and 3’ termini, which were subsequently defined by performing both 5’- and 3’-rapid amplification of cDNA ends (RACEs); each of these RACEs was followed by cDNA cloning and sequencing of ~20 randomly picked clones. (A) Genomic organization of the three ZIKV strains. (B) Pairwise comparison of the complete nucleotide (nt) and deduced amino acid (aa) sequences of the three ZIKV genomes. (C) Phylogenetic tree based on the nucleotide sequence of 29 ZIKV genomes, including the 15 complete (MR-766, green; P6-740, orange; PRVABC-59, red; and 12 others, black) and 14 near-complete (gray) genomes, with JEV K87P39 included as an outgroup. Bootstrap values from 1000 replicates are shown at each node of the tree. The scale bar represents the number of nucleotide substitutions per site. The strain name is followed by a description in parenthesis of the country, year, and host of isolation and the GenBank accession numbers. Note that MR-766 has been fully sequenced in this study and by three other groups (designated MR-766/CDC, MR-766/NIID, and MR-766/USAMRIID).
3.2. Development of Full-Length Infectious cDNA Clones

Three full-length infectious ZIKV cDNAs for the MR-766, P6-740, and PRVABC-59 strains, each capable of serving as a template for the rescue of molecularly cloned ZIKVs were constructed (Figure 3-2A). In each strain, the 10,807-nt vgRNA, excluding the 5’ and 3’ termini, was cloned as three overlapping cDNAs of 4.5-5.0 kb into the single-copy BAC vector pBeloBAC11 to ensure the stable maintenance of cloned cDNAs during propagation in *E. coli*. Each of the 5’- and 3’-terminal regions (0.9 and 0.6 kb, respectively) was cloned into the high-copy vector pRS2 to facilitate the introduction of a bacteriophage SP6 promoter immediately upstream of the viral 5’-end and a unique restriction endonuclease recognition site (*PsrI* for MR-766, *BarI* for P6-740 and PRVABC-59) just downstream of the viral 3’-end. Both the SP6 promoter and the unique restriction site were engineered so that *in vitro* run-off transcription could be used to produce m$^7$G-capped synthetic RNAs bearing authentic 5’ and 3’ ends of the vgRNA. In the last cloning step, a set of the five overlapping ZIKV cDNAs was sequentially assembled by joining at four natural pre-existing restriction sites in the viral genome to generate the full-length ZIKV cDNA without introducing any point mutations for cloning. Using this BAC-based cloning strategy, a panel of three full-length ZIKV cDNAs, designated pBac/MR-766, pBac/P6-740, and pBac/PRVABC-59 was created.

To evaluate the functionality of the three full-length ZIKV BACs, the viability of the synthetic RNAs transcribed *in vitro* was determined from each BAC by measuring their specific infectivity after RNA transfection into Vero cells. To prepare a DNA template for *in vitro* run-off transcription, the three full-length ZIKV BACs were first
linearized by digestion with \( Psrl \) (for pBac/MR-766) or \( Barl \) (for pBac/P6-740 and pBac/PRVABC-59). Each was then used as a template for a run-off transcription reaction using SP6 RNA polymerase in the presence of the \( m^7GpppA \) cap structure analog. After removal of the DNA template by DNase I digestion, Vero cells were transfected with the RNA transcripts, quantifying their infectivity as the number of plaque-forming units (PFU) per \( \mu g \) of transfected RNA. In all three BACs, the RNA transcripts invariably had an infectivity of 8.1-8.6\( \times 10^5 \) PFU/\( \mu g \) and were capable of producing a stock titer of infectious ZIKVs in culture medium that reached 1.3-5.0\( \times 10^6 \) PFU/ml at 36 hours (h) after transfection (Figure 3-2B). Each of the three recombinant BAC-derived ZIKVs (designated by the prefix “r”) formed plaques with a consistent size among each strain, with mean diameters of 5.7 (rMR-766), 1.6 (rP6-740), and 5.2 (rPRVABC-59) mm (Figure 3-2C).
Figure 3-2. A trio of functional ZIKV cDNAs is created for the rescue of three molecularly cloned genetically divergent strains: rMR-766, rP6-740, and rPRVABC-59. (A) Construction of three full-length ZIKV cDNAs as BACs for MR-766, P6-740, and PRVABC-59. In all three cases, each vgRNA (top panel) was first subcloned into five overlapping cDNAs (middle panel), which were then joined at four shared restriction sites as indicated to assemble its full-length cDNA (bottom panel). Presented below the three full-length cDNAs are the sequences corresponding to the 5’ and 3’ termini conserved in all three ZIKVs (black lowercase), an SP6 promoter placed just upstream of the viral genome (magenta uppercase), and a run-off site positioned immediately downstream of the viral genome (PsrI or BsrI, blue uppercase). Marked below the sequences are the transcription start (white arrowhead) and run-off (black arrowhead) sites. (B) Functionality of the three full-length ZIKV cDNAs. After linearization with PsrI or BsrI, as appropriate, each full-length cDNA was used as a template for in vitro transcription with SP6 RNA polymerase in the presence of the dinucleotide cap analog m’GppA. Capped RNA transcripts were transfected into Vero cells to determine the number of infectious centers (plaques) counterstained with crystal violet at 5 days after transfection (RNA infectivity). At 36 h post-transfection, culture supernatants from RNA-transfected cells were harvested to estimate the level of virus production by plaque assay on Vero cells (Virus yield). (C) Plaque morphology. The average plaque sizes were estimated by measuring 20 representative plaques.
Full characterization of the reverse genetics system addressed three key aspects that are important for reliable and efficient recovery of infectious viruses from the cloned cDNAs: (1) Specific infectivity requires the *in vitro* run-off transcription of RNA from a full-length ZIKV cDNA (Figure 3-3). Using pBac/PRVABC-59, the full-length cDNA itself was not infectious but was required as the template for transcription, since the presence of DNase I in the transcription reaction eliminated infectivity. After transcription, DNase I treatment had no effect on infectivity, when compared to the intact reaction mixture, but RNase A treatment abolished infectivity. (2) An RNA cap structure is necessary for the full infectivity of *in vitro*-generated RNAs (Figure 3-4). Uncapped RNA transcripts synthesized in the absence of the m\(^7\)GpppA cap structure analog from each of the three full-length ZIKV cDNAs always had an infectivity of 2.6-4.3×10\(^2\) PFU/μg, >3 logs lower than those of their m\(^7\)G-capped RNA counterparts (6.9-8.4×10\(^5\) PFU/μg). (3) Full-length ZIKV BACs are stable during propagation in bacteria (Figure 3-5). Experimentally, a single colony of *E. coli* DH10B carrying each of the three full-length ZIKV BACs was grown in liquid 2xYT medium overnight and then serially passaged for 4 days by diluting it 10\(^6\)-fold daily, such that each passage represented ~20 generations [45]. In all three cases, no differences in specific infectivity of the RNA transcripts made from the BAC plasmids extracted from passages 0, 2, or 4 were observed. In sum, genetically stable BAC-based reverse genetics platforms were established for the recovery of three molecularly cloned, genetically distinct ZIKVs.
Figure 3-3. Infectious RNA is derived by transcription from a full-length ZIKV cDNA clone. The BarI-linearized pBac/PRVABC-59 (250 ng) was used as the template DNA in a 25-μl transcription reaction with SP6 RNA polymerase to synthesize capped RNA in the absence (without treatment) or presence (DNase during) of DNase I. After completion of the transcription, some reaction mixtures were treated with DNase I (DNase after) or RNase A (RNase after) for 30 min at 37°C. A control reaction was carried out in parallel in the absence of SP6 RNA polymerase (without SP6 Pol). (A) DNA/RNA integrity. A 2-μl aliquot of each reaction mixture was separated on a 0.6% agarose gel containing ethidium bromide to visualize the DNA template and RNA transcripts. (B) DNA/RNA infectivity. A 20-μl portion of each reaction mixture was transfected directly into Vero cells, and infectious centers (plaques) were counted after counterstaining with crystal violet at 5 days post-transfection. ND, not detected.
Figure 3-4. A 5’ cap on the in vitro transcribed ZIKV RNA maximizes its specific infectivity. Each of the three Psrl/BarI-linearized full-length ZIKV cDNAs (250 ng), as indicated, was used as a DNA template in a 25-μl transcription reaction with SP6 RNA polymerase in the presence or absence of the dinucleotide cap analog m7GpppA. (A) RNA integrity. A 2-μl aliquot of the reaction mixtures was run on a 0.6% agarose gel containing ethidium bromide to visualize the DNA template and RNA transcripts. (B) RNA infectivity. A 20-μl portion of the reaction mixtures was transfected into Vero cells, and infectious centers (plaques) were counted after counterstaining with crystal violet at 5 days post-transfection.
To test whether the genetic variation in ZIKV can have differential effects on its replicability and cytopathogenicity, monkey kidney-derived Vero cells were infected at a MOI of 1, then the replicative and cytopathic properties of the three cloned cDNA-derived ZIKVs (rMR-766, rP6-740, and rPRVABC-59) were compared to those of the uncloned parental ZIKVs (MR-766, P6-740, and PRVABC-59) used for cDNA construction. In all three strains, there were no noticeable differences between the cloned and uncloned viruses in the accumulation of vgRNA over the first 24 hours post-infection (hpi) (Figure 3-6A), paralleling the kinetics of viral growth and cytopathic effect (CPE)
of the first 3 days post-infection (dpi) (Figure 3-6B) and the average sizes of the anti-ZIKV NS1 (α-ZNS1) antibody-reactive plaques stained at 4 dpi (Figure 3-6C). However, clear differences were observed among the three strains, for both the cloned and uncloned viruses, in their replicability and cytopathogenicity (Figure 3-6A-C): (1) rMR-766/MR-766 displayed the fastest rate of RNA replication, induced complete lysis of the infected cells by 36 hpi, achieved the highest virus titer of $2.0-3.3 \times 10^7$ PFU/ml at 36-48 hpi, and formed the largest plaques of 6.3-mm diameter. (2) rP6-740/P6-740 had the slowest rate of RNA replication, did not cause complete CPE until 72 hpi, reached its maximal virus titer of $1.1-1.2 \times 10^7$ PFU/ml at 60-72 hpi, and generated the smallest plaques of 2.4-mm diameter. (3) rPRVABC-59/PRVABC-59 had a rate of RNA replication slightly slower than rMR-766/MR-766 but faster than that of rP6-740/P6-740; it caused complete CPE by 48 hpi, with a peak virus titer of $0.9-1.4 \times 10^7$ PFU/ml at 36-48 hpi, and produced plaques of 5.9-mm diameter.
Figure 3-6. ZIKV replicability and cytopathogenicity in cell cultures depends on the particular combination of virus strain and host cells. (A-C) Replicative and cytopathic properties of three cloned cDNA-derived ZIKVs (rMR-766, rP6-740, and rPRVABC-59) and their uncloned parental ZIKVs (MR-766, P6-740, and PRVABC-59) in Vero cells. Cells were infected with each of the six ZIKVs (MOI=1). (A) At the time points indicated after infection, cells were lysed to examine the accumulation levels of vgRNA by real-time RT-PCR with a ZIKV-specific fluorogenic probe, (B) and supernatants were collected to analyze the production levels of progeny virions by plaque assay on Vero cells. hpi, hour post infection. At the indicated time points, cells were examined microscopically for the degrees of ZIKV-induced CPE (−, 0%; +, 0-25%; ++, 25-50%; ++++, 50-75%; ++++, 75-100% cell death). (C) At 5 days post-infection, cell monolayers maintained under a semisolid overlay medium were immunostained with rabbit α-ZNS1 antiserum to visualize the infectious plaques.
The replicative and cytopathic potential of the three cDNA-derived ZIKVs were analyzed in 16 other animal cell lines from 11 different species that are potentially relevant to ZIKV pathogenesis and transmission, over the first 4 days after infection of the cells with each virus at an MOI of 1. The data revealed seven distinct patterns of viral growth kinetics and cytopathogenesis, depending on a combination of the viral strain and host cell line (Figure 3-7 and Figure 3-8): (1) In all three human cell types (embryonic kidney HEK, hepatocarcinoma Huh-7, and neuroblastoma SH-SY5Y), rMR-766 and rP6-740 grew equally well, to maximum titers of $10^7$-$10^8$ PFU/ml at 48-72 hpi, but rPRVABC-59 always grew at a slower rate, attaining a peak titer 1-2 logs lower than that of the other two strains at 72-96 hpi (HEK and SH-SY5Y) or reaching a peak titer similar to that of the other two strains only at 96 hpi (Huh-7); all three ZIKVs induced cell death, with a correlation between the degree of CPE and the magnitude of viral replication. (2) In swine testis (ST) and equine skin (NBL-6) cells, the three ZIKVs replicated to their peak titers of $10^6$-$10^7$ PFU/ml at 48 hpi, with differential growth rates similar to those seen in Vero cells (rMR-766, fastest; rP6-740, slowest; rPRVABC-59, intermediate) that paralleled the kinetics of CPE development. (3) In sheep fetal fibroblast (SFF-6) and A. albopictus (C6/36) cells, the three ZIKVs shared a superimposable growth curve, characterized by a steady increase in virus titers up to $\sim 10^7$ PFU/ml by 96 hpi, except for rP6-740, which had an exponential growth during 24-48 hpi in C6/36, but not SFF-6 cells. None of the three ZIKVs produced any visible CPE. (4) In goat fetal fibroblast (GFF-4), canine kidney (MDCK), and feline kidney (CRFK) cells and in all three mouse cell types (C57BL/6-derived embryonic fibroblast MEF, NIH/Swiss-derived embryonic
fibroblast NIH/3T3, and motor neuron-like hybrid NSC-34), rMR-766 was the fastest-growing, reaching its highest titer of $10^6$-$10^7$ PFU/ml at 48-96 hpi; rPRVABC-59 was the slowest-growing, gaining a maximum titer of only $10^3$-$10^4$ PFU/ml during the same period; and rP6-740 was intermediate in growth rate. However, none of these viruses produced visible CPE. (5) In chicken embryo fibroblast (CEF) cells, both rMR-766 and rP6-740 had a relatively long lag period of 36 h, followed by a gradual increase in virus titer up to $10^5$-$10^6$ PFU/ml by 96 hpi; in contrast, rPRVABC-59 grew extremely poorly, resulting in a slow decrease in virus titer to 45 PFU/ml by 96 hpi. No CPE was observed for any of the three ZIKV-infected CEF cells. (6) In bovine turbinate (BT) cells, the three ZIKVs showed substantial differences in growth kinetics, reaching a plateau at 96 hpi, with peak titers of $4.4\times10^5$ (rMR-766), $5.0\times10^4$ (rPRVABC-59), and $8.8\times10^2$ (rP6-740) PFU/ml. However, no visible CPE was induced in any of the ZIKV-infected BT cells. (7) In bovine kidney (MDBK) cells, the titers of all three ZIKVs declined to undetectable levels at 60-96 hpi, with no observable signs of viral CPE.
Figure 3-7. Replicability and cytopathogenicity of the three cloned cDNA-derived ZIKVs in a wide range of animal cells. Each virus was used to infect the cell lines (MOI=1) specified in the figure. At the indicated time points, cells were examined microscopically for the degrees of ZIKV-induced CPE (–, 0%; +, 0-25%; ++, 25-50%; ++++, 50-75%; +++++, 75-100% cell death), and supernatants were assayed for virus production by plaque assay on Vero cells. hpi, hour post-infection.
Figure 3-8. ZIKV replicability and cytopathogenicity in cell cultures depends on the particular combination of virus strain and host cells. Each of three molecularly cloned ZIKVs (rMR-766, rP6-740, and rPRVABC-59) was used to infect the indicated cells at an MOI of 1. At the time points marked in the figure, cells were examined microscopically to determine the degrees of ZIKV-induced CPE (−, 0%; +, 0-25%; ++, 25-50%; ++++, 50-75%; ++++, 75-100% cell death), and supernatants were harvested to evaluate the levels of virus production by plaque assay on Vero cells. hpi, hour post-infection.
Although MDBK cells were not susceptible to ZIKV infection, they were shown to be permissive for ZIKV RNA replication, by using (1) single cell-based immunofluorescence (Figure 3-9A) and flow cytometry (Figure 3-9B) assays to determine the number of cells expressing ZIKV proteins (E or NS4A), when MDBK cells were either infected with each of the three cDNA-derived ZIKVs or transfected with each of the three infectious RNAs transcribed \textit{in vitro} from their corresponding cDNAs; and (2) total cell lysate-based immunoblot analyses to assess the accumulation levels of ZIKV NS1 protein in the virus-infected vs. RNA-transfected MDBK cells (Figure 3-9C). In all these experiments (Figure 3-9 A-C), a ZIKV-susceptible cell line (Vero) was used as a control. The results postulate that MDBK cells might lack one or more host factors required for ZIKV entry; alternatively, they might have a general defect in the clathrin-dependent endocytic pathway that ZIKV utilizes for internalization [56]. Thus, the functional integrity of the clathrin-dependent endocytic pathway in MDBK cells was investigated, by analyzing the susceptibility of these cells to infection by two other enveloped RNA viruses whose entry depends on clathrin-mediated endocytosis: bovine viral diarrhea virus (BVDV) and vesicular stomatitis virus (VSV). In contrast to their resistance to ZIKV infection, MDBK cells were highly susceptible to infection with both BVDV and VSV, as demonstrated by their plaque formation and high level of progeny virion production (Figure 3-10). These results indicate that the cellular machinery associated with the clathrin-dependent endocytic pathway is functional in MDBK cells, and they support the hypothesis that MDBK cells lack a host factor(s) promoting ZIKV entry.
Figure 3-9. MDBK cells are permissive for ZIKV RNA replication but are not susceptible to infection with the virus. MDBK cells were mock-infected or infected with rMR-766, rP6-740, or rPRVABC-59 at an MOI of 3 (for virus infection experiments), or mock-transfected or transfected with 3 μg of synthetic RNAs transcribed in vitro from their respective infectious cDNAs (for RNA transfection experiments). At the indicated time points, the expression of three ZIKV proteins (E, NS1, and NS4A) within the cells was analyzed by confocal microscopy for E (A), flow cytometry for NS4A (B), and immunoblotting for NS1 (C). The insets in panel A show enlarged views of the boxed areas with the fluorescence of propidium iodide (PI)-stained nuclei excluded. In all experiments, ZIKV-susceptible Vero cells were included in parallel. hpi, hour post-infection; hpt, hour post-transfection.
3.4. Viral Gene Products and Their Related Species

To identify all viral proteins produced by rMR-766, rP6-740, and rPRVABC-59, total cell lysates of mock- and ZIKV-infected Vero cells were examined in two series of immunoblotting experiments. The first series, probed with 15 JEV region-specific rabbit antisera (Figure 3-11), originally produced to detect all JEV gene products [30], which were estimated to have the potential for cross-reactivity with their ZIKV counterparts, given the significantly high levels (35-71%) of amino acid sequence identity between

![Figure 3-10](image.png)

**Figure 3-10.** MDBK cells are highly susceptible to infection with both BVDV and VSV. MDBK cells were infected at an MOI of 1 with BVDV (strain NADL) or VSV (strain Indiana). (A) Viral replication. At 36 h post-infection, culture supernatants were tested to measure the infectious virus yields by plaque assay on MDBK cells. (B) Representative plaques. At 3 days post-infection, cell monolayers maintained under a semisolid overlay medium were counterstained with crystal violet to visualize the infectious plaques.
their antigenic regions (Figure 3-12A). Indeed, six (α-JE<sup>N-term</sup>, α-JNS1<sup>C-term</sup>, α-JNS2B, α-JNS3<sup>C-term</sup>, α-JNS5<sup>N-term</sup>, and α-JNS5<sup>C-term</sup>) of the 15 antisera showed moderate-to-strong cross-reactivity with their respective ZIKV gene products, but the remaining nine had no reactivity (Figure 3-12B). To cover the remaining undetected parts of ZIKV ORF, seven ZIKV region-specific rabbit antisera were generated, using rPRVABC-59 as the viral strain of choice (Figure 3-13), immunizing the rabbits with five bacterially expressed glutathione-S-transferase (GST) fusion proteins (α-ZC, α-ZM, α-ZE, α-ZNS4A, and α-ZNS4B) or two chemically synthesized oligopeptides (α-ZNS1 and α-ZNS2B). In all cases, the 19- to 51-aa antigenic regions of ZIKV were selected to have relatively low levels (16-42%) of amino acid sequence identity with those of JEV (Figure 3-14A). The resulting seven ZIKV region-specific antisera were used for a second series of immunoblots, in which their respective ZIKV gene products were detected (Figure 3-14B). In all immunoblots, two additional cell lysates were included (as a reference for JEV proteins) from Vero cells infected with the virulent JEV strain SA<sub>14</sub> or its attenuated strain SA<sub>14-14-2</sub>; both JEVs share the same genome-wide viral protein expression profile, except that the NS1' protein is expressed only by SA<sub>14</sub> [49].
Figure 3-11. Details of the 15 JEV region-specific rabbit antisera used to detect their antigenically cross-reactive ZIKV counterparts. A collection of 15 rabbit antisera covering nearly all parts of the JEV protein-coding regions were raised by immunization with 14 E. coli-expressed glutathione-S-transferase (GST) fusion proteins (α-JC, α-JPr, α-JM, α-JEN-term, α-JEC-term, α-JNS1N-term, α-JNS1C-term, α-JNS1FS, α-JNS3N-term, α-JNS3C-term, α-JNS4A, α-JNS4B, α-JNS5N-term, and α-JNS5C-term) or with a keyhole limpet hemocyanin (KLH)-conjugated synthetic oligopeptide (α-JNS2B). The nucleotide (nt) and amino acid (aa) positions of the viral antigenic regions (blue) are based on the complete genomic sequence of JEV SA14 (GenBank accession no. KU323483; see also Figure 3-12A). The working dilutions of the rabbit antisera used in our study are given.
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<th>Dilution</th>
<th>Antigen: Synthetic Peptide</th>
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**Figure 3-12.** A sub-set of 15 JEV region-specific polyclonal antibodies detects the cross-reactive ZIKV E, NS1, NS2B, NS3, NS5, and their related species in ZIKV-infected cells. (A) Schematic illustration showing the antigenic regions recognized by 15 JEV region-specific rabbit antisera. The 10,977-nt vgRNA of JEV SA\textsubscript{14} has a 95-nt 5’NCR, a 10,299-nt ORF, and a 583-nt 3’NCR (top panel). The ORF encodes a 3,432-aa polyprotein that is processed by viral and cellular proteases into at least 10 mature proteins (middle panel). Marked on the polyprotein are one or two transmembrane domains (vertical black bar) at the C-termini of three structural proteins (C, prM, and E) and at the junction of NS4A/NS4B, as well as four N-glycosylation sites (asterisk) in the pr portion of prM (\textsuperscript{15}NNT), E (\textsuperscript{154}NYS), and NS1 (\textsuperscript{130}NST and \textsuperscript{207}NDT). During viral morphogenesis, prM is cleaved by furin protease into a soluble pr peptide and a virion-associated M protein. NS1’ is the product of a -1 ribosomal frameshift (F/S) event that occurs at codons 8-9 of NS2A, adding a 52-aa C-terminal extension to the NS1 protein. The bottom panel displays the antigenic regions (horizontal blue bar) recognized by 15 JEV region-specific rabbit antisera and the aa sequence identities (% in parentheses) between the corresponding JEV SA\textsubscript{14} and ZIKV PRVABC-59 regions. (B) Identification of viral proteins in ZIKV-infected cells by immunoblotting. Vero cells were mock-infected or infected at MOI 1 with each of three ZIKVs (rMR-766, rP6-740, and rPRVABC-59) or two JEVs (SA\textsubscript{14} and SA\textsubscript{14}-14-2, for reference). At 20 h post-infection, total cell lysates were separated by SDS-PAGE on a glycine (Gly) or tricine (Tri) gel and analyzed by immunoblotting with each of the 15 JEV region-specific rabbit antisera or \alpha-GAPDH rabbit antiserum as a loading and transfer control. Molecular size markers are given on the left of each blot, and major JEV proteins for reference are labeled on the right. Provided below each blot are the estimated molecular sizes of the predicted ZIKV proteins, and marked on the blot are the predicted (yellow or pink dot) and unexpected (white circle) proteins. CHO, N-glycosylation.
Figure 3-13. Details of the seven ZIKV region-specific rabbit antisera used to identify ZIKV gene products and their related species. (A) A panel of seven rabbit antisera, each recognizing a 19- to 51-aa region defined in the ZIKV protein-coding sequences, were generated using five *E. coli*-expressed glutathione-S-transferase (GST) fusion proteins (α-ZC, α-ZM, α-ZE, α-ZNS4A, and α-ZNS4B) or two keyhole limpet hemocyanin (KLH)-conjugated synthetic oligopeptides (α-ZNS1 and α-ZNS2B) as antigens. The nucleotide (nt) and amino acid (aa) positions of the viral antigenic regions (magenta) are based on the complete genomic sequence of ZIKV PRVABC-59 (GenBank accession no. KX377337; see also Figure 3-14A). The working dilutions of the rabbit antisera used in this study are presented. (B) Production of five GST-tagged recombinant proteins. GST fusion proteins were expressed from pGex-4T-1 vector in *E. coli* BL21 and purified from bacterial lysates by affinity chromatography using glutathione-Sepharose. Purified proteins were resolved by SDS-PAGE on a glycine gel and stained with Coomassie blue.
Figure 3-14. A panel of seven ZIKV region-specific polyclonal antibodies identifies ZIKV C, prM/M, E, NS1, NS2B, NS4A’, NS4B, and their related species in ZIKV-infected cells. (A) Schematic illustration showing the antigenic regions recognized by seven ZIKV region-specific rabbit antisera. The 10,807-nt vgRNA of ZIKV PRVABC-59 consists of a 107-nt 5’NCR, a 10,272-nt ORF, and a 428-nt 3’NCR (top panel). The ORF encodes a 3,423-aa polyprotein that is predicted to be cleaved by viral and cellular proteases into at least 10 mature proteins (middle panel). Marked on the polyprotein and its products are one or two transmembrane domains (vertical black bar) at the C-termini of three structural proteins (C, prM, and E) and at the junction of NS4A/NS4B, as well as four N-glycosylation sites (asterisk) in the pr portion of prM (70NTT), E (154NDT), and NS1 (130NNS and 207NDT). The bottom panel shows the antigenic regions (horizontal magenta bar) recognized by seven ZIKV region-specific rabbit antisera and the aa sequence identities (% in parentheses) between the corresponding ZIKV PRVABC-59 and JEV SA14 regions. (B) Identification of viral proteins in ZIKV-infected cells by immunoblotting. Vero cells were mock-infected or infected at MOI 1 with each of three ZIKVs (rMR-766, rP6-740, and rPRVABC-59) or two JEVs (SA14 and SA14-14-2, for comparison). At 20 h post-infection, total cell lysates were separated by SDS-PAGE on a glycine (Gly) or tricine (Tri) gel and analyzed by immunoblotting with each of the seven ZIKV region-specific rabbit antisera. Molecular size markers are given on the left of each blot, and major ZIKV proteins are labeled on the right. Provided below each blot are the estimated molecular sizes of predicted ZIKV proteins, and marked on the blot are the predicted (yellow or pink dot) and unexpected (white circle) proteins. CHO, N-glycosylation.
The immunoblot analysis used a battery of 13 ZIKV antigen-reactive region-specific rabbit antisera to create a full catalog of viral gene products and their related species, except for the predicted 24-kDa NS2A (Figure 3-12 and Figure 3-14): (1) α-ZC recognized the 13-kilodalton (kDa) C protein, with no accumulation of the further-processed 12-kDa C’ (see below for description of virion-associated proteins), but with appearance of one or two cleavage products of 10-11 kDa in rPRVABC-59- or rP6-740-infected cells, respectively; however, this antiserum did not react with any of the C-related proteins of rMR-766. (2) α-ZM reacted strongly with the 9-kDa M protein and its 24-kDa precursor prM, with the ratio of M:prM varying, depending on the viral strain; the observed size of prM was 5 kDa larger than its predicted size, consistent with an addition of N-glycans at Asn-70 (70NTT) to its pr domain [57] that is conserved in all three ZIKVs. Also, the α-ZM reacted weakly with at least two minor proteins of 15 and 19 kDa. (3) α-JE N-term/α-ZE detected four E-related proteins (of 54/56, 43/45, 24/26, and 14 kDa). Among these, the first three proteins from rP6-740 were all 2 kDa smaller than those from rMR-766 and rPRVABC-59, in agreement with a missense mutation of the N-glycosylation site at Asn-154 (154NDT→NDI) in the E protein of rP6-740 relative to that of rMR-766 and rPRVABC-59 [19, 20]. Notably, the full-length 54/56-kDa E protein on the gel was pushed down by a large amount of cellular protein migrating just above it, forcing it to run faster than its actual size. (4) Both α-JNS1 C-term and α-ZNS1 identified the 45-kDa NS1 exclusively. This protein was 5 kDa larger than predicted by its amino acid sequence because of the addition of N-glycans at Asn-130 (130NNS) and Asn-207 (207NDT), both of which are conserved in all three ZIKVs [58, 59]. These data also
showed that only NS1, and not its frameshift product NS1’, was produced by all three ZIKVs. The lack of NS1’ expression corroborated the finding that ZIKV lacks the two-component ribosomal frameshift signal found in JEV and WNV (Figure 3-15). (5) \(\alpha\)-JNS2B/\(\alpha\)-ZNS2B revealed the 14-kDa NS2B, together with an unexpected protein of 11 kDa at a barely detectable level. (6) \(\alpha\)-JNS3\textsuperscript{C-term} recognized the 69-kDa NS3; it also reacted more strongly with a major cleavage product of 34 kDa, representing the C-terminal half of the full-length NS3 [30], and less intensely with at least seven minor proteins of 33-60 kDa. Intriguingly, \(\alpha\)-JNS3\textsuperscript{C-term} detected a species with a mass of 85 kDa, corresponding to the calculated size of an NS2B-3 or NS3-4A/4A’ processing intermediate. (7) \(\alpha\)-ZNS4A did not detect the predicted 16-kDa NS4A but did predominantly recognize its further-processed 14-kDa NS4A’, which ran as a single species in tricine-SDS-PAGE but migrated as a doublet in glycine-SDS-PAGE. Unexpectedly, this antiserum also identified two clusters of multiple protein bands, one at 29 kDa (NS4A\textsuperscript{p29}) and the other at 35 kDa (NS4AB\textsuperscript{p35}, which also reacted with \(\alpha\)-ZNS4B; Figure 3-16). (8) \(\alpha\)-ZNS4B stained the predicted major 27-kDa NS4B, along with two unexpected minor proteins at 11 kDa (NS4B\textsuperscript{p11}) and 35 kDa (NS4AB\textsuperscript{p35}, which again reacted with \(\alpha\)-ZNS4A; Figure 3-16). (9) \(\alpha\)-JNS5\textsuperscript{N-term} and \(\alpha\)-JNS5\textsuperscript{C-term} reacted with the predicted 103-kDa NS5.
Figure 3-15. ZIKV lacks the ribosomal frameshift signal directing the expression of NS1'. (A) Nucleotide sequence alignment for seven major mosquito-borne flaviviruses (17 strains total). The consensus sequence is shown on top, and residues that match the consensus are hidden as dots to emphasize residues that differ from the consensus. (B) Predicted RNA folding involved in JEV NS1' frameshifting and its ZIKV counterpart. RNA secondary structures with pseudoknots are predicted using the IPknot program. Highlighted are the primary sequences and secondary structures important for the expression of JEV NS1': the heptanucleotide slippery sequence (blue), stem-loop 1 (SL1, orange), stem-loop 2 (SL2, magenta) and pseudoknot base-pairing (green). Also, indicated is the silent point mutation G3599A (red circle) that is sufficient to abolish the synthesis of JEV SA14 NS1'.
Figure 3-16. Multiple NS4A- and NS4B-related proteins are accumulated in ZIKV-infected cells. Vero cells were mock-infected or infected at an MOI of 1 with each of three ZIKVs (rMR-766, rP6-740, and rPRVABC-59) or two JEVs (SA14 and SA14-14-2). (A) At 20 h post-infection, total cell lysates were separated by glycine- or tricine-SDS-PAGE and analyzed by immunoblotting with α-ZNS4A or α-ZNS4B. (B) Two sets of the same lysates from mock- and rPRVABC-59-infected Vero cells were run side-by-side in a glycine or tricine gel and transferred to a single membrane. The membrane was split into two parts, each stained with either α-ZNS4A or α-ZNS4B. In parallel, an aliquot of the same rPRVABC-59-infected cell lysate was also included in between the two sample sets, and the corresponding membrane strip was probed with a mixture of both α-ZNS4A and α-ZNS4B to serve as a reference for all the immunoreactive proteins. Provided below the blot are the molecular weights of predicted ZIKV NS4A, NS4A’ and NS4B proteins, and marked on the blot are the predicted (yellow or pink dot) and unexpected (white circle) proteins.
In addition to the three full-length structural proteins (C, prM/M, and E) of ZIKV, their multiple smaller products were accumulated to lower but still significant amounts in Vero cells infected with each of the three ZIKVs, with nearly the same protein expression profile (Figure 3-14). To define the actual viral structural proteins incorporated into ZIKV particles, rPRVABC-59 was used to profile all the structural proteins associated with extracellular virions, which were purified by pelleting through a 20% sucrose cushion. Then a comparison of the extracellular virion-associated proteins with their cell-associated counterparts was shown by immunoblotting with α-ZC, α-ZM, and α-ZE (Figure 3-17). The purified ZIKV particles were shown to contain (1) the 12-kDa C’ protein, which appeared as a closely spaced doublet with the lower band being more prominent than the upper band and migrating in a gel marginally faster than one cell-associated major 13-kDa C protein, but slower than the other cell-associated minor 10-kDa C-derived cleavage product; (2) the 9-kDa M protein and a trace amount of its glycosylated precursor prM, which appeared as two bands, the slightly less intense and faster one migrating with a mass of 23-24 kDa and the slightly more intense and slower one at 25-26 kDa, reflecting the trimming of high mannose and the addition of more complex sugars to the cell-associated 24-kDa prM protein during virus release through the cellular secretory pathway [60]; and (3) the glycosylated 58-kDa E protein, which ran slightly slower than the cell-associated 56-kDa E protein, again reflecting the difference in its glycosylation status. Collectively, these results demonstrate the extracellular ZIKVs to be composed of three post-translationally modified full-length structural proteins, excluding their smaller species.
Figure 3-17. Profiling of virion-associated ZIKV proteins compared to their cell-associated counterparts. Vero cells were left uninfected (Uninf) or infected (Inf) with ZIKV rPRVABC-59 at an MOI of 1. For cell-associated viral proteins, total cell lysates were prepared by lysing the cell monolayers at 20 h post-infection. For virion-associated viral proteins, cell culture supernatants were collected at the same time point, and extracellular virions were pelleted by ultracentrifugation through a 20% sucrose cushion. Equivalent portions of total cell lysates and pelleted virions were resolved by SDS-PAGE on a glycine (Gly) or tricine (Tri) gel and analyzed by immunoblotting with α-ZC, α-ZM, or α-ZE. Molecular weight markers are shown on the left of each blot. The molecular weights of predicted C, C’, prM, M, and E proteins are indicated below each blot. Marked on each blot are the predicted (yellow or pink dot) and unexpected (white circle) proteins. CHO, N-glycosylation.

3.5. Neuropathogenicity in Outbred CD-1 Mice

The virulence of rMR-766, rP6-740, and rPRVABC-59 was compared in CD-1 mice at three different ages (1, 2, and 4 weeks) by examining two neuropathogenic properties: (1) neuroinvasiveness (the ability to penetrate the central nervous system from a peripheral site), quantified by generating the dose-dependent survival curve and
determining the lethal dose 50\% (LD\textsubscript{50}) after an intramuscular (IM) inoculation; and (2) neurovirulence (the ability to establish a lethal infection within the central nervous system), quantified by creating the dose-dependent survival curve and measuring the LD\textsubscript{50} after an intracerebral (IC) inoculation. For both IM and IC inoculations, the appropriate dose ranges for calculating the LD\textsubscript{50} values were determined, to optimize the study designs prior to the performance of full-scale experiments. For these pilot experiments, all three age groups of the mice were injected with a maximum dose of each virus: \(1.2 \times 10^5\) PFU/mouse for IM inoculations and \(3.6 \times 10^4\) PFU/mouse for IC inoculations. If necessary, a series of large-scale dose-response studies was performed, inoculating groups of the mice at 1, 2, and 4 weeks of age via the IM or IC route with serial 10-fold dilutions of the virus. Following infection, the mice were monitored daily for mortality, weight loss, and other clinical signs of illness over 20 days.

Comparative assessments of the dose-dependent survival curves and LD\textsubscript{50} values revealed the following (Figure 3-18A): (1) rMR-766 exhibited age-dependent neuroinvasiveness, as evidenced by an IM LD\textsubscript{50} of 90.2 PFU for 1-week-old mice and \(>1.2 \times 10^5\) PFU for 2- and 4-week-old mice, yet it displayed a high level of neurovirulence at all three ages, as evidenced by an IC LD\textsubscript{50} of \(<3.6, 3.6, \text{and } 5.7\) PFU for 1-, 2-, and 4-week-old mice, respectively. (2) rP6-740 showed barely detectable neuroinvasiveness in 1-week-old mice, with only 1 or 3 of 10 infected mice dying when inoculated with the two highest doses, \(3.6 \times 10^3\) or \(3.6 \times 10^4\) PFU/mouse, respectively (IM LD\textsubscript{50}, \(>3.6 \times 10^4\) PFU). Similarly, it had no detectable neuroinvasiveness in 2- and 4-week-old mice, with no infected mice dying even when inoculated with the highest dose, \(1.2 \times 10^5\) PFU/mouse.
However, rP6-740 showed age-dependent neurovirulence, as it was highly neurovirulent in 1-week-old mice (IC LD50, <3.6 PFU) but non-neurovirulent in 2- and 4-week-old mice (IC LD50, >3.6×10^4 PFU). (3) rPRVABC-59 was essentially non-neuroinvasive and non-neurovirulent, regardless of the mouse age, with its IM and IC LD50 values estimated to be greater than the highest dose used for each route of infection, without a single death. Of the three ZIKVs, therefore, rMR-766 was the most virulent, rPRVABC-59 was the least virulent, and rP6-740 showed intermediate virulence.

Moreover, not only the lethal virulence displayed by rMR-766 and rP6-740 was recognized, but also the non-lethal virulence exhibited by all of the three ZIKVs, including rPRVABC-59. This effect was most prominent in 1-week-old mice (Figure 3-18B). The lethal virulence was invariably associated with a sharp drop in the body weight of infected mice that began ~3 days prior to death, in conjunction with clinical signs. It began with decreased activity, ruffled fur, and hunched posture, and often progressed to tremors and hind limb paralysis. Various viral loads were detected postmortem in the brains of all mice that died (8.0×10^3-3.9×10^8 PFU/brain). Non-lethal virulence, in contrast, was characterized by an initial weight loss of various degrees, albeit without obvious clinical signs, and a subsequent recovery to some extent that was not complete. At the end of the study, no infectious ZIKV was detected in the brains of any of the mice that survived. In both the lethal and non-lethal virulent cases, no changes in body temperature were observed. Altogether, in CD-1 mice, the three ZIKVs had a wide range of virulence, depending on the virus strain, mouse age, and route of infection.
Figure 3-18. Three molecularly cloned ZIKVs display a full range of variation in neuropathogenicity for outbred CD-1 mice in an age-dependent manner. Groups of CD-1 mice (n = 8-10, half male, half female) were mock-inoculated or inoculated at 1, 2, and 4 weeks of age via the intramuscular (IM) or intracerebral (IC) route with a maximum dose of $3.6 \times 10^4$ or $1.2 \times 10^5$ PFU, or serial 10-fold dilutions of rMR-766, rP6-740, or rPRVABC-59. (A) Survival curves were generated by the Kaplan-Meier method, and LD$_{50}$ values were determined by the Reed-Muench method and are presented in the bottom left corner of each curve. (B) Weight changes are plotted, with each mouse represented by one color-coded line. dpi, days post-infection.
To compare the contributions of the host IFN response to the virulence of rMR-766, rP6-740, and rPRVABC-59, their neuroinvasiveness and neurovirulence were examined using groups of 4-week-old A129 (IFNAR−/−) mice and groups of age-matched wild-type inbred C57BL/6J mice as a control (Figure 3-19A). In the control mice, rMR-766 was non-neuroinvasive (IM LD$_{50}$, >1.2×10$^5$ PFU) but neurovirulent (IC LD$_{50}$, 7.8 PFU). In contrast, both rP6-740 and rPRVABC-59 were non-neuroinvasive (IM LD$_{50}$, >1.2×10$^5$ PFU) as well as non-neurovirulent (IC LD$_{50}$, >3.6×10$^4$ PFU), in agreement with the data obtained in age-matched outbred CD-1 mice (Figure 3-18A). In A129 mice, however, the neurovirulence of all three ZIKVs was increased dramatically, and they became highly neurovirulent (IC LD$_{50}$, <3.6 PFU), with median survival times estimated to be 4 (rMR-766), 5 (rP6-740), and 7 (rPRVABC-59) days, with a lethal dose of 3.6×10$^2$ PFU/mouse. Similarly, the neuroinvasiveness of the three ZIKVs was also elevated but to different degrees, as evidenced by the estimated IM LD$_{50}$ of <1.2 (rMR-766), 576.1 (rP6-740), and >1.2×10$^5$ (rPRVABC-59) PFU. Noticeably, rPRVABC-59 was nearly non-neuroinvasive in A129 mice. This finding prompted further testing to determine the neuroinvasiveness of rPRVABC-59, as compared to that of the other two ZIKVs, in 4-week-old AG129 (IFNAR−/−/IFNGR−/−) mice (Figure 3-19A). In AG129 mice, all three ZIKVs were highly neuroinvasive (IM LD$_{50}$, <1.2 PFU), although the median survival times for the three viruses varied from 7 (rMR-766) to 12 (rP6-740) and 13 (rPRVABC-59) days, with a lethal dose of 1.2×10$^2$ PFU/mouse. Furthermore, in all three mouse strains (C57BL/6J, A129, and AG129), the two LD$_{50}$-based neuropathogenic properties of
the three ZIKVs were always corroborated by the decreases in body weight (Figure 3-19B), accompanied by the typical clinical signs seen in CD-1 mice. In all the mice that died, various viral loads were detected in their brains postmortem, with higher loads being found in the absence of IFN signaling, i.e., $4.7 \times 10^4 - 2.0 \times 10^8$ PFU/brain for C57BL/6J, $1.3 \times 10^6 - 1.0 \times 10^9$ PFU/brain for A129, and $8.5 \times 10^6 - 3.6 \times 10^9$ PFU/brain for AG129. In the case of all mice that survived, however, no infectious ZIKV was detected in the brain at the end of the study. Taken together, these data show a full range of variation in IFN sensitivity among the three cloned ZIKVs in mice.
Figure 3-19. Three molecularly cloned ZIKVs show a full spectrum of variation in IFN sensitivity in mice lacking type I or both type I and II IFN receptors. Groups of 4-week-old C57BL/6J (n = 8), A129 (n = 5), or AG129 (n = 5) mice, approximately half of each sex, were mock-inoculated or inoculated through the intramuscular (IM) or intracerebral (IC) route with a maximum dose of $3.6 \times 10^4$ or $1.2 \times 10^5$ PFU, or serial 10-fold dilutions of rMR-766, rP6-740, or rPRVABC-59. (A) Survival curves were created by the Kaplan-Meier method, and LD$_{50}$ values were calculated by the Reed-Muench method and are given in the bottom left corner of each curve. (B) Weight changes are plotted, with each mouse indicated by one color-coded line. NT, not tested; dpi, days post-infection.
4. DISCUSSION

This report highlights the development of three full-length infectious ZIKV cDNAs as BACs for each of three spatiotemporally distinct and genetically divergent ZIKV strains [46]: MR-766 (Uganda, 1947), P6-740 (Malaysia, 1966), and PRVABC-59 (Puerto Rico, 2015). Additionally, 13 ZIKV region-specific polyclonal rabbit antisera capable of identifying all the viral structural and nonstructural proteins and their related species, except for NS2A were produced. The functional cDNAs and antibodies in combination with various cell culture and murine model systems, have demonstrated that the three molecularly cloned cDNA-derived ZIKVs have nearly the same genome-wide viral protein expression profile but differ dramatically in their replicability and neuropathogenicity (neuroinvasiveness and neurovirulence), depending on the particular combination of viral and host genetic backgrounds, as well as in the presence or absence of type I/II IFN signaling. In particular, the results demonstrate that type I IFN regulates ZIKV neuroinvasiveness in a virus strain-dependent manner. In all, these reagents offer a new toolbox for viral genome engineering and protein analysis. Together with a roster of in vitro and in vivo infection models, these tools will not only provide an ideal platform for defining the viral and host genetic factors that contribute to ZIKV replication and pathogenesis at the cellular and organismic levels but also offer promising new avenues for developing and testing an effective, critically needed vaccine or antiviral against ZIKV.
The advent of functional cDNA-based reverse genetics has revamped the field of RNA viruses [61]. For flaviviruses, however, the cloned cDNAs are commonly unstable because of the toxicity of their prM-E genes in host cells, posing a major technical challenge to functional cDNA construction [62]. In the present study, a complete cDNA copy of the ZIKV vgRNA was cloned into a BAC vector that is capable of stably housing a DNA fragment of >300 kb in bacteria [63], and has previously been done for JEV [45, 51]. In the case of all three ZIKVs (MR-766, P6-740, and PRVABC-59), the structural and functional integrity of their full-length BACs remained stable for at least 80 generations of growth in *E. coli*. To date, the BAC cloning technology has been applied to constructing full-length infectious cDNAs for ~10 members of three plus-strand RNA virus families (*Flaviviridae*, *Arteriviridae*, and *Coronaviridae*), all of which have a large genome size of 11-31 kb [44]. Moreover, site-directed mutagenesis to introduce a point mutation(s) into each of the three infectious ZIKV cDNAs was performed (data not shown), indicating that targeted mutations can be engineered by manipulating the infectious ZIKV BACs in *E. coli*. Thus, this BAC-based reverse genetics for ZIKV will facilitate genetic studies of both viral RNA elements and gene products associated with all aspects of ZIKV biology.

A strategy was formulated to assemble three full-length infectious ZIKV cDNAs, each capable of generating m⁷G-capped *in vitro*-transcribed RNAs identical in nucleotide sequence to their respective vgRNAs, particularly regarding the 5’- and 3’-end sequences. On the 5’ side, an SP6 promoter sequence (5’-ATTTAGGGGACACTATA, with transcription starting at the underlined G) was positioned upstream of the first
adenine nucleotide of the viral genome to incorporate the dinucleotide cap analog m⁷GpppA in SP6 RNA polymerase-driven in vitro transcription reactions. The importance of an m⁷G cap at the 5’-end of transcribed RNAs in maximizing RNA infectivity was shown by uncapped RNAs derived from each of the three functional ZIKV cDNAs always having an infectivity >3 logs lower than that of their m⁷G-capped counterparts. On the 3’ side, a unique restriction endonuclease recognition sequence, Psrl [(N₇↓N₁₂)GAACN₆TAC(N₁₂↓N₇)] or Barl [(N₇↓N₁₂)GAAGN₆TAC(N₁₂↓N₇)], was placed downstream of the last thymine nucleotide of the viral genome. The use of Psrl/Barl for cDNA linearization is particularly advantageous because both are extremely rare-cutting endonucleases that cut out their recognition sequences after any nucleotide, which makes this approach applicable for all plus-strand RNA viruses, regardless of the identity of the nucleotide at the 3’ end of the viral genome. RNA transcripts with 11 ZIKV-unrelated nucleotides hanging on their 3’ ends were ~1 log less infectious than those with authentic 3’ ends (data not shown), indicating the importance of the authentic 3’ end for the production of infectious ZIKV RNAs.

Several functional cDNAs for ZIKV have hitherto been made using two different strategies, depending on the vector adopted to clone its full-length cDNA and the method applied to create the viral 5’ and 3’ ends: (1) The low-copy plasmid pACYC177 (~15 copies/cell) has been utilized to house a complete cDNA flanked by a 5’ bacteriophage T7 promoter and a 3’ hepatitis delta virus ribozyme (HDVr). This T7-HDVr system, analogous to the SP6-Psrl/Barl system created in this study, requires an in vitro transcription and transfection of transcribed RNAs into cells for virus recovery. This
“RNA-initiated” approach has been implemented to clone the vgRNA of the 2010 Cambodian FSS13025 strain [64]. To circumvent the need for a single plasmid containing a full-length cDNA, *in vitro* ligation of two or four cDNA fragments pre-cloned individually into the low-copy pACYC177 or high-copy pUC57 (500-700 copies/cell) plasmid, although relatively inefficient, has been done to generate a full-length cDNA template prior to *in vitro* transcription using the T7-HDVr system for the Ugandan MR-766 (1947), French Polynesian H/PF/2013 (2013), Puerto Rican PRVABC-59 (2015), and Brazilian SPH2015 (2015) and BeH819015 (2015) strains [65, 66]. (2) The low-copy pACNR1811 (10-20 copies/cell) or high-copy pcDNA6.2 (500-700 copies/cell) plasmid is used to house a full-length cDNA containing one or two artificial introns to restrict its instability during propagation in *E. coli*. In this case, a eukaryotic RNA polymerase (RNAP) II-dependent cytomegalovirus (CMV) promoter is positioned before the viral 5’ end, and a pair of HDVr and an SV40 poly(A) signal/RNAP II terminator are placed after the viral 3’ end. Unlike the SP6-PsrI/BarI system, the CMV-HDVr system requires transfection of cells with a plasmid carrying the intron-bearing full-length cDNA. This “DNA-initiated” approach has been applied to clone the vgRNA of the Ugandan MR-766 (1947) and Brazilian Paraiba (2015) strains [67, 68]. Alternatively, a circular form of the intronless full-length cDNA for the 2015 Brazilian Natal strain has been generated by PCR-mediated joining of eight overlapping cDNA fragments that are pre-cloned individually into the high-copy pUC plasmid [69]. Although far less efficient, a similar PCR-based method has also been reported that uses three overlapping cDNA fragments covering the vgRNA with no joining of these fragments into a circular cDNA [70]. In the
present study, a single plasmid-based RNA-initiated reverse genetics system developed for ZIKV, not only maximizes the stability of its cloned cDNA but also simplifies the synthesis of infectious RNAs *in vitro*.

ZIKV circulates in a sylvatic cycle between nonhuman primates (NHPs) and forest-dwelling mosquitoes, as well as in an urban cycle between humans and town-dwelling mosquitoes [12]. Apart from NHPs, however, information is scarce on any potential animal hosts or reservoirs for ZIKV transmission. Using the three cDNA-derived genetically distinct ZIKVs, their ability to infect and replicate in 17 animal cell lines from 12 different species (monkeys, humans, mosquitoes, mice, cows, pigs, sheep, goats, horses, dogs, cats, and chickens) was evaluated. The data showed that ZIKV has a broad cell tropism *in vitro*, being capable of establishing productive infection in 16 of the 17 cell lines tested, although its growth rate and ability to induce CPE varied widely depending on both the specific virus strain and host cell line. Of particular note, all three ZIKVs grew readily in both porcine ST and equine NBL-6 cells, with their growth kinetics similar to those observed in simian Vero cells; generally they also replicated and spread equally well in ovine SFF-6 cells and in aedine C6/36 cells. These results raise an interesting question as to whether three agriculturally important domestic animals, pigs, sheep, and horses, play any role in ZIKV transmission or can potentially serve as sentinel animals for ZIKV surveillance. Moreover, a nonsusceptible bovine MDBK cell line had a block in ZIKV entry but fully supported the subsequent post-entry steps; however, this cell line remained highly susceptible to infection by two other enveloped RNA viruses, BVDV [71] and VSV [72] which, like ZIKV [56], enter cells through the clathrin-
dependent endocytic pathway. This ZIKV-nonsusceptible bovine cell line thus offers a unique opportunity to identify the host factors involved in ZIKV entry.

This work is the first to generate such a large panel of 13 ZIKV region-specific antibodies that can identify nearly all the viral gene products and their related species in infected Vero cells and define all three structural proteins associated with extracellular virions. The data are in overall good agreement with the current model for flavivirus gene expression [21], but they also revealed the following unexpected findings: (1) While the full-length 13-kDa C and its one or two processed 10- to 11-kDa proteins were accumulated intracellularly, the extracellular virion-associated C’ protein appeared as a tightly spaced 12-kDa doublet. (2) For each of the two viral surface glycoproteins (24-kDa prM and 54/56-kDa E), two or three smaller products were also cell-associated but not virion-associated. (3) Only the 45-kDa NS1, and not its theoretically frameshift-derived product NS1’, was expressed. (4) In addition to the intact 14-kDa NS2B, its processed 11-kDa product was also stained, although weakly. (5) The full-length 69-kDa NS3 was processed to yield multiple truncated species of 33-60 kDa, of which the C-terminal 34-kDa fragment was the most prominent species. (6) The predicted 16-kDa NS4A was completely undetectable, but three unexpected NS4A-related proteins were readily identified, i.e., a major doublet at 14 kDa (NS4A’) and two minor protein clusters at 29 kDa (NS4A<sup>p29</sup>) and 35 kDa (NS4AB<sup>p35</sup>). (7) Not only the predicted 27-kDa NS4B but also two unexpected NS4B-related proteins were observed, one at 11 kDa (NS4B<sup>p11</sup>) and the other at 35 kDa (NS4AB<sup>p35</sup>). Although the importance of these findings for ZIKV biology requires further investigation, the results provide a solid foundation for the study
of viral replication and pathogenesis, virus-host interactions, and host responses to viral infection at both the cellular and organismic levels.

Much progress has been made over the past year in developing animal models (i.e., mice and NHPs) for ZIKV [42]. To date, the mouse is the most feasible small animal that mimics aspects of ZIKV infection in humans, albeit with some limitations resulting from species differences in innate immunity, reproductive system, and fetal development. Previously, no productive infection was detected when several strains of immunocompetent adult mice were inoculated peripherally with diverse ZIKVs, but robust peripheral ZIKV infection causing substantial morbidity and mortality was observed in both immunocompromised adult and immunocompetent neonatal mice [73-82]. However, the large variation in ZIKV pathogenicity among the previous studies, were conducted by inoculating a variety of ZIKVs into different strains of mice via various routes. In the current report, ZIKV neuropathogenicity in immunocompetent CD-1 mice at 1, 2, and 4 weeks of age can only be defined in the context of a virus-host combination, as evidenced by comparison of the neuroinvasiveness and neurovirulence of the three molecularly cloned, genetically distinct ZIKVs: (1) rMR-766 exhibited neonate-specific age-dependent neuroinvasiveness but displayed a high level of neurovirulence at all three ages. (2) rP6-740 had little-to-no neuroinvasiveness at all three ages but possessed neonate-specific age-dependent neurovirulence. (3) rPRVABC-59 was non-neuroinvasive and non-neurovirulent at all three ages. Also, marked differences in IFN sensitivity among the three ZIKVs were shown: In 4-week-old A129 (IFNAR−/−) mice, the three ZIKVs were uniformly neurovirulent but varied in neuroinvasiveness (rMR-
766, neuroinvasive; rP6-740, intermediate; and rPRVABC-59, almost non-neuroinvasive); however, all three ZIKVs, including rPRVABC-59, were neuroinvasive in age-matched AG129 (IFNAR<sup>−/−</sup>/IFNGR<sup>−/−</sup>) mice. Consistent with previous work, a greater susceptibility and more severe disease in AG129 mice than in A129 mice was noted [66, 74, 79, 83-85]. In all fatal cases, the mortality was related to the productive infection in the brain, coupled with tremors, ataxia, and hind limb paralysis.
5. CONCLUSIONS

The emergence of ZIKV in new zones and potential for endemic establishment, threatens public health infrastructure without ongoing investigations to identify ZIKV determinants of disease. ZIKV infectious clones permit the elucidation of virus components contributing to replication and pathogenesis among susceptible and non-susceptible hosts. This study developed three ZIKVs clones as BACs, in which site-directed mutagenesis supports changes to the virus genome, enabling analysis of molecular components participating in the ZIKV life-cycle. Recombinant viruses mimicked the parental virus phenotype in monkey Vero cells, affirming the utility of a reverse genetics system in replicating virus characteristics in vitro. Variable phenotypic differences among many cell lines representing an array of hosts, merits additional investigation of the susceptibility of organisms whose cells accommodated effective replication and pathogenesis. ZIKV gene specific antibodies revealed genome organization following the prevailing expression pattern of other flaviviruses. Nevertheless, noted deviations from the flavivirus model and between ZIKV strains probably contributed to distinguishing characteristics, but confirmation of related virus gene product roles requires supplementary examination. In vivo studies highlighted the significance of an intact immune response, which is likely connected to the increased mortality observed among younger animals. Additionally, signs of disease more closely modeled the neurotropic focus of ZIKV infections among some reported human cases. In summary, these findings demonstrate that ZIKV replication and pathogenesis is
influenced by the virus and host genetic profiles, coupled with the existence or lack of
immune system interferon signaling.

Use of these newly developed comparative functional genomics and immunologic
tools, combined with various cell culture and mouse infection model systems, will
facilitate further research leading to ZIKV disease prevention and therapy, as well as an
in-depth understanding of ZIKV biology.
6. REFERENCES


genetic systems, construction techniques and applications: a historical

63. Shizuya, H.; Birren, B.; Kim, U. J.; Mancino, V.; Slepak, T.; Tachiiri, Y.; Simon,
M., Cloning and stable maintenance of 300-kilobase-pair fragments of human
DNA in Escherichia coli using an F-factor-based vector. *Proc Natl Acad Sci U S A*

64. Shan, C.; Xie, X.; Muruato, A. E.; Rossi, S. L.; Roundy, C. M.; Azar, S. R.; Yang,
Y.; Tesh, R. B.; Bourne, N.; Barrett, A. D.; Vasilakis, N.; Weaver, S. C.; Shi, P. Y.,
An Infectious cDNA Clone of Zika Virus to Study Viral Virulence, Mosquito
Transmission, and Antiviral Inhibitors. *Cell Host Microbe* 2016, 19, (6), 891-900.

65. Widman, D. G.; Young, E.; Yount, B. L.; Plante, K. S.; Gallichotte, E. N.;
M.; Baric, R. S., A Reverse Genetics Platform That Spans the Zika Virus Family
Tree. *MBio* 2017, 8, (2).

Romo, H.; Nguyen, C.; Ruckert, C.; Brault, A. C.; Bowen, R. A.; Stenglein, M.;
Geiss, B. J.; Ebel, G. D., Development and Characterization of Recombinant
Virus Generated from a New World Zika Virus Infectious Clone. *J Virol* 2017, 91,
(1).

Laassri, M.; Chumakov, K.; Pletnev, A. G., A Full-Length Infectious cDNA Clone
of Zika Virus from the 2015 Epidemic in Brazil as a Genetic Platform for Studies

68. Schwarz, M. C.; Sourisseau, M.; Espino, M. M.; Gray, E. S.; Chambers, M. T.;
Tortorella, D.; Evans, M. J., Rescue of the 1947 Zika Virus Prototype Strain with a
Cytomegalovirus Promoter-Driven cDNA Clone. *mSphere* 2016, 1, (5).

Suhrbier, A.; Khromykh, A. A., De Novo Generation and Characterization of New
Zika Virus Isolate Using Sequence Data from a Microcephaly Case. *mSphere*
2017, 2, (3).

70. Atieh, T.; Baronti, C.; de Lamballerie, X.; Nougairede, A., Simple reverse


