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Brief Report

Construction and Rescue of a DNA-Launched DENV2 Infectious Clone

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Abstract: Flaviviruses represent a large group of globally significant, insect-borne pathogens. For many of these viruses, there is a lack of antivirals and vaccines. Thus, there is a need to continue the development of tools to further advance our efforts to combat these pathogens, including reverse genetics techniques. Traditionally, reverse genetics methods for flaviviruses rely on producing infectious RNA from in vitro transcription reactions followed by electroporation or transfection into permissive cell lines. However, the production of Zika virus has been successful from CMV promoter-driven expression plasmids, which provides cost and time advantages. In this report, we describe the design and construction of a DNA-launched infectious clone for dengue virus (DENV) serotype 2 strain 16681. An artificial intron was introduced in the nonstructural protein 1 segment of the viral genome to promote stability in bacteria. We found that rescued viruses maintained the ability to form plaques and replicate efficiently in commonly used cell lines. Thus, we present a rapid and cost-effective method for producing DENV2 strain 16681 from plasmid DNA. This construct will be a useful platform for the continued development of anti-DENV therapeutics and vaccines.

Keywords: positive-strand RNA virus; flavivirus; dengue virus; infectious clone; reverse genetics

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1. Introduction

Flaviviruses are a large group of insect-borne viruses that represent a significant risk to human health throughout most of the world. Dengue virus (DENV) is transmitted through the bite of infected mosquitos in tropical and sub-tropical regions [1,2]. More than 3 billion people live in regions that put them at risk of being infected with DENV, making it the most prevalent insect-borne virus in the world [3,4]. Disease from DENV infection can range from mild, febrile illness to hemorrhagic fever or shock that can lead to death [5]. To date, there are no approved antivirals for DENV, and the vaccine is not approved for use in children and the elderly, which are populations with the highest risk of severe disease [6,7]. Thus, there is a need to continue to develop tools that can be used to advance our efforts in antiviral and vaccine research.

Reverse genetics systems are important tools for virology research because they help to maintain viral genome sequence integrity and provide a platform to study specific mutations. Traditionally, flaviviruses have been rescued from cDNA clones expressed from a plasmid using in vitro transcription [8–15]. However, this can be a time-consuming process. Several flaviviruses have been successfully rescued from cytomegalovirus (CMV) promoter-driven plasmids after direct transfection of permissive cells [16–20]. A common issue observed during the production of flavivirus cDNA clones is the instability of the genome in bacteria due to the presence of cryptic promoters [17,21]. This was overcome for ZIKV cDNA clones by introducing synthetic introns into the genomic sequence, which force the viral polyprotein open reading frame (ORF) out of frame [19,20]. DENV2 has previously been rescued from a DNA-launched infectious clone that was stabilized through the addition of seven repeats of a tetracycline response element upstream of a minimal CMV promoter, which requires the addition of doxycycline and co-transfection of a pTet-Off

plasmid to rescue virus [17]. Alternatively, DENV has been successfully rescued from a bacterial artificial chromosome (BAC) system [22]. However, these BAC systems can be complicated and time-consuming to produce.

In this report, we describe the construction and rescue of DENV serotype 2 strain 16681 (DENV2) from a CMV promoter-driven cDNA clone. We found that the introduction of a synthetic intron into NS1 stabilized the plasmid in bacteria and that virus was efficiently rescued from transfected cells using a cost-effective transfection reagent. Further, the rescued virus retained plaque-forming capacity and growth in commonly used cell lines. Together, we show that this system provides a rapid and cost-effective alternative to the traditional RNA-launched cDNA infectious clones.

2. Materials and Methods

2.1. Cell Culture

Human embryonic kidney (HEK) 293T cells, U2OS cells, and C6/36 cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (P/S). Human hepatoma cells (Huh7) were maintained in DMEM supplemented with 10% FBS, 9 g/L glucose, and 100 U/mL P/S. VeroE6 cells were maintained in modified Eagle's media (MEM) supplemented with 10% FBS and 100 U/mL P/S. Baby hamster kidney (BHK-21 clone 15 were a generous gift from Dr. Douglas Brackney, Connecticut Agricultural Experiment Center) cells were maintained in MEM supplemented with 10% FBS, 1× non-essential amino acids, 1× sodium pyruvate, and 100 U/mL P/S. All mammalian cells were maintained in humidified incubators at 37 °C. C6/36 cells were maintained in humidified incubators at 28 °C.

2.2. Construction of Infectious Clone Plasmid

Figure 1 and Table 1 summarize the plasmid design and construction of pcDNA6.2 DENV2 16681. The vector was obtained from PCR using Vector-F and Vector-R primers with the pcDNA6.2 ZIKV-MR766-HDVr plasmid [20]. This PCR introduced homologous sequences corresponding to the 5′UTR (22 nt) and 3′UTR (18 nt) of DENV2 into the linearized vector. The sequence encoding the DENV2 5′UTR was obtained as a gBlock (IDT) flanked by sequences homologous to the vector (22 nt) and PCR A fragment (24 nt). PCR A was obtained from DENV2 16681 CprME plasmid (a gift from Theodore Pierson, NIH) using PCR-A_F and PCR-A_R [23]. The synthetic intron was obtained as a gBlock (IDT) flanked by homologous sequences to PCR A (43 nt) and PCR B (20 nt). PCR B was amplified from the subgenomic DENV2 replicon pcDNA3.1 DENVrepGFP/Zeo (a gift from Theodore Pierson, NIH) using PCR-B_F and PCR-B_R, which introduced sequence homologous to the vector at the 3′ end (15 nt) [23]. PCR products and gBlocks were assembled using NEB HiFi Assembly, according to the manufacturer's protocol. Reactions were transformed in Stbl2 competent cells (Invitrogen), according to the manufacturer's protocol, and grown at 30 °C. Full plasmid sequencing was performed using Plasmidsaurus.

2.3. Preparation of Virus Stocks

Laboratory stocks of DENV2 16681 (DENV2, a gift from Dr. Carolyn Coyne, Duke University) were prepared from C6/36 cells at 33 °C, as previously described [24]. DENV2 was rescued from HEK 293T cells transfected with pcDNA6.2 DENV2 16681 plasmid using polyethylenimine (PEI, 25 kDa) at a 1:1 ratio of DNA (ug) to 1 mg/mL PEI stock (µL) [25]. Virus-containing supernatants were harvested at various times post-transfection and assayed for infectious viruses. Viral titers were determined by fluorescent focus forming unit assays, as previously described [26]. Briefly, 10-fold dilutions of virus-containing supernatant were used to infect VeroE6 cells in 96-well plates. At ~48 hpi, cells were fixed in 4% paraformaldehyde diluted in PBS, permeabilized with 0.1% Triton 100-X diluted in PBS, and viral E protein was detected using cell culture derived monoclonal antibody against flavivirus envelope protein, 4G2 (1:50), and AlexaFluor-conjugated secondary

antibodies (Invitrogen, 1:1000). Fluorescent foci were counted using an inverted fluorescent microscope, Olympus IX83.

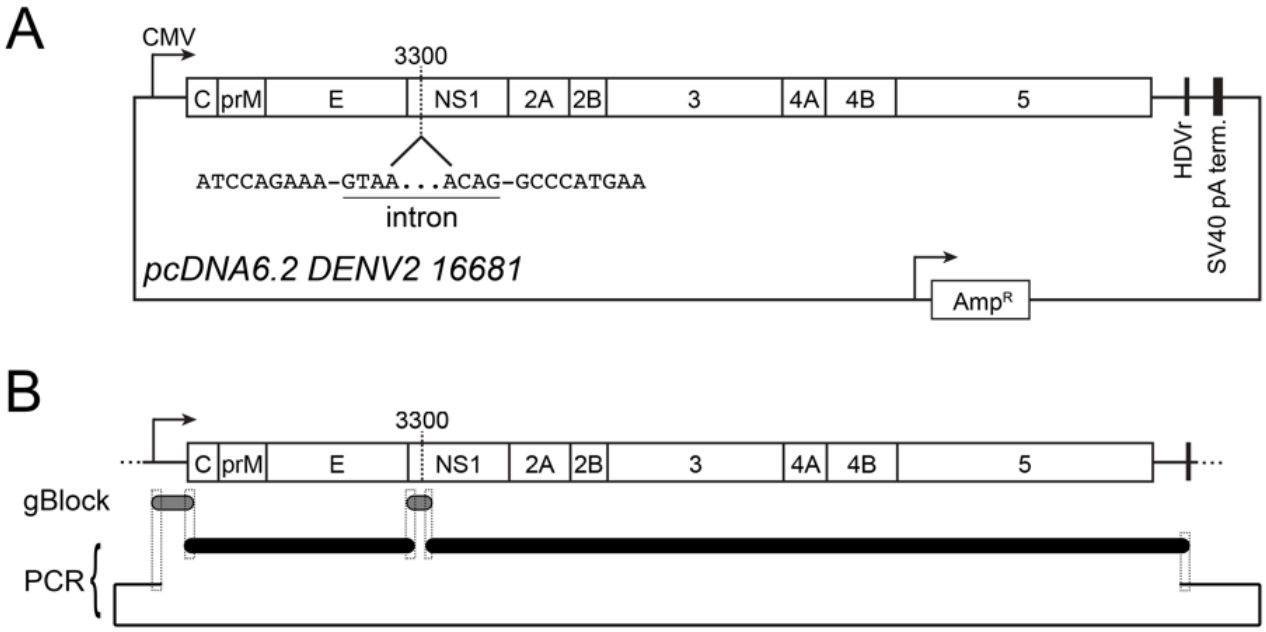

Figure 1. Design of pcDNA6.2 DENV2 16681. Schematics of a CMV promoter-driven cDNA clone of DENV2 strain 16681. (**A**) Plasmid map of pcDNA6.2 DENV2 16681. The viral genome lies between a CMV promoter and HDVr, followed by an SV40 polyA termination sequence. The location of the artificial intron is indicated at nucleotide 3300. The underlined sequence corresponds to the 5′ and 3′ ends of the intron. (**B**) Construction of pcDNA6.2 DENV2 16681. Overlapping regions between gBlocks (grey) and PCR products (black) are shown by dashed line boxes.

Table 1. Primer and gBlock sequences.

Name	Sequence (5′ to 3′)	Template
Vector_F	GTTGAATCAACAGGTTCTGGCCGGCATGGTCCCAGCCTC	pcDNA6.2-ZIKV MR766-HDVr
Vector_R	CGTAGACTAACAACTCGGTTCACTAAACGAGCTCTGC	
DENV 5′UTR	GCAGAGCTCGTTTAGTGAACCGAGTTGTTAGTCTACGTGGACCGACA-AAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTT-TTTAATTAGAGAGCAGATCTCTGATGAATAACCAACGGAAAAAGGCG	gBlock
PCR-A_F	GATCTCTGATGAATAACCAACGGAAAAAGGCG	pcDNA3.1 DENV 16681 CprME
PCR-A_R	CACAACGCAACCACTATCGGCCTGCACCATGACTCCCAAATAC	
NS1-Intron	GTATTTGGGAGTCATGGTGCAGGCCGATAGTGGTTGCGTTGTGAGCT-GGAAAAACAAAGAACTGAAATGTGGCAGTGGGATTTTCATCACAGAC-AACGTGCACACATGGACAGAACAATACAAGTTCCAACCAGAATCCCC-TTCAAAACTAGCTTCAGCTATCCAGAAAGTAAGTATCAAGGTTACAAG-ACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAA-GACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACTTT-GCCTTTCTCTCCACAGGCCCATGAAGAGGGCATTTGTGGAATCCGCTC-AGTAACAAGACTGGAGAATCTGATGTGGAAACAAATAACACCAGAAT-TGAATCACATTCTATCAGAAAATGAGGTGAAGTTAACTATTATGACAG-GAGACATCAAAGGAATCATGCAGGCAGGAAAACGATCTCTGCGGCC-TCAGCCC	gBlock
PCR-B_F	GATCTCTGCGGCCTCAGCCCACTGAGCTGAAG	pcDNA3.1 DENVrepGFP/Zeo
PCR-B_R	GGGACCATGCCGGCCAGAACCTGTTGATTCAACAGCACC	

Underlined sequences indicate homologous sequences used in HiFi assembly.

2.4. Virus Infections

Multicycle replication kinetics were determined in the indicated cell lines that were infected with 0.01 FFU/cell in a 6-well plate. At indicated times post-infection, 50 µL of supernatant was collected and stored at −80 °C until being titrated by FFU assay.

2.5. Immunoblots

Lysates from transfected HEK 293T cells were lysed in 1× RIPA + protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysates were clarified by centrifugation at 4 °C and 12,000× *g* for 10 min. Clarified lysates were separated by SDS PAGE using a 4–20% Tris-glycine polyacrylamide pre-cast gel (BioRad, Hercules, CA, USA) and transferred to nitrocellulose membranes. Following 30 min of blocking in PBS + 10% non-fat milk, membranes were probed using rabbit anti-DENV NS3 (1:2000; GeneTex, Irvine, CA, USA) and mouse anti-actin (1:10,000; ProteinTech, Rosemont, IL, USA) diluted in PBST + 5% BSA. Proteins were visualized using near-infrared dye-conjugated secondary antibodies (LiCor, Lincoln, NE, USA) diluted in PBST + 5% non-fat milk and imaged on an Odyssey CLx imaging system (LiCor).

2.6. Immunofluorescence Microscopy

Transfected 293T cells seeded on poly-D-lysine coated 24-well plates were fixed at the indicated times post-infection using PBS + 4% paraformaldehyde for 10 min, permeabilized with PBS + 0.1% Triton 100-X for 10 min, washed in PBS, and incubated with mouse anti-dsRNA antibody (Kerafast) for 1 h. Following primary antibody incubation, cells were washed in PBS ×3 and incubated with AlexaFluor-conjugated secondary antibodies (Invitrogen). Following washing, samples were incubated with PBS + 300 nM DAPI and imaged on an IX83 Olympus inverted fluorescent microscope using a 10× objective.

2.7. Splicing Assay

RNA was extracted from virus-containing supernatants using the PureLink Viral RNA Mini Kit (Invitrogen). Next, cDNA was generated using 1 µL of RNA in a reverse transcription reaction containing Protoscript III (Invitrogen) and an antisense NS1-specific primer (5′-AGCAGCTGTGACCAAGGAGTT-3′). PCR was performed with NS1-specific sense (5′-CACCATGGATAGTGGTTGCGTTGTG-3′) and antisense (described above) primers using cDNA, an NS1-expressing plasmid (positive control), or the pcDNA6.2 DENV2 16681 plasmid (negative control). Amplicon sizes were determined by separating reactions on a 2% agarose gel. PCR products from the rescued virus were submitted for Sanger sequencing (UAB Genomics Core) and aligned using Benchling.

2.8. Plaque Assay

Dilutions of DENV2 were absorbed on BHK-21 clone 15 cells seeded in a 6-well plate for 1 h at 37 °C, with gentle rocking every 15 min. Infected cells were overlayed with growth media containing 2% FBS and 1.2% cellulose (colloidal, microcrystalline, Sigma-Aldrich). Cells were incubated for 6 days, followed by rinsing with PBS and staining with 10% ethanol + 0.05% crystal violet. Plaque assay plates were imaged using a Bio-Rad Gel Doc EZ Imager and plaque sizes were measured using ImageJ v1.53.

2.9. Statistics

Statistical analyses were performed using Prism 9 Software (GraphPad, San Diego, CA, USA).

3. Results and Discussion

3.1. Design and Construction of a DNA-Launched DENV2 16681 Infectious Clone

To simplify the production of DENV2, we sought to design a CMV promoter-driven infectious clone. We introduced the DENV2 16681 genome (NC_001474.2) into a modified pcDNA6.2 expression plasmid between the CMV promoter and a hepatitis delta

ribozyme (HDVr), which self-cleaves upon transcription to maintain 3′ UTR sequence integrity (Figure 1A) [20]. Initial attempts to obtain clones containing the viral genome were unsuccessful due to large deletions starting in the nonstructural protein 1 (NS1) segment of the genome. Previous reports have demonstrated the instability of flavivirus genomes in plasmids due to the presence of cryptic promoters [17,21,27]. However, ZIKV has been successfully rescued via the insertion of a synthetic intron into the viral genome, which forces the viral ORF out of frame [19,20]. Thus, we took advantage of this strategy for the rescue of DENV2 clones by introducing an artificial intron at nucleotide 3300 of the DENV2 genome (Figure 1A). We successfully obtained a DNA-launched infectious clone through the assembly of gBlock DNA fragments, PCR-derived fragments of the viral genome, and a PCR-derived pcDNA6.2 vector containing the HDVr (Figure 1B and Table 1). Sequencing of recovered plasmid confirmed the correct assembly of fragments containing no errors compared to the NC_001474.2 GenBank entry.

3.2. Rescue of DENV2 16681

Next, we sought to rescue the virus from our constructed plasmid. Supernatants from HEK 293T cells transfected with pcDNA6.2 DENV2 16681 plasmid showed the presence of infectious DENV2 starting at 48 h post-transfection (~2×10^3 FFU/mL). The amount of virus in the supernatant increased up to the final time point of 120 h when titers reached 2×10^6 FFU/mL (Figure 2A). We further validated the production of DENV2 via immunoblot analysis of lysates from transfected HEK 293T cells. Consistent with our titer data, we observed an increase in nonstructural protein 3 (NS3) over time, with the peak expression occurring at 120 h post-transfection (Figure 2B). The presence of NS3 in cell lysates also indicates successful splicing of the recombinant genome because expression of this protein is dependent on the removal of the artificial intron. This was confirmed by performing RT-PCR on RNA from virus-containing supernatants that indicated that the size of NS1 is the same between parental and rescued virus, which both migrate at the same rate as the product from an NS1 expression plasmid but not the intron-containing infectious clone plasmid (Figure 2C). This finding was further confirmed through the sequencing of the PCR product from rescued virus, which shows the absence of the intron (Figure 2D). Furthermore, immunofluorescence shows an increase in dsRNA in transfected HEK 293T cells from 48 to 120 h post-transfection, which indicates the presence of DENV2 replication intermediates (Figure 2E). Interestingly, we found that rescued virus produced plaques with a more consistent size compared to the parental stock virus, suggesting that rescued virus stock has less genetic variability (Figure 2F,G). Together, these results show infectious DENV2 can be rescued from a CMV promoter-driven expression plasmid.

3.3. Characterization of Rescued DENV2 16681

Next, we compared the replication kinetics of rescued DENV2 to parental DENV2 in various cell lines used in the literature. We performed multicycle replication assays in VeroE6, U2OS, and Huh7 cells from 0 to 6 days post-infection (dpi) or C6/36 cells at 28 °C from 0 to 10 dpi using rescued and parental virus. We observed similar replication kinetics in VeroE6 cells, which are commonly used for the titration of viruses (Figure 3A). However, we found that rescued virus reached peak titers more rapidly in U2OS and Huh7 cells (Figure 3B,C). Conversely, we observed that rescued virus had a significant growth delay in C6/36 cells (Figure 3D). Differences in virus titer from infected U2OS, Huh7, and C6/36 cultures could suggest the presence of tissue culture adapted mutants that are present in the stock parental virus compared to the rescued virus, which originated from a single genomic sequence. Overall, our results suggest rescuing DENV2 from pcDNA6.2 DENV 16681 is a rapid and cost-effective method for generating laboratory stocks of the virus.

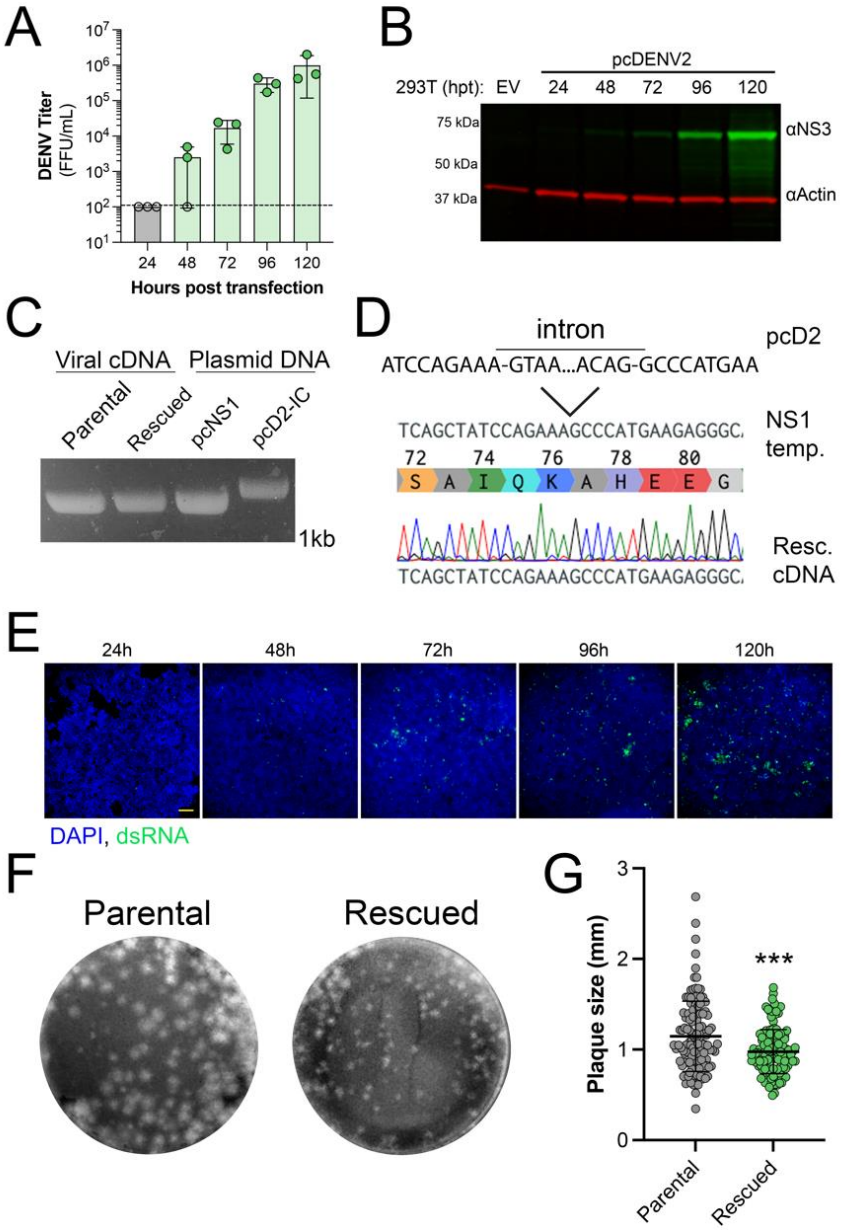

Figure 2. Rescue of DENV2. Validation of rescued virus. (**A**) Titer of DENV2 from pcDNA6.2 DENV 16681 transfected HEK 293T cells at indicated times post-transfection. Data represent the average FFU/mL ± SD of three independent transfections. Open circles represent samples below the limit of detection, shown by the dashed line. (**B**) Representative immunoblot of lysates from transfected HEK 293T cells collected at the indicated hours post-transfection (hpt). DENV NS3 is shown in green and actin is shown in red. The experiment was performed three independent times. (**C**) Representative agarose gel of NS1 PCR products for viral cDNA (parental and rescued) or plasmid controls (pcDNA-NS1 or pcDNA6.2 DENV2 16681). (**D**) Sequence alignment of the portion of NS1 containing the synthetic intron, WT NS1, and sequencing results of rescued virus cDNA, including the chromatogram. (**E**) Immunofluorescence staining of transfected HEK 293T cells fixed at the indicated time post-transfection. Viral dsRNA replication intermediate is shown in green and nuclei stained with DAPI are shown in blue. Scale bar represents 100 µm. Representative images are shown from one of three independent experiments. (**F**) Representative images of plaque assays in BHK-21 cells. (**G**) Sizes for individual plaques (mm), $n = 128$, produced from parental and rescued virus infection. Individual plaque sizes are shown as data points with the average ± SD. Significance was determined by Student's *t*-test, *** $p < 0.0001$.

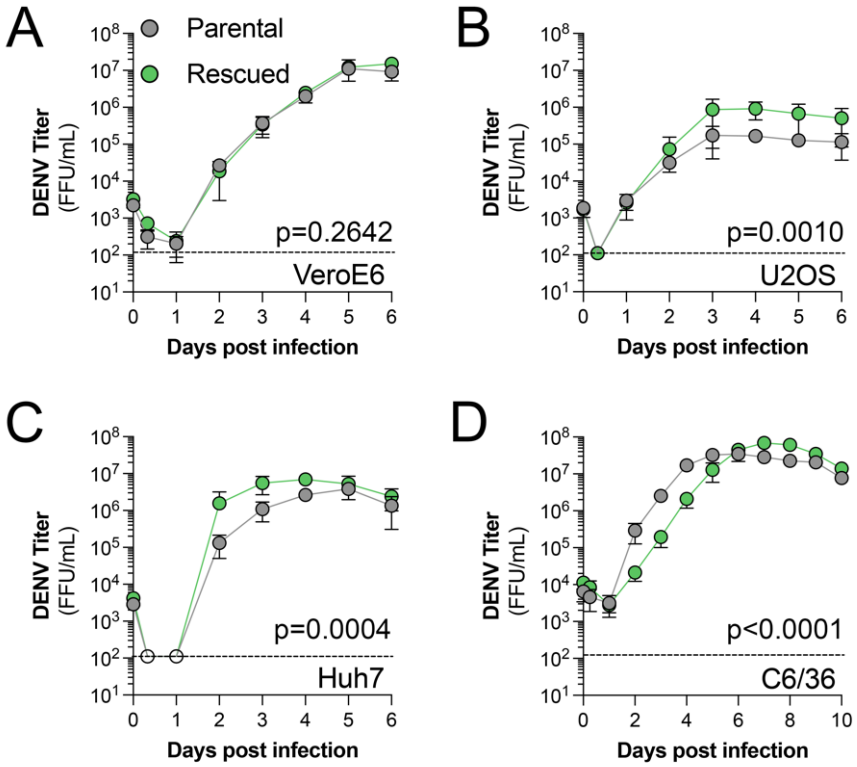

Figure 3. Replication kinetics of rescued virus in commonly used cell lines. (**A**–**D**) Indicated cell lines were infected with parental or rescued virus at an MOI of 0.01 for the indicated times. Supernatants were collected from the indicated time points and titrated on VeroE6 cells. Data are shown as the average FFU/mL ± SD of three independent experiments. Significance was determined using a 2-way ANOVA.

4. Conclusions

Historically, cDNA clones of flaviviruses have been rescued via in vitro transcription systems [21]. However, these systems can be time-consuming and expensive. Thus, we developed a CMV promoter-driven plasmid to rescue DENV2 strain 16681 via direct transfection, similar to plasmids reported for other flaviviruses [17,19,20]. Additionally, we utilized the inexpensive PEI transfection reagent, which further decreases the cost of rescuing the virus. Using this strategy, we were able to successfully rescue the infectious DENV2 virus that resembles the parental virus; however, rescued virus produced more consistently sized plaques and slight differences in growth in cell culture. This platform will streamline the production and analysis of DENV2 for a variety of applications, including the development of antiviral therapeutics and vaccines.

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References

1. Pierson, T.C.; Diamond, M.S. The Continued Threat of Emerging Flaviviruses. *Nat. Microbiol.* **2020**, *5*, 796–812. [CrossRef] [PubMed]
2. Diamond, M.S.; Pierson, T.C. Molecular Insight into Dengue Virus Pathogenesis and Its Implications for Disease Control. *Cell* **2015**, *162*, 488–492. [CrossRef] [PubMed]
3. Stanaway, J.D.; Shepard, D.S.; Undurraga, E.A.; Halasa, Y.A.; Coffeng, L.E.; Brady, O.J.; Hay, S.I.; Bedi, N.; Bensenor, I.M.; Castañeda-Orjuela, C.A.; et al. The Global Burden of Dengue: An Analysis from the Global Burden of Disease Study 2013. *Lancet Infect. Dis.* **2016**, *16*, 712–723. [CrossRef] [PubMed]
4. Bhatt, S.; Gething, P.W.; Brady, O.J.; Messina, J.P.; Farlow, A.W.; Moyes, C.L.; Drake, J.M.; Brownstein, J.S.; Hoen, A.G.; Sankoh, O.; et al. The Global Distribution and Burden of Dengue. *Nature* **2013**, *496*, 504–507. [CrossRef]
5. Simmons, C.P.; Farrar, J.J.; van Vinh Chau, N.; Wills, B. Dengue. *N. Engl. J. Med.* **2012**, *366*, 1423–1432. [CrossRef]
6. Hadinegoro, S.R.; Arredondo-García, J.L.; Capeding, M.R.; Deseda, C.; Chotpitayasunondh, T.; Dietze, R.; Hj Muhammad Ismail, H.I.; Reynales, H.; Limkittikul, K.; Rivera-Medina, D.M.; et al. Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N. Engl. J. Med.* **2015**, *373*, 1195–1206. [CrossRef]
7. Troost, B.; Smit, J.M. Recent Advances in Antiviral Drug Development towards Dengue Virus. *Curr. Opin. Virol.* **2020**, *43*, 9–21. [CrossRef]
8. Rice, C.M.; Grakoui, A.; Galler, R.; Chambers, T.J. Transcription of Infectious Yellow Fever RNA from Full-Length CDNA Templates Produced by in Vitro Ligation. *New Biol.* **1989**, *1*, 285–296.
9. Lai, C.J.; Zhao, B.; Hori, H.; Bray, M. Infectious RNA Transcribed from Stably Cloned Full-Length CDNA of Dengue Type 4 Virus. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5139–5143. [CrossRef]
10. Kapoor, M.; Zhang, L.; Mohan, P.M.; Padmanabhan, R. Synthesis and Characterization of an Infectious Dengue Virus Type-2 RNA Genome (New Guinea C Strain). *Gene* **1995**, *162*, 175–180. [CrossRef]
11. Gualano, R.C.; Pryor, M.J.; Cauchi, M.R.; Wright, P.J.; Davidson, A.D. Identification of a Major Determinant of Mouse Neurovirulence of Dengue Virus Type 2 Using Stably Cloned Genomic-Length CDNA. *J. Gen. Virol.* **1998**, *79*, 437–446. [CrossRef] [PubMed]
12. Polo, S.; Ketner, G.; Levis, R.; Falgout, B. Infectious RNA Transcripts from Full-Length Dengue Virus Type 2 CDNA Clones Made in Yeast. *J. Virol.* **1997**, *71*, 5366–5374. [CrossRef] [PubMed]
13. Zhu, W.; Qin, C.; Chen, S.; Jiang, T.; Yu, M.; Yu, X.; Qin, E. Attenuated Dengue 2 Viruses with Deletions in Capsid Protein Derived from an Infectious Full-Length CDNA Clone. *Virus Res.* **2007**, *126*, 226–232. [CrossRef]
14. Khromykh, A.A.; Westaway, E.G. Completion of Kunjin Virus RNA Sequence and Recovery of an Infectious RNA Transcribed from Stably Cloned Full-Length CDNA. *J. Virol.* **1994**, *68*, 4580–4588. [CrossRef] [PubMed]
15. Mandl, C.W.; Ecker, M.; Holzmann, H.; Kunz, C.; Heinz, F.X. Infectious CDNA Clones of Tick-Borne Encephalitis Virus European Subtype Prototypic Strain Neudoerfl and High Virulence Strain Hypr. *J. Gen. Virol.* **1997**, *78*, 1049–1057. [CrossRef]
16. Pierson, T.C.; Diamond, M.S.; Ahmed, A.A.; Valentine, L.E.; Davis, C.W.; Samuel, M.A.; Hanna, S.L.; Puffer, B.A.; Doms, R.W. An Infectious West Nile Virus That Expresses a GFP Reporter Gene. *Virology* **2005**, *334*, 28–40. [CrossRef]
17. Pu, S.Y.; Wu, R.H.; Tsai, M.H.; Yang, C.C.; Chang, C.M.; Yueh, A. A Novel Approach to Propagate Flavivirus Infectious CDNA Clones in Bacteria by Introducing Tandem Repeat Sequences Upstream of Virus Genome. *J. Gen. Virol.* **2014**, *95*, 1493–1503. [CrossRef]
18. Khromykh, A.A.; Varnavski, A.N.; Sedlak, P.L.; Westaway, E.G. Coupling between Replication and Packaging of Flavivirus RNA: Evidence Derived from the Use of DNA-Based Full-Length CDNA Clones of Kunjin Virus. *J. Virol.* **2001**, *75*, 4633–4640. [CrossRef]
19. Tsetsarkin, K.A.; Kenney, H.; Chen, R.; Liu, G.; Manukyan, H.; Whitehead, S.S.; 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 of Virus-Host Interactions and Vaccine Development. *MBio* **2016**, *7*, e01114-16. [CrossRef]
20. 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*, e00246-16. [CrossRef]
21. Aubry, F.; Nougairède, A.; Gould, E.A.; de Lamballerie, X. Flavivirus Reverse Genetic Systems, Construction Techniques and Applications: A Historical Perspective. *Antivir. Res.* **2015**, *114*, 67–85. [CrossRef] [PubMed]
22. Usme-Ciro, J.A.; Lopera, J.A.; Enjuanes, L.; Almazán, F.; Gallego-Gomez, J.C. Development of a Novel DNA-Launched Dengue Virus Type 2 Infectious Clone Assembled in a Bacterial Artificial Chromosome. *Virus Res.* **2014**, *180*, 12–22. [CrossRef]

23. Ansarah-Sobrinho, C.; Nelson, S.; Jost, C.A.; Whitehead, S.S.; Pierson, T.C. Temperature-Dependent Production of Pseudoinfectious Dengue Reporter Virus Particles by Complementation. *Virology* **2008**, *381*, 67–74. [CrossRef] [PubMed]
24. Vasilakis, N.; Fokam, E.B.; Hanson, C.T.; Weinberg, E.; Sall, A.A.; Whitehead, S.S.; Hanley, K.A.; Weaver, S.C. Genetic and Phenotypic Characterization of Sylvatic Dengue Virus Type 2 Strains. *Virology* **2008**, *377*, 296–307. [CrossRef]
25. Boussif, O.; LezoualC'H, F.; Zanta, M.A.; Mergny, M.D.; Scherman, D.; Demeneix, B.; Behr, J.P. A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in Vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301. [CrossRef] [PubMed]
26. Payne, A.F.; Binduga-Gajewska, I.; Kauffman, E.B.; Kramer, L.D. Quantitation of Flaviviruses by Fluorescent Focus Assay. *J. Virol. Methods* **2006**, *134*, 183–189. [CrossRef]
27. Li, D.; Aaskov, J.; Lott, W.B. Identification of a Cryptic Prokaryotic Promoter within the CDNA Encoding the 5′ End of Dengue Virus RNA Genome. *PLoS ONE* **2011**, *6*, e18197. [CrossRef]