Generation of an infectious Negev virus cDNA clone

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The newly proposed genus Negevirus consists of insect-only viruses isolated from mosquitoes and sandflies. Here, we report the successful construction of a full-length infectious cDNA clone of Negev virus (NEGV) strain M30957. Viral RNA was transcribed in vitro and virus was readily rescued with or without the use of a cap analogue. These results strongly suggest that NEGV, and likely other members within the genus, is a non-segmented, single-stranded, positive-sense RNA virus.

The genus Negevirus consists of insect-only viruses isolated from mosquitoes and sandflies. The first member of the proposed genus Negevirus, Negevirus virus (NEGV), was initially isolated from Anopheles coustani mosquitoes collected in the Negev desert, Israel, in 1983. NEGV has subsequently been isolated from Culex species collected at various localities in Texas (Vasilakis et al., 2013). NEGV is unable to infect mammalian cells whereas it readily infects insect cells with peak titres of ~10^10 p.f.u. ml^-1. It causes extensive cytopathic effects (CPEs) within 12–24 h of infection in the C6/36 and C7/10 lines of Aedes albopictus mosquito cells. Additionally, Aedes aegypti mosquitoes are susceptible to oral infection (Vasilakis et al., 2013).

Reverse genetics is a powerful molecular tool that has facilitated the understanding of many aspects of genomic organization, viral replication and pathogenesis, and has revolutionized vaccine development (Wimmer et al., 2001). Briefly, full-length viral genome was assembled in three fragments (Fig. 1). NEGV RNA was isolated from cell culture supernatant using the QIAamp Viral RNA Mini kit (Qiagen). cDNA was produced via reverse transcription by using random hexamers and SuperScript III (Invitrogen). cDNA fragments were amplified via PCR with NEGV-specific primers containing endonuclease restriction sites and Phusion DNA polymerase (New England Biolabs) (Fig. 1). Amplified PCR products were purified using the QIAquick Gel Extraction kit (Qiagen), digested with restriction endonucleases and cloned into pRS2 plasmid (a derivative of pUC19) (Fig. 1). Viral genome was placed under the control of an SP6 RNA polymerase promoter at the 5′ end and included a 3′ poly(A) tail with a downstream NotI linearization site for in vitro transcription (Fig. 1). Full-length plasmid cDNA was confirmed via Sanger sequencing.

To rescue recombinant NEGV progeny, linearized plasmid was purified via phenol/chloroform extraction and 1 µg was utilized for the transcription reaction: 0.5 mM NTP, 0.5 mM m7G(5′)ppp(5′)G RNA cap analogue (New England Biolabs), 0.5 µl RNase inhibitor and 1.25 µl SP6 polymerase (Ambion) in 25 µl total volume. Viral RNA transcripts were prepared in vitro with and without the m7G(5′)ppp(5′)G cap analogue (NEGV + cap and NEGV –cap, respectively). Transcription was performed for 2 h at 42 °C and then the transcription mixture placed on ice. Freshly confluent C7/10 cells seeded overnight were gently scraped into single-cell suspensions, washed five times with...
1 × PBS and resuspended in 450 µl 1 × PBS. Cells were mixed with each transcription mixture (10 µg RNA), placed in 2 mm electroporation cuvettes and immediately electroporated (BTX ECM-830 Square Wave Electroporator; Harvard Apparatus) using the following conditions: 680 V, pulse length 99 μs, interval between pulses 200 ms and five pulses. Following electroporation, C7/10 cells were placed at 28 ºC with 5 % CO₂ and observed for CPEs. Recombinant NEGV progeny were readily produced with substantial CPEs by 12 h post-electroporation (h.p.e.) (Fig. 2a). Virus progeny produced plaques on ~80 % confluent C7/10 cell monolayers seeded overnight in six-well plates. Briefly, duplicate wells were infected with 0.1 ml aliquots from serial 10-fold dilutions in growth medium, then 0.4 ml of growth medium was added to each well to prevent cell desiccation and virus was adsorbed for 1 h. Following incubation, the virus inoculum was removed, and cell monolayers were overlaid with 3 ml of a 1:1 mixture of 2 % tragacanth and 2× modified Eagle’s medium with 5 % FBS, 2 % tryptose phosphate broth solution and 2 % penicillin/streptomycin. Cells were incubated at 28 ºC with 5 % CO₂ for 36 h for plaque development, the overlay was removed and monolayers were fixed with 3 ml 10 % formaldehyde in PBS for 30 min. Cells were stained with 2 % crystal violet in 30 % methanol for 5 min at room temperature; excess stain was removed under running water and plaques were counted. The successful recovery of recombinant NEGV was confirmed via full-length Sanger
transcribed RNA (Ahlquist et al., 1984; Dasmahapatra et al., 1986; Polo et al., 1997; Pugachev et al., 1997; Rice et al., 1987). Therefore, our results strongly suggested the presence of an internal ribosome entry site (IRES) at the 5’ end of the NEGV genome, which would mediate cap-independent translation of the viral ORF to initiate replication. Indeed, analysis of 234 nt in the 5’ UTR of the NEGV genome using mfold (Zuker, 2003) predicted a highly organized RNA secondary structure (Fig. 2c) typical of IRES elements used by other positive-strand RNA viruses (Liu et al., 2009).

To assess potential phenotypic differences between the parental and clone-derived viruses (rescued with or without the use of the cap), one-step replication curves were performed with the parental and recombinant viruses in 50% confluent C7/10 monolayers, in triplicate, with a m.o.i. of 10 p.f.u. per cell. Regardless of the rescue conditions, the replication kinetics of recombinant viruses were nearly identical to those of the WT parent with peak titres of $1.0 \times 10^9$ p.f.u. ml$^{-1}$ (Fig. 3a).

Next, we compared replication of the recombinant viruses at the initial steps after cell transfection with transcribed RNA with and without the use of the cap. C7/10 cells were electroporated with 8 µg RNA in duplicate, transcribed with or without the cap as described above and samples of culture supernatants were collected every hour for the first 5 h, and then at 12 and 24 h.p.e. Interestingly, infectious virus was first detected at 1 h.p.e. for NEGV + cap, but only at 5 h.p.e. for NEGV − cap (Fig. 3b). Also, there was an estimated 2–3 h delay for the burst of virus production in NEGV − cap compared with NEGV + cap. However, both viruses reached the same peak titre of $10^9$ p.f.u. ml$^{-1}$ at 24 h.p.e. A possible explanation of this outcome is that IRES-mediated translation of viral proteins is less efficient than cap-mediated translation, which would result in a delay in initial accumulation of viral proteins that form replicative complexes. However, when those complexes begin to produce normally capped viral genomes, the rate of viral protein production increases, which in turn increases the rate of viral replication to the level observed after electroporation of capped RNA.

In summary, we describe the generation of an infectious cDNA clone of NEGV, the first isolated member of the newly proposed genus Negevirus. NEGV rescued in vitro in mosquito cells produced CPEs, plaque morphology and replication kinetics similar to the original virus. Remarkably, viable virus was readily obtained without the use of a cap analogue for in vitro genomic RNA synthesis, although its initial replication after electroporation was delayed compared with the capped RNA counterpart. Considering the highly organized predicted RNA secondary structure of the 5’ UTR, these data suggest the presence of an IRES at the 5’ end of the NEGV genome, which mediates low-efficiency translation. Additional experiments are needed to confirm the putative IRES and evaluate its role in NEGV replication. Our data suggest that genomes in the genus Negevirus likely consist of non-segmented, single-stranded, positive-sense RNA. The infectious NEGV cDNA clone we developed can be utilized to further characterize this newly discovered genus to elucidate many aspects of its biology, particularly its distinctive host specificity, and its potential for use in vector control or as a vehicle for vaccine production.
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References


