Functions and requirements of conserved RNA structures in the 3' untranslated region of Flaviviruses

Agostinho Gonçalves Costa da Silva, P.

Chapter 5

Characterization of a stable full-length cDNA clone for the transcription of infectious RNA of a Flavivirus with no known vector

Patrícia A. G. C. Silva[#]
Xiaohong Jiang[#]
Tim. J. Dalebout
Charles M. Rice
Peter J. Bredenbeek
[#]These authors contributed equally

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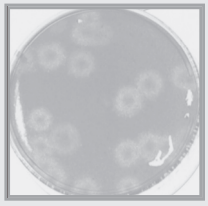
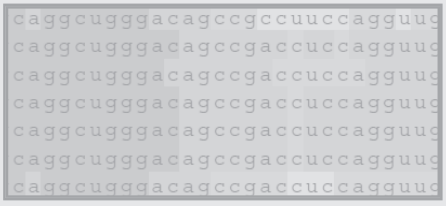



ABSTRACT

The *Flavivirus* genus can be divided into three different groups depending on the vector of transmission: i) mosquito-borne, ii) tick-borne, and iii) no known vector (NKV) flaviviruses. The third group is less-well studied, which in part is due to the lack of full-length cDNA clones that can be used for the transcription of infectious RNA. In this chapter the construction and characterization of a stable infectious full-length cDNA clone of the NKV Modoc virus (MODV) is described. The full-length MODV cDNA was constructed in the low copy number plasmid pACNR1180. An initial screening of plasmids containing full-length MODV cDNAs for the production of infectious RNA transcripts resulted in four plasmids whose transcripts yielded immunofluorescent positive cells. Subsequent analysis revealed that only one of these clones could be used for the production of transcripts with a high enough specific infectivity to allow semi-first cycle analysis of virus replication. This clone (pACNR-FLMODV6.1) was shown to be genetically stable in *E. coli*. The viruses derived from this clone showed similar plaque morphology and growth kinetics as the parental MODV virus. This first infectious cDNA clone for a NKV flavivirus will be a valuable tool to increase our understanding of general and virus-specific characteristics in flavivirus biology.

INTRODUCTION

Phylogenetic analysis of members of the Flavivirus genus revealed three clusters of related viruses that largely coincided with the route of transmission. Apart from the clusters of mosquito- and tick-borne flaviviruses, which contain important human pathogens, the third cluster comprises a less-well studied group of viruses that have been exclusively isolated from bats and rodents and for which no arthropod vector has been implicated in transmission [1,2]. An increasing number of these no known vector (NKV) flaviviruses has been sequenced [3-5], revealing a similar organization of the approximately 10.5 kb long NKV genome as that of the arthropod-borne flaviviruses. The viral genome encompasses one large open reading frame that encodes the following proteins: (5') C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (3'). Similar to other flaviviruses, protease and NTPase/helicase/RNA triphosphatase domains were found in the NS3 N-terminal and C-terminal regions, respectively, of Modoc virus (MODV) [5]. Furthermore, the N-terminal region of the MODV NS5 protein was shown to encode motifs important for methyltransferase activity, while the C-terminal region encoded highly conserved RNA-dependent RNA polymerase (RdRp) domains [5]. The crystal structure of the methyltransferase domain of MODV NS5 was recently determined [6].

The genomic RNA is not polyadenylated; instead it terminates with a large 3' stem-loop structure (SL) that is characteristic for all flaviviruses. Apart from this 3' SL structure, the 5' and 3' UTRs of NKV viruses contain RNA elements that have also been identified and characterized in the UTRs of arthropod-borne flaviviruses, like the conserved sequence (CS) 2 and complementary sequences predicted to be involved in circularization of the viral genome [4,5]. Recently, it has been shown that cells infected with NKV flaviviruses also produce a small subgenomic flavivirus RNA (sfRNA) [7] as was initially reported for arthropod-borne flavivirus infections [8-13]. Synthesis of this sfRNA results from incomplete degradation of the genomic RNA by the host ribonuclease XRN1 [10,12,7].

NKV flaviviruses are divided into three groups: i) the Entebbe bat virus group, which includes viruses isolated from bats like the Entebbe bat virus, Sokuluk virus and Yokose virus (YOKV), ii) the Modoc virus group that comprises viruses isolated from rodents such as Modoc virus (MODV) and Apoi virus (APOIV) and iii) the Rio Bravo virus group encompassing viruses like Rio Bravo virus (RBV) and Montana myotis leukoencephalitis virus (MMLV) isolated from bats [14]. Little is know about how NKV flaviviruses spread among their hosts. It has been postulated that NKV viruses are transmitted by nasal or oral contact between infected and uninfected animals [15-17]. Initially it was thought that these viruses were unable to infect arthropods due to a block at the level of entry. However, recently it has been shown that at least for MODV, the inability to infect arthropods is not at the level of entry, but at a later stage of the viral life cycle [18].

MODV was initially isolated from white-footed deer mouse (*Peromyscus maniculatis)* in Modoc County in California [19] and was shown to cause a persistent infection in rodents [20]. MODV has not been implicated in human disease, although there has been an indirect reference to an apparently fatal infection of a boy. A serological study provided evidence for the occurrence of natural infection without disease among human inhabitants of Alberta [17]. MODV is neuroinvasive and causes lethal encephalitis in SCID mice and hamsters similar to flaviviral encephalitis in humans, making MODV a potential model virus to study flaviviruses infections [21]. The viral prM and/or E proteins have been shown to be important for the neuroinvasive characteristics of MODV in SCID mice [22]. Sequence analysis and comparison of the MODV 3' UTR with the 3' UTR of APOIV, RBV and MMLV revealed four regions with similar secondary RNA structures [4].

The construction of full-length flavivirus genome cDNAs that can be used for the *in vitro* transcription of infectious RNA can be rather challenging, as the plasmid backbone used and/or the bacterial host strain selected can greatly influence the outcome [23]. For some of these viruses like Kunjin virus (KUNV) [24], West Nile virus (WNV) [25], some dengue (DENV) strains [26,27], and tick-borne encephalitis virus (TBEV) [28], this approach has been straight forward enabling the construction of stable, full-length genome cDNA copies in *Escherichia coli*. For other flaviviruses, like YFV and JEV, the *in vitro* production of infectious RNA transcripts required the use of labor-intensive *in vitro* ligation procedures [29-31] or the use of low copy number plasmid vectors [32,33] to circumvent the genetic instability of the full-length cDNA insert in the *E. coli* host. This report describes the construction and characterization of the first stable full-length NKV cDNA clone. RNA transcribed from this MODV clone yielded infectious virus upon transfection of BHK cells. Furthermore, the virus derived from the clone showed similar growth kinetics when compared with the wild-type virus.

MATERIAL AND METHODS

Cell culture and virus

The origin and culture conditions of the BHK-21J cells that were used throughout this study were described before [32]. The Modoc virus strain M455 was obtained from Prof. J. Neyts (Leuven, Belgium) during collaborative research [22] and was originally purchased from the American Tissue Culture Collection (ATCC, Manassas, USA). Stocks of MODV M455 were produced by infecting BHK-21J cells at a multiplicity of infection (MOI) of 0.1 in PBS containing 2% fetal calf serum (FCS) for 1 hr and subsequent incubation at 37°C and 5% CO_2 in DMEM/2%FCS. After 3 to 4 days, depending on the severity of the cytophatic effect (CPE), the medium was harvested and centrifuged at 3000 x g for

5 min to remove cell debris. The supernatant was used as a virus stock. Stocks of the cDNA-derived viruses were obtained by electroporating BHK-21J cells with full-length RNA transcripts [34]. For analysis of the viral growth kinetics, BHK-21J cells were infected at an MOI of 1; medium was subsequently collected and replaced by the same volume of fresh medium at the indicated times. MODV titers were determined by plaque assays on BHK-21J cells as described previously [34], except for the agarose in the overlay, which was replaced by Avicel [35].

Recombinant DNA techniques and plasmid constructions

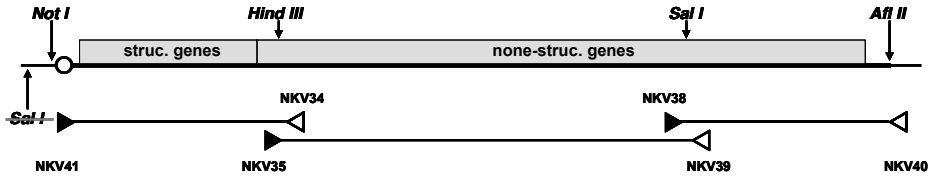
General standard nucleic acid methodologies were used throughout this study [36] unless described in more detail. Chemically competent *E. coli* DH5α cells were used for cloning [37]. Nucleotide numbering of the various constructs containing MODV-derived inserts and the resulting full-length clones was based on the MODV sequence deposited in GenBank (AJ242984; [5]).

MODV cDNA was prepared using a one-step RT-PCR system containing a modified M-MLV reversed transcriptase for the cDNA reaction and a mixture of Taq polymerase and *Pyrococcus* GB-D polymerase (Invitrogen, Carlsbad, USA) for the PCR. The RT-PCR reactions contained 1 μg of total RNA that was isolated from MODV-infected cells at 36 hr post infection (p.i.), using reaction conditions as suggested by the supplier. Oligonucleotides were designed based on the published MODV sequence. The most 5' oligonucleotide (NKV41, fig.1) contained the T7 Φ2.5 promoter [38], so that T7 RNA polymerase driven transcription would start on the "A" residue that is the first nucleotide of the MODV genome. In the oligonucleotide that hybridized to the extreme 3' end of MODV (NKV40, fig. 1) the complement of the two last viral nucleotides were fused to 3'TAAG 5' to yield an unique *Afl II* restriction enzyme site. Relative positions of the oligonucleotides used for the cDNA reconstruction of MODV are depicted in fig. 1.

RNA transcription

Plasmid DNA for *in vitro* run-off RNA transcription was purified using the Nucleobond AX DNA isolation kit. pACNR-FLMODV plasmids were linearized with *Afl II* and purified by proteinase K treatment and phenol/chloroform extraction. Approximately 2 μg of linearized DNA was used as a template for *in vitro* transcription using the Ampliscribe T7 high yield transcription kit (Epicentre, Madison, USA). For the production of 5'-capped full-length MODV transcripts, UTP, GTP and CTP concentration was 7.5 mM, whereas the ATP concentration was 2 mM. G(5')ppp(5')A (NEB, Ipswich, USA) was added as RNA cap

A)



B)

Name	Sequence	Position	Remarks
NKV34	CTGCCAGGAAAGACCATTGCGGCCAG	2719 – 2694	sense
NKV35	GGTATGGGGGTACATGTACTACCCAG	2454 – 2480	anti-sense
NKV38	GTTCCTCTGGAGACCGCATGGCCGGTGT	7716 – 7689	sense
NKV39	GGGCATTTGTTGGAGTGCCCCCACACT	7536 – 7563	anti-sense
NKV40	AGCGCTTAAGCGGAGGTCATATTCATGACCACACAG	10600 – 10573	Contains *Afl II* run-off site sense
NKV41	GACGCGGCCGCAGTAATACGACTCACTATTAGTTGATCCTGCCAGCGGTGGGTCGCTAC	1 – 29	Contains *Not I* site and T7 RNA pol. ϕ 2.5 promoter. anti-sense

Fig. 1. Schematic representation of the construction of pACNR-MODV.
A) The large boxes represent the viral ORF encoding the structural and non-structural proteins. The oligonucleotides that were used to generate the cDNA fragments for constructing the clone are indicated by triangles. The open circle upstream the MODV insert represents the T7 Φ2.5 promoter. The restriction sites that were used to assemble the clone as well as the deleted *Sal I* site are indicted. **B)** Table with detailed information over the sequence, position and orientation of the oligonucleotides that were used in the construction of this clone.

analog to a final concentration of 6 mM. After 2 hr of incubation at 42°C, DNase I was added and the incubation was continued for another 15 min at 37°C. The RNA transcripts were subsequently purified by LiCl precipitation and the concentration was determined by spectrophotometry.

RNA transfection and analysis of viral RNA synthesis

BHK-21J cells were transfected with 5 µg of full-length MODV as described previously [34]. For RNA analysis, 2.5 ml (approximately 1.5 x 10^6 cells) of the transfected BHK-21J cell suspension was seeded in a 10 cm^2 plate. Total RNA was isolated from the transfected cells at 30 hr post electroporation (p.e). Analysis of RNA synthesis by [^{3}H]-uridine labelling was performed as described before [32]. Trizol (Invitrogen, Carlsbad, USA) was used for

cell lysis and subsequent RNA isolation. [^{3}H]-Uridine labelled RNAs were denatured with glyoxal and analyzed on 0.8% agarose gels [36].

Immunofluorescence

At times indicated in the legend of the figures, control and infected cells were washed once with PBS and prepared for immunofluorescence as described previously [32]. Commercially available immune ascitic fluid obtained from mice infected with MODV (ATCC, Manassas, USA) was used in a 1:1000 dilution as primary antibody.

RESULTS AND DISCUSSION

Construction of a full-length MODV cDNA for the *in vitro* transcription of infectious RNA

It is notoriously difficult to construct full-length cDNAs for the *in vitro* transcription of infectious RNA for many flaviviruses due to genetic instability of these clones in *E. coli* [29-31,33]. Therefore, the pACNR1180-derived vector backbone, previously used for the successful construction of a stable full-length YFV-17D clone [32], was selected as the vector for the MODV cDNA inserts. pACNR-FLYF17D$_a$, in which the standard *Xho I* run-off site is replaced by *Afl II*, was cut with *BamH I* and religated. Subsequently the *Sal I* site upstream the Sp6 promoter in this plasmid was inactivated by site-directed mutagenesis. The resulting plasmid pACNR-FLYF17D$_a$ Δ2576-9294 contained unique *Not I*, *Hind III, Sal I* and *Afl II* restriction enzyme sites that were used to assemble the MODV full-length cDNA.

Figure 1 is a schematic representation of the MODV genome and the position of the oligonucleotides used to prime RT-cDNA reactions on MODV RNA isolated from infected cells; the three PCR products that were used to assemble the full-length MODV cDNA are also depicted. Oligonucleotide NKV41 contained the ϕ2.5 promoter for T7 RNA polymerase fused to the most 5′ 19 nucleotides of MODV, whereas in oligonucleotide NKV40, the 28 3′ nucleotides of MODV were fused to a unique *Afl II* recognition sequence, serving as the transcription run-off site. Construction of the full-length MODV clone in pANCR-FLYF17D$_a$ Δ2576-9294 was performed in three steps in the 5′ to 3′ direction during which the remaining YFV-17D sequences were replaced by MODV cDNAs fragments. First, the 2597 bp fragment obtained after *Not I* and *Hind III* digestion of the NKV41 – NKV39 PCR product was cloned into *Not I – Hind III* digested pANCR-FLYF17D$_a$ Δ2576-9294. Subsequently, the 5063 bp *Hind III – Sal I* fragment and the 3′ *2952 bp Sal I – Afl II* fragment were cloned stepwise, to complete the construction of the full-length MODV cDNA. Many

plasmids harbouring what appeared to be a full-length MODV genome cDNA insert based on various restriction enzyme digests were obtained using this strategy.

Identification and characterization of an infectious MODV cDNA clone

Plasmids that appeared to contain a full-length MODV genome cDNA insert were used to produce full-length genomic MODV RNAs by *in vitro* RNA transcription. The RNA transcripts were electroporated into BHK-21J cells; immunofluorescence assay (IFA) were performed 48 hr p.e. for MODV antigen expression. Four (clone numbers 2.4, 4.2, 6.1 and 8.7) out of the ten original clones yielded a clearly positive signal in IFA (data not shown). ^{3}H-uridine labelling of cells electroporated with *in vitro* transcribed RNA of these four plasmids revealed that only clone 6.1 allowed effective first cycle analysis of RNA synthesis (fig. 2). No MODV genome-sized ^{3}H-labeled RNA could be detected in cells electroporated with RNA transcripts from clones 2.4, 4.2 or 8.7. From these results it was concluded that pACNR-FLMODV6.1 contained the most robust template for the *in vitro* production of infectious MODV RNA. This clone and its infectious RNA transcripts were characterized in more detail and compared to the parental MODV virus.

The genetic stability of the full-length MODV6.1 clone in *E.coli* DH5α was analyzed by repeated passaging, i.e., growing the bacteria for more than 12 hr in 2 ml of LB medium plus 50 μg/ml ampicilline, followed by a streak on selective medium to obtain a single colony for the next cycle. After the 10th streak, a bacterial colony was picked and used to prepare plasmid DNA (pACNR-FLMODV6.1-p10). The purified plasmid DNA was digested with various restriction enzymes as an indicator for its genetic stability. No difference in the restriction pattern was observed from the plasmid at passage 10 compared to the original pACNR-FLMODV6.1 DNA (data not shown). Full-length MODV RNA transcripts were then prepared from both pACNR-FLMODV6.1 and pACNR-FLMODV6.1-p10 and

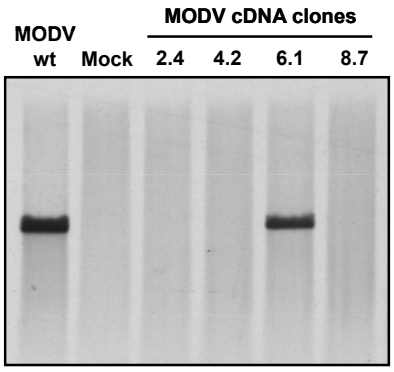


Fig. 2. Analysis of viral RNA synthesis in BHK-21J cells transfected with transcripts of pACNR-MODV clones 2.4, 4.2, 6.1 and 8.7.
BHK-21J cells that were electroporated with full-length MODV transcripts were labelled with ^{3}H-uridine from 24 – 30 hr post transfection. Total RNA was isolated and analyzed after denaturation by agarose gel electrophoresis as described in the Materials and Methods section. BHK-21J cells infected with the parental MODV virus (wt) were used as a control.

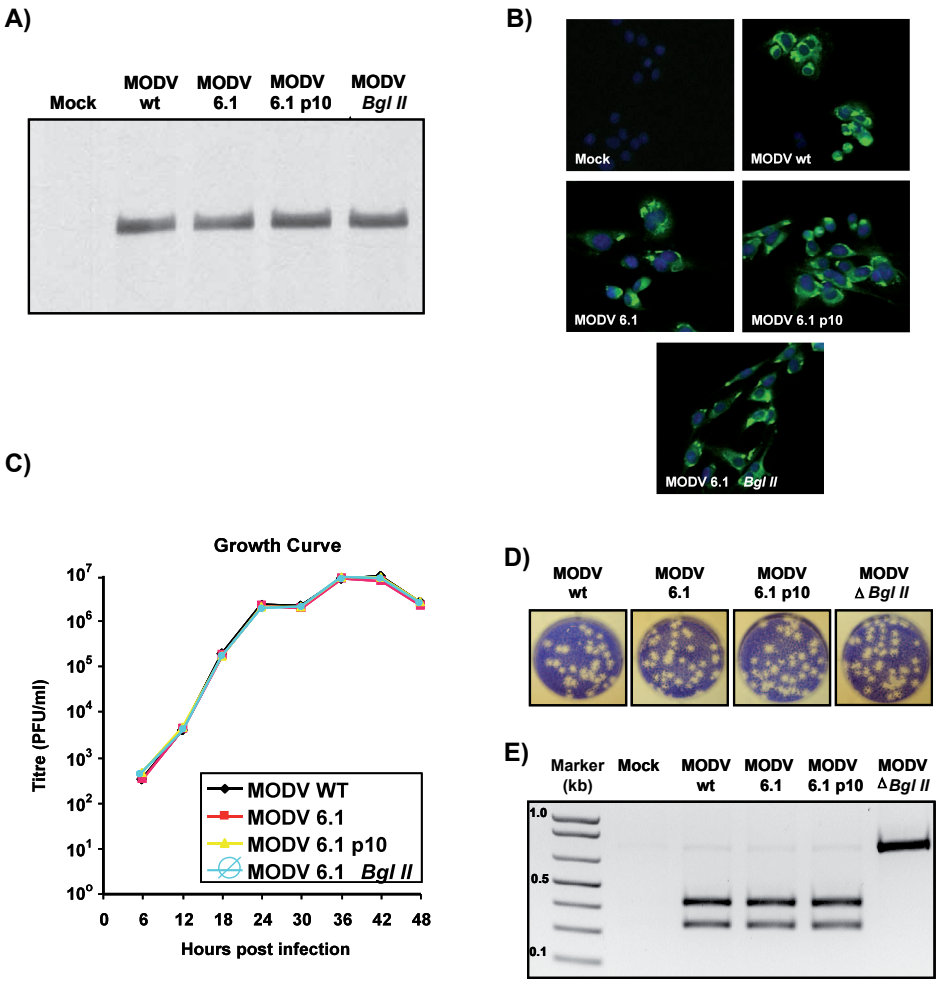


Fig. 3. Characterization of the pACNR-MODV6.1 infectious cDNA clone.
A) Viral RNA synthesis in BHK-21J cells transfected with *in vitro* transcribed RNA of the pACNR-MODV 6.1, 6.1-p10 and 6.1Δ*Bgl II*. Transfected cells were labelled with ^{3}H-uridine from 24 - 30 hr post transfection. Total RNA was isolated and analyzed after denaturation by agarose gel electrophoresis as described in the Materials and Methods section. BHK-21J cells were infected with the parental MODV virus as a control. **B)** Immunofluorescence staining of BHK-21J cells that were infected with wt-MODV or transfected with full-length MODV RNA derived from pACNR-MODV6.1 and related plasmids. Cells were fixed at 30 hr p.e. and stained with the MODV hyperimmune serum. **C)** Viral growth kinetics; BHK cells were infected with a MOI of 1; the medium of the infected cells was harvested at the indicated times p.i. and the viral titer was determined by plaque assays. **D)** Plaque morphology of the wild-type MODV, pACNR-MODV6.1, pACNR-MODV6.1 p10 and pACNR-MODV Δ*Bgl II* in BHK-21J cells. **E)** *Bgl II* restriction analysis of the RT-PCR products generated from RNA isolated from BHK-21J cells infected with the indicated viruses. The sizes of the DNA marker (lane 1) are given on the left side.

electroporated into BHK-21J cells. As a control, BHK cells were infected in parallel with the parental MODV virus. Electrophoresis of ^{3}H-uridine-labelled total intracellular RNA isolated from MODV-6.1-p10 transfected cells, showed the synthesis of MODV genome RNA that co-migrated with the ^{3}H-uridine labelled genome RNA isolated from MODV-infected or MODV6.1 RNA transfected cells (fig. 3.A; lanes 2, 3 and 4). IFA on the electroporated cells with the MODV hyperimmune serum as the primary antibody indicated similar transfection efficiency with pACNR-FLMODV6.1 and pACNR-FLMODV6.1-p10 (fig. 3.B). These results confirmed high specific infectivity as well as genetically stability of pACNR-FLMODV clone 6.1. Furthermore, viruses derived from both pACNR-FLMODV6.1 and pACNR-FLMODV6.1-p10 showed similar growth kinetics when compared to the wt MODV (fig. 3.C). No differences in plaque morphology were observed (fig. 3.D).

In order to rule out the possibility of accidental cross-contamination of BHK-21J cells with the parental MODV virus, thus the obtained results, a mutation was introduced at position 8756 of pACNR-FLMODV6.1, abolishing the *Bgl II* as a genetic marker. The mutation was silent and did not impair viral RNA synthesis (fig. 3.A, lane 5), infectivity of the RNA transcript (fig. 3.B) or viral growth kinetics (fig. 3.C). The wt MODV, MODV6.1, MODV6.1-p10 and MODV6.1Δ*Bgl II* virus stocks harvested for the growth curve experiment (fig. 3.C) at 48 hr p.i. were used to infect BHK-21J cells at an M.O.I. 5. At 30 hr p.i., total intracellular RNA was isolated; a fragment spanning MODV nts. 8435 to 9176 was amplified by RT-PCR, followed by *Bgl II* digestion. As shown in fig. 3.E, the MODV6.1Δ*Bgl II* still lacks this restriction site, demonstrating that the infectious clone was stable and the viruses generated by transfection of permissive cells with transcripts of pACNR-FLMODV6.1 were indeed derived from the cDNA.

The nucleotide sequence of clone 6.1 was determined. Compared to the MODV sequence AJ242984 available in GenBank, 22 nucleotide differences were identified in clone 6.1. As summarized in table 1, these differences were present throughout the coding sequence and five of them corresponded to silent mutations. Although the E protein is the most variable between flaviviruses, only one amino acid difference was observed when comparing the MODV GenBank sequence with clone 6.1. Apart from a high variation in NS5, the largest protein, a relatively high number of amino acid changes were detected in the rather small and generally well conserved NS4B protein (around 254 amino acids). The function of NS4B in the flavivirus life cycle remains to be established, although studies have implicated the involvement of NS4B in modulating the host interferon response [39,40]. None of the mutations found in NS3 or NS5 were part of the functional domains proposed for these viral proteins [5]. Taken together, clone pACNR-MODV6.1 generated in this study can yield viable and infectious MODV particles, and is comparable to wild-type MODV regarding infectivity, plaque morphology and growth kinetics.

Table 1. Summary of the nucleotide differences between the MODV AJ242984 and the MODV clone 6.1 sequences. Changes are grouped by encoded viral proteins. Positions, actual nucleotide change as well as the amino acid substitutions are shown. Nucleotide or amino acid to the right indicates MODV NCBI AJ242984 followed by the nucleotide or amino acid encountered in the MODV6.1 genome.

Gene	Position	Nucleotide	Amino Acid
prM	610	U → C	silent
Env	1543	U → G	Phe → Leu
NS1	2776	C → U	silent
	3089	C → A	Gln → Arg
NS2A	3529	A → G	Ile → Met
NS2B	4410	A → G	Glu → Gly
NS3	4861	A → G	silent
	6312	U → A	Leu → Gln
NS4B	6837	U → C	Ile → Thr
	7098	G → A	Ser → Asn
	7444	C → G	silent
	7445	C → G	Leu → Val
	7503	U → A	Leu → His
NS5	7756	G → A	silent
	7767	G → C	Ser → Thr
	7938	G → C	Arg → Thr
	8141	C → G	Arg → Ala
	8142	G → C	Arg → Ala
	8612	C → G	Gln → Glu
	8920	U → A	Ser → Arg
	9120	A → G	Lys → Arg
	9990	A → G	Asp → Gly

The strategy to clone the MODV inserts directly into the low copy number plasmid pACNR1180, which is known to accept viral sequences that are not well tolerated by *E. coli* [41,32] probably contributed significantly to the successful construction of clone 6.1. pACNR-FLMODV6.1 proved to be stable upon repeated passages in *E.coli*. Attempts to clone the full-length MODV insert from pACNR-FLMODV6.1 into high copy number plas-

mids like pBluescribe or pUC has met no success thus far and only resulted in plasmids that contained recombined MODV inserts harbouring deletions (unpublished results). The pACNR-FLMODV6.1 clone will be a useful tool to increase our understanding of the molecular determinants important for flaviviral replication and tropism. The biological relevance of predicted conserved RNA structures in NKV viruses can now be studied using a reverse genetic approach. Another useful application for this clone is the construction of chimeric viruses between NKVs and arthropod-borne flaviviruses that can provide valuable insights into host-range restrictions and pathogenicity, as illustrated by studies using a yellow fever virus in which prM and E were replaced by the analogous proteins of MODV [22,18]. In addition, this clone and the viruses derived from it are likely to be useful in the screening of antiviral compounds. A very convenient model to monitor MODV infection in the Syrian hamster has been developed to test small molecules with anti-flavivirus activity in a relevant small animal model [21]. The availability of this MODV infectious clone will be a valuable addition to these studies by allowing a more comprehensive analysis of viral resistance.

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