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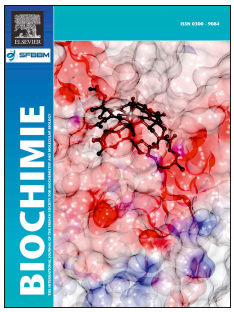

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Title

Template requirements of Zika RNA polymerase during *in vitro* RNA synthesis from the 3'-end of virus minus-strand RNA.

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Abstract

As ZIKV continues to spread, many “unknowns” remain and research is needed to advance the understanding of this important pathogen. Viral RNA dependent-RNA polymerases (RdRp) are validated targets for inhibitors of the replication of several viruses. Several studies have set up *in vitro* enzymatic assays of the RdRp of the Zika virus for testing of candidate inhibitors. While most of these studies use short synthetic polymers, we have shown in a previous work that the Zika polymerase domain is capable of a *de novo* synthesis of the viral genome using the natural viral RNA as template. Here we have studied the role of the sequences at the 3’end of the minus-strand RNA in the initiation of the RNA synthesis by the Zika isolated RdRp. Our results strongly suggest that the region containing the 105 first nucleotides from the 3’ end of the minus-strand RNA is important for initiation of the positive RNA synthesis. This indicates that this region displays all the primary and secondary structures to be efficiently recognized by the recombinant RdRp *in vitro*. Moreover, we show that the 46 nucleotides are sufficient to initiate RNA synthesis. In addition, the ZIKV polymerase domain poorly replicated the RNA of other RNA viruses and appeared highly selective for its own RNA.

Key words

Zika virus; RNA polymerase; *in vitro* assay; template requirement.

1. Introduction

Zika virus (ZIKV) is a *Flavivirus*, which together with the genus *Pestivirus* and *Hepacivirus*, integrates the *Flaviviridae* family [1]. Flaviviruses such as Dengue, Japanese encephalitis, West Nile, Yellow Fever, and ZIKV have become an increasing public health concern over the last decades since their global incidence has grown dramatically [2]. ZIKV was first identified during the monitoring of Yellow Fever in 1947 in Uganda [3], then detected in Micronesia in 2007, French Polynesia in 2014, and finally Latin America in 2015 [4].As for other flaviviruses, ZIKV is transmitted to humans by an arthropod vector, namely mosquitoes from the *Aedes* genius. Some transmission directly through sexual contact has been reported [5]. Infectious virus can be detected in semen and vaginal secretions, along with other body fluids. Therefore, ZIKV has the unusual capacity among arboviruses to persist, in at least a limited manner, as a sexually transmitted infection.

Initially, ZIKV infection was considered a minor ailment as most individuals were asymptomatic (in approximately 80% of cases). However, during the 2015 outbreak in Brazil, the incidence of microcephaly surged with a 20-fold increase in the number of cases observed [6]. Later studies revealed that pregnant women infected with ZIKV can transmit the virus to the fetus, causing fetal loss, microcephaly and other serious brain defects in newborns. Similarly, neurological affections were also reported in some infected adults developing Guillain-Barre syndrome (GBS) [7]. GBS is clinically defined as an acute peripheral neuropathy causing limb weakness that progresses over a time period of days or, at the most, up to 4 weeks. The prevalence of GBS is about 20-fold higher in Zika infected patients [7], which strongly suggests that the ZIKV infection could be a strong trigger for the development of GBS. Other neurological complications associated with ZIKV infection including encephalitis, meningoencephalitis, and acute myelitis have also been reported [8].

The geographical distribution of ZIKV has expanded globally. The explosive spread of the virus through the Americas and the increasing evidence of neuropathic effects associated with ZIKV infections have caused great concern and prompted the WHO to declare Zika a global public health emergency of International Concern (PHEIC) on February 1st, 2016 [9].ZIKV spread is predicted to continue due to favorable conditions that include a large, mobile population, favorable climate for vector transmission, and widespread occurrence of mosquito species capable of ZIKV transmission [3].

Despite all the possible tragic consequences of ZIKV infection in humans, there is currently no specific vaccine or treatment to stop the global spread of this pathogen [10]. Therefore, there is an urgent need to study the molecular and cellular replication of the virus in order to develop a targeted therapy. The ZIKV genome is a single-stranded (ss) positive sense (+) RNA of ~10.7 kilobases in length that contains one large open reading frame flanked by highly structured 5′ and 3′ untranslated regions (UTRs). The 5′UTR contains a methylated 5′ cap structure essential for translation [11]. The genomic RNA encodes a single polyprotein that is cleaved to form seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and three structural proteins (Capsid, precursor-Membrane (prM), Envelope (E))]. Non-structural proteins assemble into replication complexes (RC) associated with cellular membranes (D) and carry out viral RNA replication [12]. First, the genomic RNA serves as template for the synthesis of a complementary strand. Second, this (-) sense RNA is a replication intermediate subsequently used to generate back large amount of (+) sense RNAs. Finally, these full-length RNAs will have three possible destinations: i) re-enter the replication loop and serve again as templates for the generation of additional negative copies, ii) act as messengers for protein synthesis and iii) undergo packaging inside of the capsid and assemble with other viral proteins to produce new virions [12].

The viral enzyme responsible for this RNA dependent RNA polymerase (RdRp) activity is the NS5 protein. Recent *in vitro* studies have shown that the isolated polymerase domain of NS5 (depleted from the methyl-transferase activity in its amino terminal domain) is sufficient to initiate *de novo* RNA synthesis, which is a mechanism similar to that of other members of the Flaviviridae family. In the past few years, biochemical studies have demonstrated that specific RNA structures were required for the recruitment of the polymerases on the positive strand RNA [13]. Yet, even if (+)RNA viruses share fundamental similarities in the way they replicate their genomes, the molecular determinants that modulate the RdRp activity remain largely unknown. Hence, it appears crucial to study how ZIKV polymerase and in general Flaviviruses polymerases recognize their own genomic RNAs compared to other RNAs present in the cell such as cellular mRNAs. Additionally, the replication of the negative strand to recover the positive genomic strand is poorly studied and little is known about the recognition domains of the 3'end of the negative strand by the polymerase. In the present work, using model RNAs and recombinant polymerase, we describe the selectivity of the Zika RdRp toward its own RNA, which is required to efficiently synthesize the positive viral strand.

2. Material and methods

Oligonucleotides were purchased from Eurofins Genomics. Zika RdRp was purified as described previously in [14].

2.1 Amplification of the DNA templates

Viral RNA templates were obtained by *in vitro* transcription of corresponding DNA templates. For transcription of the RNAs of ZIKV (-)5'UTR, a DNA fragment containing the ZIKV sequence 1-269 strain SPH2015 was obtained by gene synthesis. The DNA templates used for transcription of the cognate RNA were amplified by PCR using a primer with the T7 promotor sequence upstream the forward primer, and a reverse primer. The primers used are presented in Table 1. For the synthesis of Chikungunya virus RNA, the last 209 nucleotides (nucleotides 11617-11826, (5'(+)UTR), were *in vitro* transcribed. The HIV-1 RNA (3'(+)UTR) was obtained by *in vitro* transcription of the region 9406-9541 of HIV-1 NL4-3. Finally, region 1 to 157 (positive strand) of HCV H77 was cloned and transcribed in an RNA corresponding to nucleotides 9442-9549 of the (-)5'UTR. The PCR reaction was carried out by the Phusion Hot Start Flex polymerase (BioLabs). The reaction mixture was incubated at 98ºC for 30 s, followed by 30 cycles of 98ºC for 10 s, 72ºC for 30 s, and 72ºC for 6 min. The amplified DNA was purified on an Illustra MicroSpin® S-400 column followed by phenol / chloroform extraction, and finally re-suspended in 12 µl of DEPC water (water treated with diethyl pyro carbonate) for *in vitro* transcription. The PCR product was verified by agarose gel electrophoresis (0.8% w / v) in TBE buffer.

2.2 RNA templates.

In vitro transcription of the RNAs was performed using the MEGAscript® (Ambion) kit. The reaction mix containing the T7 RNA polymerase enzyme, the ribonucleotides (GTP, UTP, CTP, ATP) and the proper reaction buffer as indicated by the manufacturer was incubated at 37ºC with DNA in conditions indicated in experiments. The DNA template was then digested with RNase free DNase for 15 minutes at 37ºC. The RNAs thus obtained were extracted with phenol /chloroform and subsequently precipitated with 1 volume of isopropanol and 0.5 M of ammonium acetate. Once precipitated, the RNAs were washed with 70% ethanol, then re-suspended in 50 µl of DEPC water and finally passed through a G25 column in order to remove unincorporated ribonucleotides. The size, concentration and integrity of transcribed RNAs were

verified on a chip (RNA 6000 Nano) by capillary electrophoresis (Agilent 2100 Bio analyzer, supplemental data). All RNAs were also *in vitro* transcribed in the presence of [α-^{32}P]UTP to be used as markers.

2.3 RdRp assay.

Reactions were performed as described in [14] for 2 hours at 30°C with purified RdRp (7 pmoles) in a reaction buffer containing 20 mM Tris-HCl pH 8.0, 1 mM DTT, 3 mM $MnCl_2$ and RNasin (13.2 U, Promega), RNA templates (1.5 pmoles), rATP and rCTP (0.5 mM each), [α^{32}P]-GTP (5 µCi, 3000 Ci/mmole) and [α^{32}P]-UTP (5 µCi, 3000 Ci/mmol).

Reactions were stopped by addition of 7 M urea. Products were purified by addition of phenol/chloroforme/isoamylalcool (25:24:1, v:v:v) and subsequent nucleic acids precipitation. After washing and drying of the pellet, products were resuspended in loading buffer [formamide containing 1% sodium dodecyl sulfate, 0.25% bromophenol blue, and xylene cyanol] (volume sufficient to obtain 10,000 cpm/µl). Products (50,000 cpm) were separated on 12% polyacrylamide denaturing sequencing gels. Autoradiography was performed using an imaging plate (Fujifilm) and images were obtained with a FLA-5000 Imaging System (Fujifilm). The quantification of the synthesized products was made by densitometric measurement of the bands using Quantity One software. Synthesis in the presence of the Zika RNA template was normalized to 100%.

3. Results

In a previous work, we described the bacterial expression and purification of the NS5 isolated RdRp domain and performed the biochemical characterization of its enzymatic activity [14]. Notably, we found that the isolated RdRp domain is capable of *de novo* synthesis using specific RNA templates mimicking the natural viral UTR substrate [14]. *In vitro* and likely *in vivo*, the RdRp initiates RNA synthesis without a primer and was proficient at replicating a 153 nucleotide-long template corresponding to the 3'end of the (-) strand ZIKV RNA (Figure 1B, lane 2). This *de novo* mechanism needs specific interactions between the polymerase and viral RNA primary, secondary or tertiary structures. Our aim was to identify genomic elements of the minus strand RNA involved in the initiation of positive RNA synthesis. For this purpose, we generated RNAs corresponding to the complementary region of the 5' end of the genome by *in vitro* transcription and used them as template for replication assays using the recombinant polymerase (Table 1). In order to evaluate the capacity of the (-)UTR *strictus senso* to be

replicated by the polymerase, we produced a RNA corresponding to the complementary region of the 5'UTR, (nucleotides 1 to 105 of the negative strand, Figure 1A). This template was replicated by the polymerase, generating a major product at the expected size (Figure 1B, lane 3). Nonetheless, some minor secondary products of higher size could also be observed. Altogether, the 3'end of the minus RNA is sufficient for initiation of positive strand synthesis (Figure 1B, lane 3).

To better understand the requirements for initiation of RNA synthesis on Zika genomic RNA in the (-)5'UTR, RNA templates containing deletions of the 3'end of the minus RNA strand were used. These fragments corresponded to nucleotides 1 to 68 and 1 to 46 of the (-)5'UTR. The region 1 to 68 was successfully replicated by the polymerase as a product migrating at the expected size was observed (Figure 1B, lane 4). However, an additional band of lower mobility was also generated corresponding to an unidentified product (apparent size of approximately 90-100 nt). Reduction of the template to the 46 first nucleotides resulted in the appearance of a smear around and above the expected size of a 46 nt product (lane 5). This suggests that the region 1 to 105 allows the initiation of the positive RNA strand synthesis.

To evaluate the role of the 3' end in the initiation process, we deleted the first 31 nucleotides in 3'of the (-)5'UTR (RNA 32-105). Using this 73 nucleotide-long template, a large smear was observed as in the context of the RNA 1-46. (Figure 1B, lane 6). Thus, the extremity of the (-)5'UTR appeared to be essential to correctly replicate the template. In addition to the products corresponding to the size of the template, products of higher or lower mobility were observed. These templates could generate secondary or tertiary structures that might be responsible for the synthesis of those products.

In parallel, several studies reported that the (+)5'UTR was not sufficient to have efficient replication of HCV replicons in cells. Instead, the presence of extra sequence corresponding to the beginning of the coding sequence (corresponding to the capsid) was required to facilitate the *in vivo* replication of the flavivirus genome. To determine whether this requirement is related to the cellular context or an intrinsic feature of the polymerase, we produced a second series of templates to study *in vitro* the role of this upstream region. In details, two templates were generated harboring the (-)5'UTR plus either 46 or 162 extra-nucleotides corresponding to the complementary sequence of the capsid coding region (RNA 1-153 and 1-269, respectively). Both resulting RNAs were successfully replicated, generating a single band-forming product at the expected size (Figure 1B, lanes 2 and 9). The overall quality of the reaction appeared to be increased by the addition of these capsid-derived sequences (decrease in the quantity of synthesis intermediates, Figure 1B compare lanes 2 and 9 to lane 3,

respectively). However, the intensity of the bands was clearly lower than that of the product generated with the (-)5'UTR alone. As a control, two other RNAs were generated lacking the (-)5'UTR region, RNA 106-153 and 106-269, Figure 1A. Using RNA 106-153 as template, the polymerase produced various products as multiple bands and smears were observed but no signal could be detected at the expected size of 47 nt (Figure 1B, lane 7). A similar result was obtained using RNA 106-269 as template, with a main product having an apparent slower mobility than the expected 163 nt (+) copy (Figure 1B, lane 8). Thus, it appears that sequences downstream of the 5'UTR are not involved in the initiation process *in vitro*, but may be involved in specific structures of the RNA that may prevent shuttering.

Since the ZIKV RdRp requires some determinants on the (-)5'UTR RNA to initiate the plus strand synthesis, one would expect a high sequence selectivity for its own RNA compared to that of other viruses. To verify that hypothesis, the capacity of the ZIKV RdRp to synthesize RNA using the cognate RNA of other (+)RNA viruses was evaluated as described in Material and methods (Figure 2). The 3' (+)UTR of Chikungunya virus (last 209 nucleotides corresponding to nucleotides 11617-11826) was a sub-optimal template as only 39 % of the RNA synthesis activity was observed compared to the canonical ZIKV sequence (Figure 2B). The ZIKV polymerase was inefficient at replicating the HIV-1 LTR region (nucleotides 9406-9541 of the RNA 3' end) with only 17 % of the RNA synthesis activity as compared with the ZIKV RNA used as template, corresponding to a decrease of approximately 6-fold in the quantity of RNA produced. More drastically, an even poorer incorporation of radioelement was observed with the (-)5'UTR of the HCV genome (nucleotides 9442 to 9549 of the negative strand) used as template (Figure 2A, lane 2). Thus, the ZIKV RdRp domain appeared highly selective for RNA templates that mimicked its own RNA, which is in agreement with the existence of sequence/structural determinants. Interestingly, those determinants might be partially conserved within templates mimicking the RNA of other arboviruses such as Chikungunya.

To assess this result, chimeric RNAs were generated between the ZIKV and the HCV RNAs (Figure 3A). In agreement with previous data, the (-)5'UTR of HCV (nucleotides 9442-9549 of (-) strand) was poorly replicated by the Zika polymerase (Figure 3B, lane 7). Addition of the first 105 nucleotides of the 3'end of the ZIKV (-)5'UTR enabled the synthesis of a 262 nt long RNA by the Zika RdRp, corresponding to the expected size of the chimeric product (Figure 3B, lane 4). Thus, the addition of the first 105nt of the ZIKV (-)5'UTR upstream of the HCV RNA seemed to stimulate the use of this template. Addition of only 68 nt to the 5' end of

(-)5'UTR of HCV also restored the synthesis of a product of synthesis at 225 nt (Figure 3B, lane 5). Another template was constructed where the 68 nucleotides of ZIKV (-)5'UTR were replaced by the 46 nucleotides corresponding to the ZIKV (-)5'UTR RNA. Figure 3B, lane 6). Still, the reaction led to the synthesis of a major product at the expected size of around 203 nt. Altogether, these results indicated that the ZIKV RdRp requires at least the 46 first nucleotides to initiate replication of the positive strand.

4. Discussion

The present study aimed at deciphering the *in vitro* mechanism of RNA synthesis initiation by the ZIKV RdRp using various templates as substrate. By analogy with other RNA viruses and particularly with viruses of the same *Flaviviridae* family, we may assume that this region of the ZIKV genome contains domains important for the initiation of the replication intermediate minus strand RNA. We show here that the 3' end of the Zika minus strand RNA is efficiently copied *in vitro* by recombinant ZIKV RdRp and gives rise to one major species of RNA. These results were obtained with templates corresponding to the first 105, 153 or 269 nucleotides located at the 3' end of the minus-strand RNA [(-)5'UTR]. Deletion of the 105 first nucleotides generated products of heterogeneous sizes, each with an electrophoretic mobility different from that of the template. When the first 68 nucleotides were used as substrate, low level of RNA synthesis at the expected size was observed with products of higher and lower mobility. Furthermore, the isolated RdRp was active using an even shorter template of 46 nt, pointing to a role for the 3' end in the substrate-enzyme recognition mechanism. Interesting results were obtained when the polymerase activity was assayed on a heterologous viral RNA. The extremities of the RNA of HIV (used as a template by the RT for the initiation of the first DNA strand, the so-called "strong stop DNA") is poorly recognized. On (-)3'end of Chikungunya Virus RNA, another positive RNA genome but not belonging to flaviviruses, the synthesis reaches 39 % of that obtained on Zika RNA. In addition, ZIKV RdRp does not recognize the 3'(-)end of HCV, another *Flavivirus*. Interestingly, the RNA synthesis was restored when the first 105, 68, or even 46 nucleotides of the Zika were added in fusion to the HCV RNA. This suggests that the addition of RNA downstream the first 46 nt of the ZIKV (-)5'UTR stimulates the use of this (-)5'UTR as starting template. Yet, the structure of this chimera might not reflect the structure of the ZIKV (-)5'UTR.

In vivo, cellular proteins and other viral enzymes that are part of the replication complex could also modify the structure of the viral template allowing a correct initiation. Flaviviruses genome can adopt a circular conformation facilitated by long-range RNA–RNA interactions between the 3' and 5' end of the genome (of same polarity). Studies of Sanford et al [15] showed

that genome circularization inhibits translation initiation on ZIKV and facilitates genome replication. The formation of the secondary structure allowing circularization mainly involves the region encompassing nucleotides 87 to 108 of the (+)5'UTR, and regions found at the 3'end of the same molecule [(+)3'UTR]. The corresponding regions of the negative genome might also be involved in such hybridization, which could explain why we found the region 1 to 105 to be important for synthesis from the (-)RNA. The region downstream from the AUG initiation codon, and particularly the region DAR ("downstream of AUG region") is also involved in hybridization, leading to circularization of the positive genome. Yet this region has been reported to be important for replication in the vector (*Aedes* mosquitoes) but not in mammalian cells. This suggested that the presence of cellular factors altered the recognition of such DAR-less substrates while the RdRp was actually proficient for RNA synthesis. Altogether, this would explain why the region upstream the (-)5'UTR is not essential for polymerisation [16].

RNA synthesis using several RNAs (RNA 1-105; RNA 1-46; RNA 32-105) as template led to the synthesis of a major product that is identical in size to the template, but also less abundant products with sizes slightly longer or shorter than the template were observed. This could reflect a mechanism of stuttering of the enzyme. The stuttering mechanism occurs after a pause of the polymerase that slips backwards together with the nascent RNA before the next base could be incorporated. This mechanism implies that the viral polymerase reiteratively inserts the same base generating a product longer than the template.

5. Conclusion

Altogether, these results indicated that the ZIKV RdRp requires a template containing at least the 46 first nucleotides from the 3' end of the minus-strand RNA to initiate the replication of the RNA. This indicates that this region displays all the primary and secondary structures required to be recognized by the recombinant RdRp.

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Authors contributions: Conceived and designed the experiments: CC, MLA, MM. Performed the experiments: CC; Analyzed the data: CC, MLA, MM; Wrote the paper: CC, MLA, MM.

Competing interest statement

The authors declare that they have no competing interests.

Figures legends

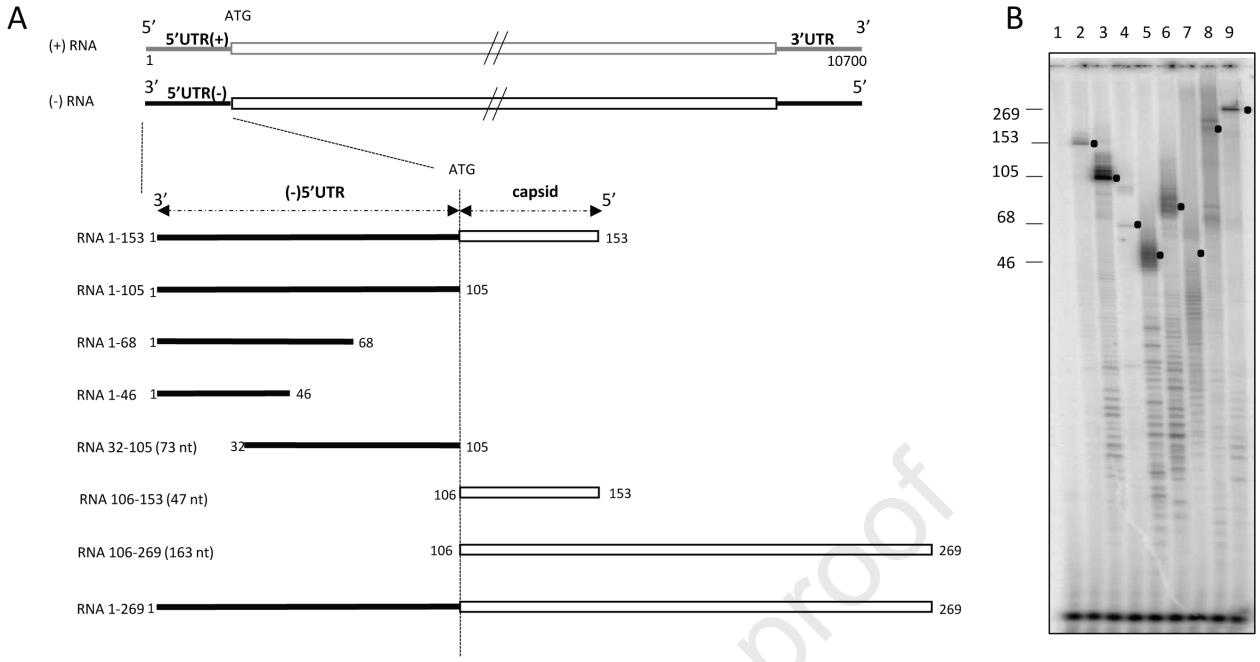
Figure 1. ZIKV RdRp *de novo* replication of various cognate RNA templates. (A) Schematic representation of templates. RNA is labelled in 3' to 5' orientation starting from nucleotide 1 of the (+) strand. (B) ZIKV RdRp activity using different RNAs as template. Lane 1: no enzyme. Lane 2: RNA 1-153, Lane 3: RNA 1-105, Lane 4: RNA 1-68, Lane 5: RNA 1-46, Lane 6: RNA 32-105; Lane 7: RNA 106-153. Lane 8: RNA 106-269. Lane 9: RNA 1-269. Black marks represent the expected migration position of the products.

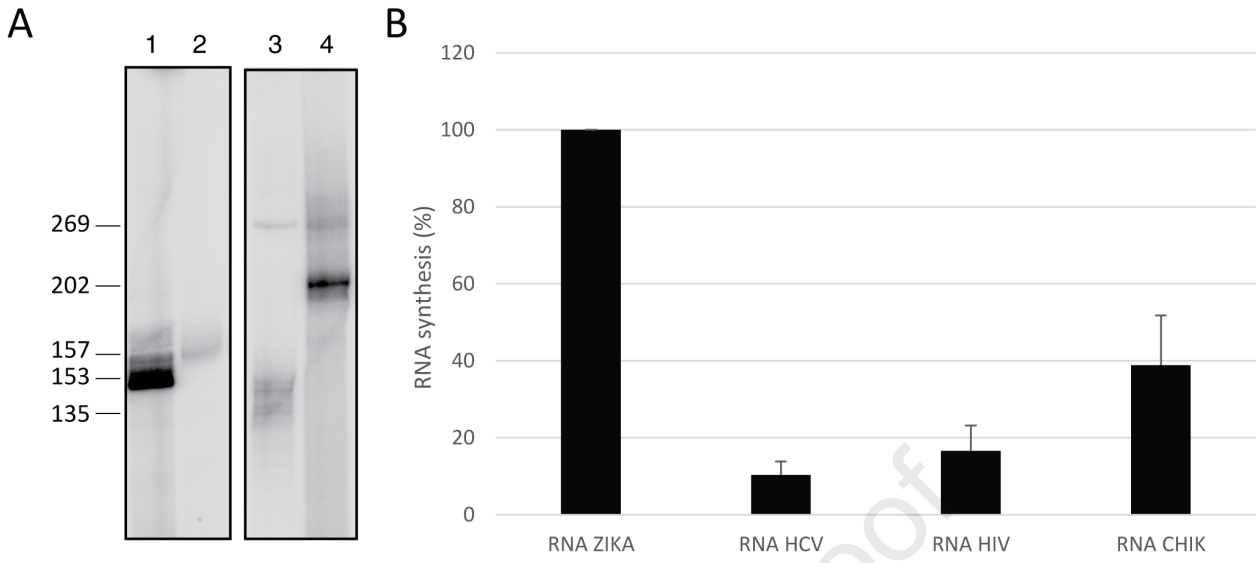
Figure 2. ZIKV RdRp activity using RNA templates deriving from other viruses. (A) Representative gels showing the activity of the ZIKV RdRp using RNA templates mimicking different viruses. The two parts of the gel are from the same gel (uncropped image of the gel is displayed in Supplemental Figure S2). Lane 1: RNA ZIKA 1-153; Lane 2: RNA (-)5'UTR HCV 1-157; Lane 3: RNA HIV 1-135; Lane 4: RNA CHIK 1-209. (B) Quantification of the level of RNA synthesis (densitometric analysis of the band at the expected size) catalyzed by ZIKV RdRp Densitometric analysis were performed using Quantity One software (Bio-Rad). Means and standard deviation derived from three independent determinations.

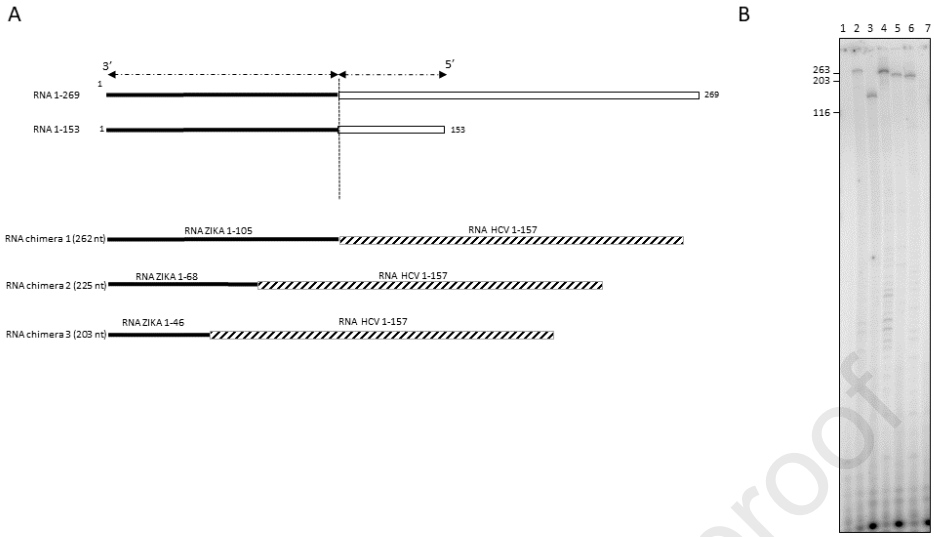
Figure 3. Effect of RNA chimeric assembly on ZIKV RdRp *in vitro* activity. (A) Schematic representation of the chimeras. (B) RNA synthesis on the ZIKV/HCV RNA chimeras. Lane 1: no enzyme. Lane 2: Zika RNA 1- 269. Lane 3: Zika RNA 1-153. Lanes 4, 5, 6: RNA chimera 1, 2, 3, respectively. Lane 7: HCV RNA 1-157.

Table 1: Synthesis of RNA templates.

	RNA template name	reverse primer	Primer S-T7	Target	Transcribed RNA	Size of the RNA template (nt)
ZIKA	1-153	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGGCATATTGACAATCC	(-)5'UTR ZIKV	10794-10641	153
	1-105	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGGACCAGAAACTCTCGTTTCCAA	(-)5'UTR ZIKV	10794-10689	105
	1-68	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGGGTTGATACTGTTGCTAGCTTTC	(-)5'UTR ZIKV	10794-10726	68
	1-46	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGGCTTCAAACTCGAACTGTCG	(-)5'UTR ZIKV	10794-10748	46
	32-105	GTTCGAGTTTGAAGCGAAAG	TAATACGACTCACTATAGGGACCAGAAACTCTCGTTTCCAA	(-)5'UTR ZIKV	10762-10689	74
	106-153	ATGAAAAACCCAAAAAAGAAATCC	TAATACGACTCACTATAGGGCATATTGACAATCC	(-)5'UTR ZIKV	10688-10641	48
	106-269	ATGAAAAACCCAAAAAAGAAATCC	TAATACGACTCACTATAGGGCTCAAAAAGGCTAGAATTGCCAAG	(-)5'UTR ZIKV	10688-10525	164
	1-269	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGGCTCAAAAAGGCTAGAATTGCCAAG	(-)5'UTR ZIKV	10794-10525	269
HCV	1-157	GCCAGCCCCCTGATGGGGG	TAATACGACTCACTATAGGTTCCGCAGACCACTATGGC	(-)5'UTR HCV	9442-9549	157
HIV	1-135	CCAGAGAGACCCAGTACAGG	TAATACGACTCACTATAGGACATCGAGCTTGCTACAAG	3'UTR (+) HIV	9406-9541	135
CHIK	1-209	GAAATATTAAAAACAAAATAACATCTCCTACGTC	TAATACGACTCACTATAGGGCTATAAAACCCCTGA	3'UTR (+) CHIKV	11617-11826	209
Chimera 2	1-68 ZIKA/1-157 HCV	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGTTCCGCAGACCACTATGGC	(-)5'UTR ZIKV / (-)5'UTR HCV		225
Chimera 3	1-46 ZIKA/1-157 HCV	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGTTCCGCAGACCACTATGGC	(-)5'UTR ZIKV / (-)5'UTR HCV		203
Chimera 1	1-105 ZIKA/1-157 HCV	GTTGTTACTGTTGCTGACTCAGAC	TAATACGACTCACTATAGGTTCCGCAGACCACTATGGC	(-)5'UTR ZIKV / (-)5'UTR HCV		262







- *The process of Zika virus (ZIKV) replication is not well understood.*
- *This work reports the characterization of the RNA requirements to serve as good template for the RNA synthesis initiation by ZIKV NS5-polymerase domain.*
- *The role of the sequences at the 3'end of the minus strand RNA was studied.*
  The region containing the first 68 nucleotides from 3' end of the ZIKV minus strand RNA is necessary and sufficient for the initiation of the positive RNA synthesis.

Competing interest statement

The authors declare that they have no competing interests.