GENETIC VARIANTS OF THE CHIKUNGUNYA VIRUS

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SUMMARY

Chikungunya virus is a mosquito-borne human pathogen and therefore must develop strategies to replicate in these two different hosts. It achieves this because its RNA genome is very prone to incorporating changes. Recombination by mould change occurs with high frequency during the synthesis of the viral genome. In this process, the viral polymerase dissociates from the template and reassociates in a second molecule to continue the synthesis of the genome. This strategy allows the virus to generate a collection of variants that make up the viral population.

Using reverse genetics experiments, we demonstrated that there are opposing requirements for viral replication in mosquito and mammalian cells. As a consequence, different viral variants are selected in each host.

To understand the mechanism of recombination, we sequenced the viral variants and identified the RNA motifs that promote template switching. This mechanism occurs most frequently in regions of the viral genome where repeated sequences and structured RNA elements are present.

Based on these findings, we proposed a model in which RNA recombination allows the chikungunya virus to change its genome to overcome species-specific barriers and be transmitted in nature.

INTRODUCTION

RNA viruses include a vast number of highly relevant emerging and re-emerging viruses, which have caused outbreaks with devastating consequences for human health and the global economy. So far in the 21st century, the WHO has declared an international epidemiological emergency due to RNA viruses on five occasions (1) . The first was in 2009 and was due to the influenza A pandemic caused by the H1N1 influenza virus, which arose from a combination of swine, avian and human influenza viruses. The second was in 2014, when the human polio virus, which had been eradicated in 1994, re-emerged. Almost simultaneously, in the period 2014-2016, the largest epidemic outbreak of Ebola virus disease occurred, which was then repeated in 2018-2020 in the Republic of the Congo. The fourth health emergency was declared in 2016 due to the reappearance of the Zika virus, an ancestral arbovirus transmitted by mosquitoes, as well as dengue and chikungunya. Finally, in 2020, the most important pandemic in our history began: that of the new SARS Cov-2 coronavirus.

It is no coincidence that the great pandemics in history have been caused by viruses with RNA genomes. These viruses encode RNA-dependent RNA polymerases (RdRp's), responsible for amplifying the viral genome. RdRp's operate with mutation rates several orders of magnitude higher than DNA polymerases that amplify the genome of cells and that of DNA viruses. (2) . This results in the continuous generation of competing viral variants within the host. Basically, the diversity of the viral genome obeys two molecular mechanisms: point mutations and genetic recombination (3-5) . Point mutations arise from the introduction of nucleotide changes by RdRp's that lack proofreading activity. In contrast, genetic recombination does not produce changes at the nucleotide level, but rather the reorganization of existing genomes to generate a new chimeric genome. These molecular mechanisms have been primarily responsible for the (re)emergence of RNA viruses and the rapid generation of new viral variants, which constitute constant threats to global public health. This fact highlights the need to study the fundamental aspects that govern the evolution of viruses with RNA genomes.

Chikungunya (CHIKV) is an RNA virus belonging to the Alphavirus genus . CHIKV is a re-emerging human pathogen transmitted by mosquitoes of the Aedes genus . Infection may be asymptomatic with development of permanent immunity or may cause (i) acute disease with high fever, joint pain, rash, nausea, vomiting and conjunctivitis; (ii) subacute, characterized by disabling joint involvement and (iii) chronic, if these symptoms persist over time (6) .

CHIKV was first isolated in Tanzania in 1952 and for more than 50 years was limited to Africa and Asia. It has been proposed that there are three original lineages of the virus: the West African lineage, the East-Central-South African lineage (ECSA), and the Asian lineage. Beginning in 2004, the ECSA lineage re-emerged on a French island in the Indian Ocean (Reunion Island) and in 2007 the first lines appeared in Europe. On the other hand, in 2013, there was an outbreak of the virus on a Caribbean island (7, 8) , but this time, the re-emergence was associated with the Asian lineage, which spread throughout the Americas and reached Argentina in 2016 (7-9) .

CHIKV has a positive-sense RNA genome with two open reading frames. The first one encodes for the proteins that form the viral replication complex, which synthesizes the negative strand and from it, the viral genome and the

subgenomic RNA. The latter corresponds to the second reading frame and encodes for the structural proteins of the virus. At the ends are the non-coding regions (5'UTR and 3'UTR). The 3'UTR of CHIKV contains a variable number of short sequence repeats (DRs for Direct Repeats) (10-12) . This feature is very striking, since viruses have very compact genomes. The number of repeats varies between viral lineages. For example, the 3'UTR of the ECSA lineage contains only two types of repeats: DR1 (two copies) and DR2 (three copies). In contrast, the Asian lineage, which was generated 100 years ago from the ECSA, contains point mutations and insertions in DR1 and DR2 [in this lineage DR (1+2)] and the duplication of a new sequence (DR3). The strain that appeared in the Caribbean Islands (CHIKV-Cbn) and spread in America, has a 3'UTR never described in nature, with three copies of DR (1+2) and two copies of DR3 (13) . The great heterogeneity of the CHIKV 3'UTR suggests that this region of the genome plays an important role during viral evolution. For this reason, we were interested in studying the evolution of the CHIKV 3'UTR during viral adaptation to its natural hosts.

We demonstrate that during adaptation to mammalian cells, template-switch recombination frequently occurs in the 3'UTR of the CHIKV genome, generating a collection of viral variants. These viral variants have different capacities to replicate in mammals and mosquitoes. Consequently, the viral population changes when the virus alternates between hosts. Furthermore, we investigate the mechanism of recombination in the CHIKV 3'UTR and identify the sequences and structures that favor it.

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MATERIALS AND METHODS

Synthesis of the viral genome

In the laboratory we have the infectious clone of the Caribbean strain of the chikungunya virus (CHIKV-Cbn), which consists of a plasmid containing the complete viral genome in the form of a DNA copy, under the promoter of the SP6 polymerase. This plasmid allows the incorporation of mutations and deletions by reverse genetics. The Mut 3'UTR construct was generated by assembly PCR, replacing the WT fragment with the fragment with the deletion of the three copies of the DR(1+2). The constructs were linearized by digestion with NotI and were used as templates for **in vitro** transcription with the SP6 polymerase, in the presence of the Cap m7G(5)ppp(5)G structure, using the mMessage Machine transcription kit (Thermo Fisher).

in vitro evolution

BHK-21 mammalian cells (Mesocricetus auratus hamster kidney , ATCC, CCL-10) were grown at 37 ◦ C in MEM alpha medium (Gibco) in a 5% CO2 atmosphere supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). C6/36 mosquito cells (Aedes albopictus , ATCC, CRL-1660) were grown at 28 ◦ C in Leibovitz L-15 medium (Gibco) supplemented with 10% FBS, 10% tryptose phosphate (Britania), 1% penicillin-streptomycin and amphotericin B (Gibco).

For RNA transfections, cell lines were grown to 60-70% confluence and transfected in 24-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Viruses were harvested after two days and a second infection was performed on the same cell lines at a multiplicity of infection (MOI) = 0.5. Viral RNAs were then extracted from culture supernatants with TRIzol™ (Invitrogen) and used for RT-PCR reactions with oligonucleotide 115 (5'-TTTTTTTTTTTTTTTTTTTTTTTGAAATAT-3', complementary to the poly(A) tail plus the last 7 nucleotides of the CHIKV genome). PCR reactions were performed using reverse oligonucleotide 115 and forward oligonucleotide 116 (5'-CTAATCGTGGTGCTATGC-3', complementary to the last portion of the coding region of the viral genome). The products were ligated into the pCR2.1-TOPO vector (Invitrogen) and used to transform XL1-Blue bacteria. Two independent experiments were performed. In each experiment, 20 clones were analyzed for WT and Mut 3'UTR viruses. The length of individual 3'UTRs was estimated by resolving the 115-116 PCR product on 1.5% agarose gels. Individual plasmid clones were sequenced by the Sanger method.

Mosquito breeding

Laboratory colonies of Aedes albopictus mosquitoes (19th generation; originally collected in Phu Hoa, Binh Duong province, Vietnam) were used. Insectary conditions for mosquito maintenance were 28 ° C, 70% relative humidity, and a 12-h light/12-h dark cycle. Adults were maintained with constant access to a 10% sucrose solution. Adult females were supplied with commercial rabbit blood (BCL, Boisset-Saint-Priest, France) twice weekly via a membrane feeding system (Hemotek Ltd).

Experimental mosquito infections

Infection assays were carried out in a biosafety level 3 (BSL-3) laboratory using 7- to 10-day-old females that were deprived of food 24 h prior to infection. Mosquitoes were offered infectious blood for 30 min via a membrane feeding system (Hemotek Ltd). Prior to blood supplementation, the viral stock was diluted in Leibovitz L-15 medium with 0.1% sodium bicarbonate (Gibco) to achieve an infectious titre of 1 × 10 6 to 1 × 10 7 plaque-forming units/ml. Following blood feeding, fully infected females were selected and incubated at 28 ° C with 70% relative humidity and a 12-h light/12-h dark cycle, with continuous access to 10% sucrose. At different times postinfection, mosquitoes were homogenized in microtubes containing steel beads (5 mm diameter) and 300 µl of DMEM supplemented with 2% FBS using a TissueLyser II instrument (Qiagen). Homogenates were clarified by centrifugation and stored at -80 ° C until processing. To analyze the CHIKV 3'UTR in viral populations, TRIzol-extracted RNAs were used in reverse transcription reactions with oligonucleotide 115 and then in PCR reactions with oligonucleotides 115 and 116. The length of individual 3'UTRs was estimated on 1.5% agarose gels. Individual plasmid clones were sequenced by the Sanger method.

Human blood and ethical declaration

Human blood used to feed the mosquitoes was obtained from healthy volunteer donors. Donor recruitment was organized by local investigators screening on the basis of medical history, laboratory results and clinical examinations. Biological samples were supplied by healthy volunteers participating in the ICAReB biobanking platform (BB-0033-00062 / ICAReB Platform / Institut Pasteur, Paris / BBMRI AO203 / [BIORESOURCE]) of the Institut Pasteur to the CoSImmGen and Diagmicoll protocols that have been approved by the French Ethics Committee (CPP) Ilede-France I. The Diagmicoll protocol was declared to the

French Ministry of Research under the reference: DC 2008-68 COL 1.

Structural analysis

CHIKV 3'UTR sequences were aligned using ClustalW2. RNA secondary structure was predicted using RNAalifold. Based on the 3'UTR alignments, RNA recombination **breakpoints were identified**.

Competition experiments

The 3'UTRs of selected recombinant viruses were introduced into the parental infectious clone. Parental and recombinant RNAs were then obtained by in vitro transcription , quantified and mixed in pairs in defined ratios. A total amount of 3 µg of transcripts per well was transfected into cultured cells in two independent experiments. Viruses were harvested from the supernatants and used to re-infect fresh cells. After one and two passages, RNA was extracted, used as a template for RT-PCR reactions and ligated into the pCR2.1-TOPO vector. The relative abundance of each virus in the population was then calculated.

RESULTS

Experimental evolution of the 3'UTR of the CHIKV genome in mammalian cells

Since different viral lineages contain different numbers of copies of the DRs in the 3'UTRs, we think that this region of the viral genome plays an important role during viral evolution. To investigate this, we performed in vitro evolution experiments . These experiments consist of successive viral passages in cells grown in culture and, at the end of these, the genome of the viral population is analyzed.

For our experiments, we used the CHIKV-Cbn variant, which arrived in the Americas in 2013 and has 3 copies of DR (1+2) and two copies of DR3 (Figure 1A). We also constructed a mutant virus containing a minimal 3'UTR (Mut 3'UTR). We synthesized in vitro the genome of the wild type virus (WT) and the Mut 3'UTR and transfected them into mammalian cells grown in culture (BHK, derived from hamster kidney). To obtain adapted viral populations, we infected fresh cells with the supernatants of infected cells (Figure 1B). After two passages in culture, we collected the supernatants, extracted the total RNA, amplified and cloned the 3'UTR of the viral genome. Representative agarose gels are shown in Figures 1C and 1D, and in each lane, the size of the 3'UTR of a variant that composes the viral population is evident. We observed that the population derived from the WT virus is formed by a collection of viral variants with deletions of the DRs in the 3'UTR, which represented 70% of the viral population. In contrast, the population derived from Mut 3'UTR was homogeneous and we did not detect variability in the 3'UTR.

These results indicate that the CHIKV 3'UTR is prone to lose DR copies during adaptation to mammalian cells, depending on its architecture. Viruses with higher DR copy numbers generate more heterogeneous viral populations than viruses containing a minimal 3'UTR.

Infection of mosquitoes with populations adapted to mammalian cells

CHIKV must constantly alternate between mosquito and mammal, which are two very different biological systems and, therefore, have different requirements for viral replication. Consequently, it is expected that mammals and mosquitoes exert different selection pressures on the viral population, modulating its composition.

To study viral population dynamics during host switching, we fed reference mosquito strains of the genus Aedes with blood infected with mammalian-adapted populations derived from either the WT or the Mut 3'UTR virus. After eight days, we extracted RNA from individual mosquitoes and analyzed the 3'UTR of the viral variants (Figure 2A).

In mosquitoes fed the WT-derived population, variants with deletions were negatively selected and came to represent

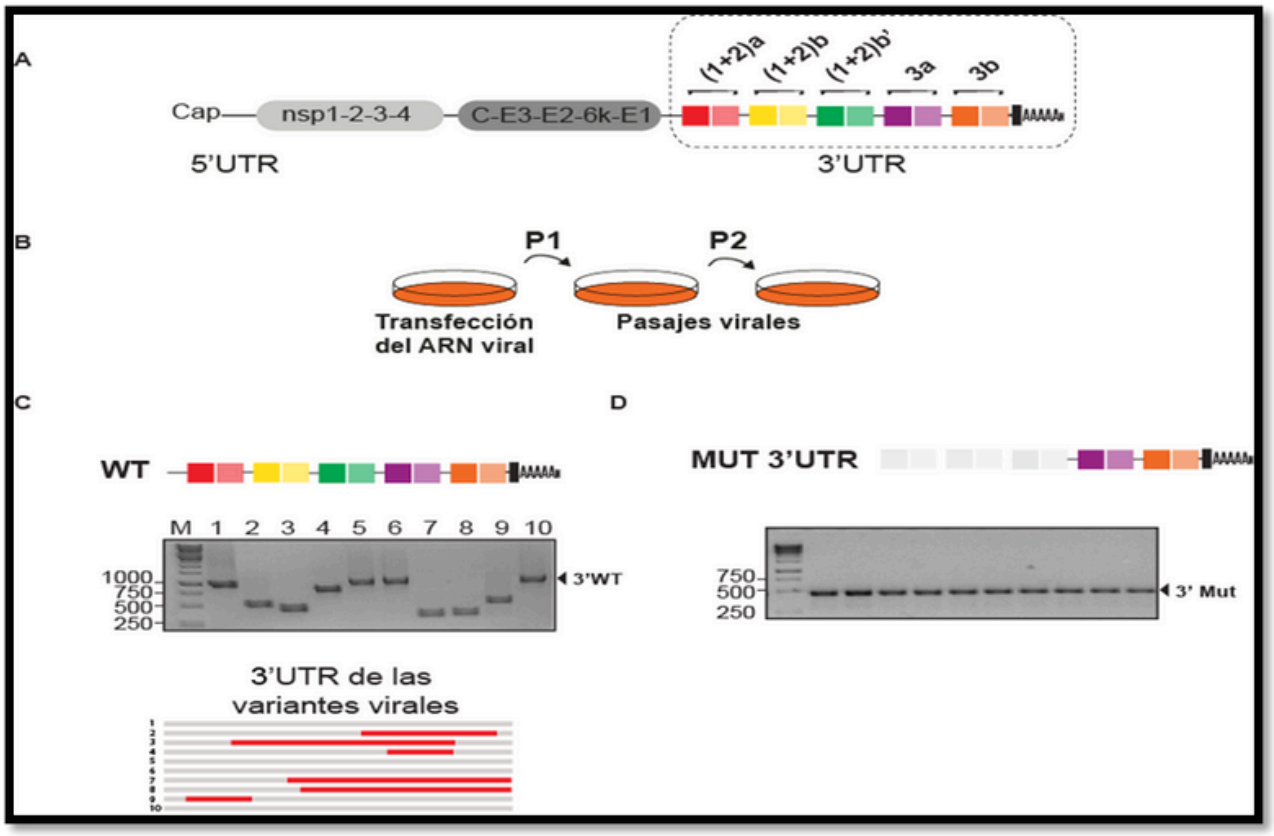
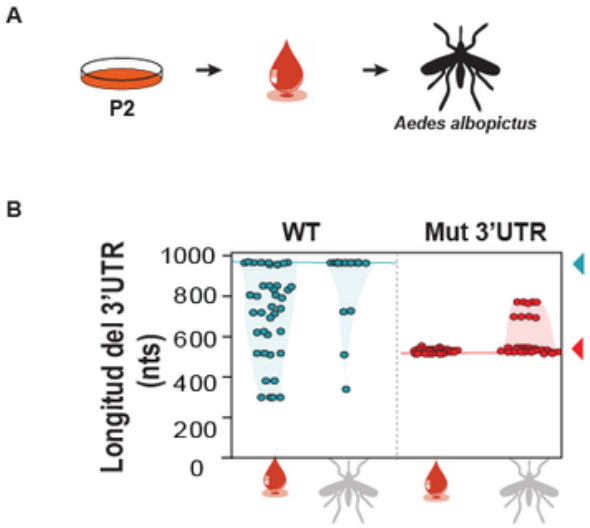


FIGURE 1

only 15% of the population (Figure 2B, left). In contrast, in mosquitoes fed the mutant virus, variants with longer 3'UTRs emerged (Figure 2B, right).

These results indicate that the CHIKV 3'UTR changes during host switching, and different viral variants are negatively and positively selected in each host. Within infected mosquitoes, viral variants with longer 3'UTRs are selected. Furthermore, after infecting mosquitoes with a virus containing a minimal 3'UTR, viral variants are generated that inserted copies of the DRs into the 3'UTR.



Viral competence assays

As we have seen, in mammalian cells, variants with deletions in the 3'UTR predominate, while in mosquitoes these variants are displaced by those with the WT 3'UTR. To examine the fitness or replicative capacity of the viral variants, we performed viral competition assays. These assays are the gold standard to determine the fitness of two viruses and consist of making a mixture of viruses compete in cell culture. The virus with the highest fitness will increase its relative abundance and may even displace the other. To perform these assays, we constructed viruses containing the 3'UTR of selected viral variants and made them compete with the WT virus. After two viral passages in mosquito cells or mammalian cells, we estimated the abundance of each one. As an example, we show the results for a representative variant (Del 3'UTR). In mammalian cells, Del 3'UTR was able to displace the WT virus, indicating that the virus with deletions has a higher fitness than the one with a WT 3'UTR. In contrast, in mosquito cells, the WT virus was

able to displace Del 3'UTR, indicating that sequence repeats confer greater fitness in this host. In addition to these two viruses, we observed that new viral variants, absent in the initial mixture, emerged in cell culture (Figure 3, in grey).

We conclude that viral variants are subject to opposite selection pressures in viral hosts: DRs are beneficial for viral replication in mosquito cells and redundant/disadvantageous in mammalian cells.

Mechanism of generation of viral variants

In previous studies in our laboratory (14) , we demonstrated that 3'UTR variants are generated by homologous recombination. In this process, the viral polymerase changes template and continues RNA synthesis. If its reassociation occurs towards the 5'UTR with respect to the dissociation point, genomes with insertions are generated, while if it occurs in a sequence towards the 3'UTR, genomes with deletions are generated (Figure 4A). Generally, this mechanism does not occur randomly, but there are sequences and/or structures that favor it. The elements that favor recombination are usually found in the vicinity of the regions that flank the deletions and/or insertions, that is, at the sites where the dissociation and/or reassociation of the viral polymerase occurred. These regions are commonly called recombination breakpoints (Figure 4B).

To investigate the recombination-promoting elements in the CHIKV 3'UTR, we sequenced the 3'UTR of the viral variants recovered in the previous assays, aligned it to the parental 3'UTR, and identified breakpoints . We observed that recombination did not occur randomly, but in defined regions of the 3'UTR. In particular, in certain viral variants recombination occurred in regions rich in adenine (A) and uracil (U), and in the vicinity of RNA secondary structures such as hairpins, long-range RNA-RNA interactions , and stem-loops (Figure 4B).

These results demonstrate that the CHIKV 3'UTR contains sequence elements and RNA structures that favor recombination, explaining its great plasticity in nature, in laboratory mosquitoes and in cell culture.

DISCUSSION

In this work, we explored the dynamics of the 3'UTR of viral populations adapted in vitro to mammalian cells and in mosquito colonies grown in the laboratory. We observed that the 3'UTR is highly plastic and dramatically changes its composition during adaptation of CHIKV to cell culture. In mammalian cells, the viral population is composed of a collection of viral variants with deletions in the 3'UTR. The diversity of the population is related to the organization of the 3'UTR: viruses with multiple copies of the DRs evolve rapidly, while those with a minimal 3'UTR are restricted in their evolutionary capacity. Interestingly, different viral lineages have different numbers of copies of the DRs in the 3'UTR, so it is possible that they differ in their evolutionary potential.

When the virus passes into mosquitoes, the viral population changes dramatically, negatively selecting viral variants with deletions. Likewise, when mosquitoes are infected with a virus containing a minimal 3'UTR, viral variants with insertions in the 3'UTR are generated.

Chikungunya virus, like other arboviruses, must constantly switch between mammals and mosquitoes, which represent two very different biological systems. To do so, it must

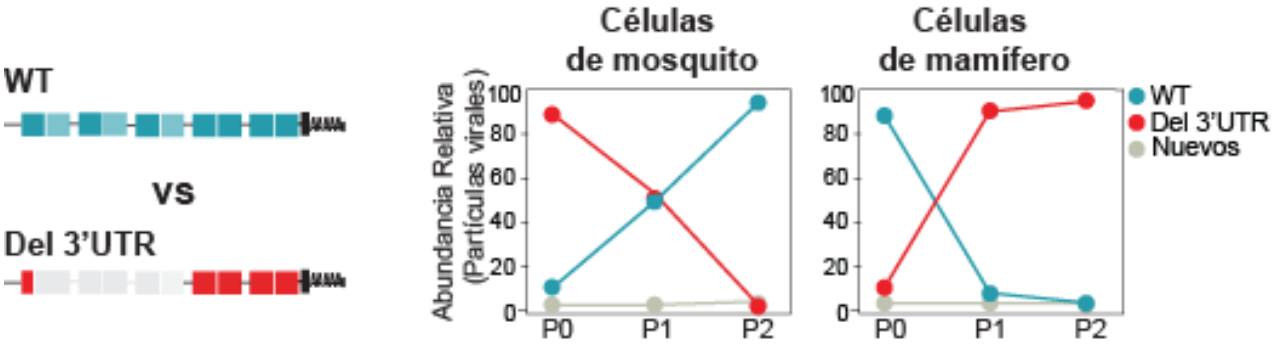


FIGURE 3

lrapidly adapt and meet the conditions that each host imposes for viral replication. Our viral competition experiments demonstrated that the 3'UTR is subject to different selection pressures in mammals and mosquitoes. This suggests that the evolutionary capacity of the virus is affected by the host in addition to being affected by intrinsic features of the viral genome. We propose, then, that homologous recombination is a viral strategy to incorporate or eliminate blocks of the DRs in the viral genome, in order to "build" a 3'UTR tailored to the host and meet the requirements that different species impose for viral replication.

For other viruses, it was observed that recombination does not occur randomly (15-18) . We then set out to identify the elements of the 3'UTR that favor recombination. We found that certain RNA motifs were found recurrently at recombination breakpoints , such as AU-rich sequences and stable RNA structures. One of the factors that keeps the replication complex associated with the RNA template is the sequence complementarity between the nascent RNA strand and the template molecule. Therefore, it is likely that when there is an abundance of AU-rich sequences in the template, this hybridization is weak and favors the dissociation of the viral replication complex, promoting the change of template by this molecular mechanism. On the other hand, RNA structures possibly offer resistance to the processivity of the replication complex and favor recombination by this mechanism. In summary, the CHIKV 3'UTR repeats contain sequence and structural elements that promote recombination through distinct molecular mechanisms. en su potencial evolutivo. Cuando el virus pasa a mosquito, la población viral cambia Ancestral CHIKV lineages have shorter 3'UTRs, whereas re-emerging lineages such as CHIKV-Cbn possess duplications of repeat sequences in the 3'UTR. Therefore, viral re-emergence seems to be associated with the incorporation of DRs in the 3'UTR (19) . Since these DRs were incorporated entirely in regions close to the domains we identified in this work, it is very likely that they were incorporated by recombination through template switching.

The possibility of modulating viral recombination is a very powerful tool. Those viruses with difficulties to recombine are restricted in their evolutionary capacity. Therefore, the incorporation of changes in the viral genome that limit recombination could be useful in the design of vaccines, since they would avoid reversions of attenuated viruses to virulent strains (20) .

On the other hand, less recombinogenic viruses would have difficulties in adapting the 3'UTR to the host, which represents an important barrier to species change.

Taken together, our findings allow us to understand the molecular basis of host switching, viral population dynamics, and the evolution of epidemic CHIKV strains, offering novel alternatives for the design of attenuated strains.

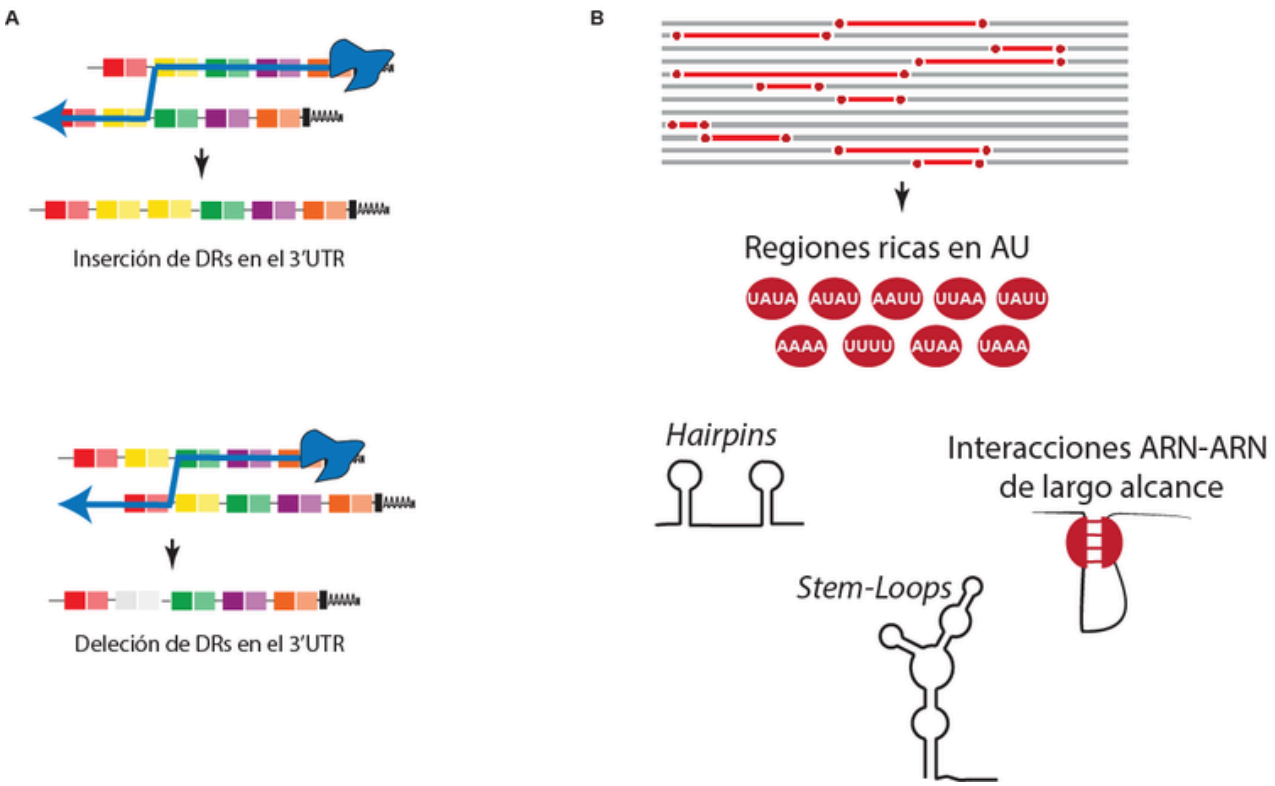


FIGURE 4

seleccionándose negativamente las variantes virales con deleciones.

Asimismo, cuando los mosquitos son infectados con un virus que contiene un 3'UTR mínimo, se generan variantes virales con inserciones en el 3'UTR.

El virus chikungunya, al igual que otros arbovirus, debe alternar constantemente entre mamíferos y mosquitos, que representan dos sistemas biológicos muy diferentes.

Para ello, debe adaptarse rápidamente y cumplir con las condiciones que cada hospedador impone para la replicación viral.

Nuestros experimentos de competencia viral demostraron que el 3'UTR está sometido a distintas presiones de selección en mamífero y mosquito.

Esto sugiere que la capacidad evolutiva del virus es afectada por el hospedador además de estar afectada por rasgos intrínsecos del genoma viral.

Proponemos entonces, que la recombinación homóloga es una estrategia viral para incorporar o eliminar bloques de las DRs en el genoma viral, con el fin de "construir" un 3'UTR a medida del hospedador y satisfacer los requerimientos que las distintas especies imponen para la replicación viral.

Para otros virus se observó que la recombinación no ocurre al azar (15-18).

Nos propusimos entonces identificar los elementos del 3'UTR que favorecen la recombinación.

Encontramos que ciertos motivos de ARN se encontraban con recurrencia en los breakpoints de recombinación, como las secuencias ricas en AU y las estructuras estables de ARN.

Uno de los factores que mantiene asociado el complejo de replicación al molde de ARN es la complementariedad de secuencias entre la hebra naciente de ARN y la molécula molde.

Por lo tanto, es probable que cuando existen en el molde una abundancia de secuencias ricas en AUs, esta hibridización sea débil y favorezca la disociación del complejo de replicación viral, promoviendo el cambio de molde por este mecanismo molecular.

En cambio, las estructuras de ARN posiblemente ofrezcan una resistencia a la procesividad del complejo de replicación y favorezcan la recombinación por este mecanismo.

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