Climate change and human globalization have spurred the rapid spread of mosquito-borne diseases to naïve populations. One such emerging virus of public health concern is chikungunya virus (CHIKV), a member of the Togaviridae family, genus Alphavirus. CHIKV pathogenesis is predominately characterized by acute febrile symptoms and severe arthralgia, which can persist in the host long after viral clearance. CHIKV has also been implicated in cases of acute encephalomyelitis, and its vertical transmission has been reported. Currently, no FDA-approved treatments exist for this virus. Recoding elements help expand the coding capacity in many viruses and therefore represent potential therapeutic targets in antiviral treatments. Here, we report the molecular and structural characterization of two CHIKV translational recoding signals: a termination codon read-through (TCR) element located between the nonstructural protein 3 and 4 genes and a programmed −1 ribosomal frameshift (−1 PRF) signal located toward the 3′end of the CHIKV 6K gene. Using Dual-Luciferase and immunoblot assays in HEK293T and U87MG mammalian cell lines, we validated and genetically characterized efficient TCR and −1 PRF. Analyses of RNA chemical modification data with selective 2′-hydroxyl acylation and primer extension (SHAPE) assays revealed that CHIKV −1 PRF is stimulated by a tightly structured, triple-stem hairpin element, consistent with previous observations in alphaviruses, and that the TCR signal is composed of a single large multibulged hairpin element. These findings illuminate the roles of RNA structure in translational recoding and provide critical information relevant for design of live-attenuated vaccines against CHIKV and related viruses.

The spread of mosquito-borne viruses has been accelerated by climate change and advances in globalization (1) One such example is chikungunya virus (CHIKV), a member of the Togaviridae family, genus Alphavirus. First identified during an outbreak in Tanzania in 1952 (2), CHIKV was implicated in large-scale outbreaks in Africa and Asia in 2004 (3). Its geographic spread has since encompassed Europe, Australia, the Pacific Islands, and the Americas (4). “Old World” alphaviruses, including CHIKV, Sindbis virus (SINV), Semliki Forest virus, and O’nyong-nyong virus, are endemic to Africa and central Asia, and infections are characterized by fever, rash, and arthritic disease. The pathogenesis of CHIKV is predominately characterized by an incubation period of 3–7 days followed by acute, febrile illness and severe arthralgia (5). Most patients recover within 2 weeks, but complications can result in debilitating sequelae persisting for years after viral clearance. Recent reports have implicated CHIKV in cases of acute encephalomyelitis (6, 7) similar to that of “New World” equine encephalitis alphaviruses, and the capability for vertical transmission during pregnancy or birth (8, 9) has been demonstrated. Currently, no United States Food and Drug Administration–approved treatments for CHIKV have been approved for civilian use.

Many RNA viruses utilize translational recoding mechanisms to expand the coding capacity of limited genome space and to optimize the stoichiometric expression of critical proteins (10). Two recoding signals have been documented in alphaviruses: those promoting termination codon read-through (TCR) and programmed −1 ribosomal frameshift (−1 PRF). The CHIKV TCR is located at an opal (UGA) termination codon in the genomic RNA beginning at nucleotide 5656, PRF). The CHIKV TCR is located at an opal (UGA) termination codon in the genomic RNA beginning at nucleotide 5656, which marks the boundary between the nsP3 and nsP4 genes (11). A closely associated downstream stimulatory element is thought to prevent efficient association of the eRF1–eRF3 complex with the ribosomes stalled at this codon, increasing the likelihood of a TCR event (12). The specific stop codon
identity for this signal is critical for optimal alphavirus functionality as substitutions to amber or ochre stop codons or an arginine read-through have been associated with lowered transmission in mosquito vectors and significantly attenuated pathogenesis (13–15). The −1 PRF signal is located in the subgenomic RNA that encodes a polyprotein that is subsequently processed into structural proteins. −1 PRF occurs at a conserved U UUU UUA slippery site sequence near the 3’ end of the 6K gene, resulting in production of a trans-frame (TF) product (16). TF then undergoes unique post-translational modifications respective to 6K and is integrated into the envelopes of mature virions (17). Although the biological function of TF is poorly understood (18), it has been hypothesized to function as an ion channel, similar to its 6K counterpart (19). A study with SINV mutants that disrupted production of TF resulted in reduced virus production in mammalian and insect cell lines that was independent of genome replication, particle infectivity, or envelope protein transport to the cellular membrane. These findings suggest potential roles for TF in virus particle assembly and budding. Notably, SINV mutants with disrupted TF production strongly attenuated the virus in mouse models (20). Similarly, ablation of −1 PRF in Venezuelan equine encephalitis virus (VEEV), a new world alphavirus, had minimal effects on viral replication but abrogated neurovirulence in mouse models (21). These findings suggest that ablation of alphavirus recoding elements could serve as the foundation for live-attenuated vaccines for this virus family.

This study describes the genetic and structural characterization of the CHIKV TCR and −1 PRF signals. We demonstrate that the bioinformatically predicted CHIKV recoding signals promote efficient levels of translational recoding in several mammalian cell lines. The observed recoding efficiencies were genetically verified through mutations used to ablate the respective TCR and −1 PRF mechanisms. Analyses of chemical modification assays identified the presence of well-organized hairpin structures downstream of the respective recoding sequences, the structures of which were tolerant of geographically isolated polymorphisms. Key mutations that significantly ablated recoding functionality were identified for both signals, laying a foundation for follow-up attenuated live virus experiments in animal models.

Results

The CHIKV translational recoding sequences are highly conserved

Alphaviruses have single-stranded, positive-sense RNA genomes that contain two large open reading frames (ORFs)
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The first ORF encodes a polyprotein that is proteolytically cleaved into the nonstructural proteins nsP1, nsP2, nsP3, and nsP4 (nsP4 encodes the viral RNA-dependent RNA polymerase or replicase). The second ORF, accessible to the translational apparatus as a subgenomic transcript, encodes a polyprotein that is subsequently processed into the C, E2, E3, 6K, and E1 structural proteins. Programmed translational recoding signals have been identified in both ORFs. In the non-structural ORF, the nsP3 gene ends with a UGA opal termination: thus, expression of the nsP4 replicase requires a programmed TCR mechanism (11). In the subgenomic RNA, a −1 PRF located in the 3′ region of the 6K viroporin gene enables synthesis of a C-terminally extended trans-frame variant dubbed TF that has been identified in the envelope of mature alphavirus particles (16). Ribosomes that have been shifted to the −1 frame to produce TF cannot translate the E1 protein.

Human globalization and climate change have facilitated the rapid global spread of CHIKV, resulting in the generation of multiple viral strains and lineages with differing degrees of pathogenicity (23–25). A prior analysis revealed considerable amounts of heterogeneity among these strains throughout their genomes (26). Consensus sequences of patient-derived CHIKV isolates from the Africa/Asia and Caribbean geographic regions (abbreviated as Af/As and Carib) were aligned to assess the conservation of the TCR and −1 PRF recoding regions (Fig. 1B). The full collection of CHIKV isolate sequences used in this study can be found in Fig. S1. The opal termination codon is universally conserved in both consensus sequences, but five polymorphisms were identified in the downstream sequence predicted to harbor the accompanying TCR stimulatory element. Similarly, full conservation of the alphaviral U UUU UUA slippery site was observed in both CHIKV −1 PRF consensus sequences. The sequences of the downstream stimulatory structures diverged by only a single nucleotide. Following this analysis, it was determined that both geographic consensus sequences for the CHIKV TCR signal would be considered for subsequent experiments, whereas only the Af sequence of the CHIKV −1 PRF signal was selected as a representative.

CHIKV-derived sequences promote efficient recoding in mammalian cell lines

The Af/As and Carib variants of the CHIKV TCR signal and the singular −1 PRF signal were cloned into Dual-Luciferase reporter vectors. The firefly luciferase ORF flanking the 3′ end of the inserted sequences was adjusted such that translation could only be achieved consequent to a termination codon read-through event or a ribosomal shift into the −1 frame, respectively (27). Dual-Luciferase reporters harboring the murine leukemia virus (MuLV) TCR and the VEEV −1 PRF signals were used as positive controls, and a reporter with a UAA termination codon inserted 5′ of the firefly luciferase ORF (5′-UAA) constituted the negative control. All three CHIKV-derived sequences promoted efficient levels of translational recoding in HEK293T and U-87 MG human astrocyte-derived cell lines (Fig. 2A). Specifically, average Af/As and Carib TCR efficiencies were in the range of 7.0 and 7.1% in HEK293T cells and 13.4 and 13.7% in U-87 MG cells, respectively. These values were very similar to those measured from the MuLV TCR control reporter. Notably, no significant differences in TCR efficiency were recorded between the Af/As and Carib geographic variants of CHIKV. Average CHIKV −1 PRF efficiency was lower, ~1.8% in HEK293T cells and 4.5% in U-87 MG cells, as compared with the VEEV −1 PRF reporter (average 2.3% in U-87MG and 4.6% in HEK293T cells). However, all of these values were statistically significantly greater than the 5′-UAA control (<1% in both cell lines). The possibility that the observed recoding efficiencies were the products of a cryptic promoter, splice site, or internal ribosome entry site was tested with two modified vector backbones that placed an in-frame termination codon either directly 5′ of the inserted test sequence or in the outgoing reading frame 3′ of the insert prior to the firefly sequence. Dual-Luciferase assays revealed that these control vectors significantly abrogated CHIKV Af/As-and Carib-mediated TCR and −1 PRF efficiencies in both cell lines, supporting the hypothesis that these are indeed legitimate viral recoding signals (Fig. 2B). As an orthogonal approach to characterizing efficient recoding, the firefly and Renilla luciferase products of transfected HEK293T cells were also visualized by immunoblotting; these data effectively recapitulated the enzymatic reporter data (Fig. 2C). Images of the original gels and blots are shown in Fig. S2.

Targeted mutations alter CHIKV recoding efficiencies

Prior studies revealed that efficient −1 PRF in encephalitic alphaviruses can be abrogated by strategically targeted slippery sequence point mutations, leading to attenuated pathogenesis (20, 21). Site-directed mutagenesis was used to test the effects of targeted mutations of CHIKV on translational recoding efficiencies in HEK293T cells. A significant decrease in efficient TCR in mammalian cell lines was observed when the termination codon was substituted with more frequently used UAA and UAG stop codons, reducing TCR from ~10% to 3.7 and 2.7% for Af/As and to 0.9 and 2.7% for Carib, respectively (Fig. 3A). Similarly, silent coding mutations to the slippery site of the −1 PRF signal intended to impede 5′ slippage of tRNA nonwobble bases (U UUU UUA→G UUC UUG) resulted in significantly decreased frameshifting efficiency (<1.0%) (Fig. 3A). Reports in the literature have also indicated that substitution of an arginine codon for the UGA codon of the CHIKV TCR signals promoted reduced viral pathogenesis (13). When the TCR opal termination codon was mutated to an AGA arginine, 83.6 and 77.0% read-through efficiencies were observed for the Af/As and to 0.9 and 2.7% for Carib, respectively (Fig. 3A). These results where replicated via Western blotting as an orthogonal means of verification (Fig. 3, C and D). Images of the original gels and blots are shown in Fig. S3.

Chemical modification analyses identify complex stem-loop elements immediately 3′ of CHIKV recoding sites

Translational recoding signals typically require the presence of highly structured RNA stimulatory elements immediately 3′ of the recoding site. These cis-acting elements are hypothesized to function as kinetic traps for ribosomes, facilitating the conditions favorable for TCR or −1 PRF (10). A diverse array of 3′ stimulatory structures have been reported, ranging from simple stem-loops to RNA pseudoknots (28). Each unique recoding
A close examination of the recoding data suggests a few notable items. With regard to the TCR reporters, the apparent “read-through” efficiency of the Arg-containing mutant was ~90%, i.e. not 100% as would be expected. This reporter retains the strong TCR stimulating stem-loop element, whereas the control 0 frame reporter merely contains the firefly luciferase genes in-frame with one another without any intervening sequence. Given that strong RNA secondary structures can induce ribosome pausing and drop-off (32–36), we specu-

**Figure 2. Characterization and validation of efficient CHIKV translational recoding in mammalian cell lines.** A, the predicted TCR and −1 PRF signals identified in Fig. 1 were cloned into pJD2257 Dual-Luciferase reporter vectors, and their ability to promote efficient recoding was monitored in HEK293T and U-87 MG cells, human kidney and astrocyte cell lines, respectively. MuLV and VEEV were used as positive controls for efficient TCR and −1 PRF as well as a negative control consisting of a UAA termination codon in the 0 frame. B, CHIKV recoding signals were cloned into dual reporter control vectors containing a premature termination codon either 5′ of the insert sequence in the 0 frame (5′ ter) or 3′ of the insert in the −1 frame (3′ ter). C, representative immunoblot of protein lysates generated from HEK293T cells transfected with the indicated reporter plasmids. rt.ctrl denotes the read-through control. The slower migrating band in the firefly luciferase-probed panel corresponds to incomplete cleavage of the Stop/Go intein that was inserted immediately 3′ of the recoding signals (n = 2). Dual-Luciferase data are presented as means with S.E. (error bars) where each point denotes a biological replicate assayed as technical triplicates. Asterisks denote statistical significance where * represents p < 0.05 and ** represents p < 0.01.
late that this structural element may cause a fraction (~10%) of elongating ribosomes to drop off of the reporter mRNA, thus accounting for the observed TCR values of less than unity. Experiments to measure the fraction of ribosomes paused at this element, their pause times, and drop-off rates are planned for the future. A second set of insights stems from the observation that the CHIKV PRF signal promotes relatively low rates of recoding (most viral frameshifting is in the range of ~5%; see Atkins et al. 37). Examination of sequences proximal to the −1 PRF signal did not reveal the potential to form either a larger stem-loop or a more complex structure, e.g. an RNA pseudoknot. However, it is possible that this element may be involved in long-range interactions that, by rendering it more difficult to unwind, would increase ribosome pause rates and thus −1 PRF efficiency (38). For example, a −1 PRF stimulating long-distance RNA–RNA interaction was first demonstrated in barley yellow dwarf virus where base pairing between an internal bulge in the −1 PRF-promoting stem-loop and a sequence

Figure 3. Mutagenesis-mediated destabilization of efficient CHIKV recoding. A, site-directed mutagenesis was used to replace the UGA termination codon (WT) in the CHIKV TCR signal with either a UAA or UAG stop codon. A slippery site mutant of the CHIKV −1 PRF signal (ssM) that replaced the U UUU UUA sequence with G UUC UUG was also created. The recoding efficiencies promoted by recoding mutants relative to their WT counterparts were assayed in HEK293T and U-87 MG cell lines. B, site-directed mutagenesis was used to substitute the UGA stop codon in both CHIKV TCR sequences with AGA. The read-through efficiency of this mutant was tested alongside the WT TCR sequences in HEK293T cells. C and D, Western blotting verification of the results described in A and B (n = 2). Sequences from MuLV and VEEV were used as positive TCR and −1 PRF controls where appropriate. Dual-Luciferase assay data are presented as means with S.E. (error bars) where each point represents a biological replicate assayed as technical triplicates. Asterisks denote statistical significance where * represents p < 0.05 and ** represents p < 0.01.
Figure 4. Structural analyses of the CHIKV recoding signals. A–C, stimulatory elements for Af/As and Carib CHIKV TCR and 1 PRF signals resolved through SHAPE. RNA templates containing the translational recoding sequences were transcribed from corresponding Dual-Luciferase reporter vectors and treated with NMI. Untreated RNAs were used as negative controls. γ-32P-radiolabeled cDNA products were separated by 8% urea-PAGE and visualized via a Fujifilm phosphorimaging system. Autoradiograms are annotated to indicate the respective sequencing lanes (G, A, U, and C), an untreated control lane (−), and the NMI-labeled experimental lane (+). Circles denote the relative reactivity of bases where white is the most unreactive and black is the most reactive. For added visual clarity of the CHIKV TCR gels, a longer run of the samples has been provided to further separate the 5’ sequence information. D and F, structures of CHIKV recoding signals derived from the above SHAPE data. Circles correspond to the previously described nucleotide reactivity scale. Polymorphisms between the Af/As and Carib consensus sequences are indicated in red. E and G, three-dimensional models of the CHIKV TCR and 1 PRF signals were generated by molecular dynamics simulations and visualized in PyMOL.
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nearly 4 kb downstream is required to promote efficient −1 PRF (39, 40). Similar long-distance interactions involving bulges located inside of −1 PRF-stimulating stem-loops have been observed in red clover necrotic mosaic virus (41), in the pea enation mosaic virus (42), and in the TCR-promoting elements of tobacco necrosis virus-D (43) and turnip crinkle virus (44). The bulged stem-loop in the CHIKV −1 PRF signal in the current study and prior demonstration of similar −1 PRF-stimulating RNA structural elements in the New World alphaviruses (21) are highly reminiscent of the plant virus recoding signals, suggesting that the alphaviral recoding elements may similarly involve long-distance RNA–RNA interactions. More broadly, it is becoming clear that dynamic long-range RNA–RNA interactions are critical for programming the fundamental molecular processes of (+)-single-stranded RNA viruses, including the switch from translation to transcription, cap-independent translation, genome circularization, replicase complex assembly, subgenomic mRNA synthesis, and repriming during discontinuous template synthesis (45, 46). Many of these are characterized by base-pairing interactions between the loops at the ends of stem-loop structures. In this respect, the forked distal tip of the CHIKV TCR signal may present a novel such interacting site. Thus, it is not unlikely that interactions between the structured recoding elements identified in the current study and distal sequence elements are involved in the dynamic programming of the CHIKV life cycle. A deeper understanding of such interactions will require studies examining RNA structural dynamics of the genomic RNA, subgenomic RNA, and (−)-strand replicative intermediates.

PRF was first discovered in retroviruses where ribosome slippage directs the synthesis of Gag-Pol polyproteins (47). Subsequent studies using totiviruses (48) and later in retroviruses (49) demonstrated that changes in −1 PRF efficiency affect virus production. From these findings, a bioeconomics model emerged in which −1 PRF rates are optimized to maximize virus particle assembly by ensuring the synthesis of the correct stoichiometric ratios of the structural Gag-derived proteins to the enzymes encoded by the Poly ORF (50). These findings engendered interest in targeting −1 PRF for antiviral therapeutics (51). In parallel, early studies examined −1 PRF signals in virus families where they do not occur between open reading frames encoding structural and enzymatic ORFs, e.g. in coronaviruses and luteoviruses. Altering −1 PRF efficiency in severe acute respiratory syndrome coronavirus severely impacted its infectivity in tissue culture, reinforcing the idea of −1 PRF as an antiviral therapeutic target (52). Lower rates of −1 PRF correlate with decreased pathogenicity in West Nile virus (53), and production of the NS1’ frameshift product is critical for neuroinvasiveness in West Nile and Japanese encephalitis viruses (54). −1 PRF also presents a target for alphaviruses: in mice, ablation of −1 PRF attenuated the symptoms of Sindbis virus (20) and VEEV (21) infections. Although less well-studied than −1 PRF, five published studies report that alteration of TCR also has negative impacts on virus replication (15, 55–58). Three of these studies (55–57) examined the impact of TCR ablation in a retrovirus (murine leukemia virus). Pertinent to this study, TCR ablation was shown to reduce the pathogenicity of two alphaviruses, O’nyong-nyong and Sindbis viruses (15, 58). Thus, both −1 PRF and TCR represent important, but underexploited targets for antiviral intervention. For example, ablation of −1 PRF and/or TCR signals may be incorporated into the design of live-attenuated virus vaccines. In parallel, elucidation of the atomic-resolution structures of these elements and of their structural dynamics may be useful in the design of therapeutic small-molecule inhibitors.

Experimental procedures

Computational prediction of CHIKV recoding signals

CHIKV sequences were imported from NCBI into the Dinman laboratory frameshifting database (http://prfdb.umd.edu) (59). The conserved opal stop codon at the 3’ end of the nsP3 gene and the U UUU UUA slippery site in the 6K gene were used to identify the sequences harboring the CHIKV TCR and −1 PRF stimulatory elements, respectively. The RNA folding algorithm NUPACK (60) was used to model potential downstream RNA secondary structures of these recoding signals, serving as guides for subsequent cloning into reporter vectors.

Dual reporter plasmid construction

Translation recoding rates were monitored using pJD2257-based Dual-Luciferase vectors, a modification of pSGDluc (61) in which a duplicate BamHI and Sall restriction site was eliminated, leaving unique sites located between the Renilla and firefly luciferase coding sequences. DNA sequences harboring the CHIKV recoding signals containing 5’ and 3’ overlapping sequences with pJD2257 were designed in silico and synthesized by Genewiz (Gaithersburg, MD) (Table S1). These were used to construct reporter plasmids by the DNA fragment assembly method (62) using the In-Fusion® HD Cloning Plus kit from Clontech (catalogue number 638911). These inserts were also cloned into control variants of pJD2257 that harbor in-frame UAA termination codons either 5’ or 3’ of the multiple cloning site (pJD2267 and pJD2269, respectively). Plasmids and sequences are described in Table S2 and are available upon request.

Cell culture

Cell lines for HEK293T (catalogue number CRL-3216) and U-87 MG (catalogue number HTB-14) were purchased through ATCC (Manassas, VA). HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Lonza) and U-87 MG cells were cultured in Eagle’s minimum essential medium (Lonza) supplemented with 1% l-glutamine, 10% fetal bovine serum, and 1× penicillin-streptomycin to obtain complete growth media. The cells were grown at 37 °C in 5% CO₂.

Plasmid transfections

HEK293T or U-87 MG cells were seeded at 0.6 × 10⁵ cells in 0.5 ml/well for 24-well plates for Dual-Luciferase transfections; and 1 × 10⁶ cell in 2 ml/well for 6-well plates for BiFl transfections in appropriate complete growth media (Dulbecco’s modified Eagle’s medium or Eagle’s minimum essential medium). Following a 24-h incubation period, control and experimental reporter plasmids were then transfected into cells using Lipo-

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fectamine 3000 transfection reagent from Thermo Fisher (catalogue number L3000015).

**Assays of translational recoding**

Translational recoding efficiencies measured using pJD2257-based reporter plasmids were assayed as described previously (21) using a Dual-Luciferase reporter assay system kit from Promega (catalogue number E1980). At 24 h post-transfection, cell culture medium was aspirated, and the cells were rinsed twice with 1× phosphate-buffered saline (PBS) before disruption with 1× Passive Lysis Buffer. Cell lysates were assayed in triplicate in a 96-well plate. Firefly and Renilla luciferase activities were quantified using a GloMax®-Multi Detection system from Promega (catalogue number E7041).

**Immunoblot analyses**

HEK293T cells were seeded at 1 × 10⁶ cells/well in 6-well plates followed by transfections with Dual-Luciferase plasmid vectors using Lipofectamine 3000 transfection reagent from Thermo Fisher. Cell were harvested 48 h post-transfection, and lysates were prepared using Nonidet P-40 lysis buffer from Boston BioProducts (catalogue number BP-119) including protease and phosphatase inhibitors from Thermo Fisher (catalogue number 78440). Protein concentrations were determined, and 15–20 μg of each sample was separated by SDS-PAGE (4–15% gels from Bio-Rad, catalogue number 4568084) and then transferred to 0.45-μm nitrocellulose membranes from Fisher (catalogue number LC2001). Post-transfer membranes were blocked with 5% nonfat skim milk in 1× PBST (1× PBS, 0.5% Tween 20) for 2 h at room temperature and then incubated overnight at 4°C with primary firefly luciferase (polyclonal anti-goat from Promega, catalogue number G7451 and lot number 7074S and lot number 26) in 5% nonfat skim milk in 1× PBST. Immunoreactive bands were detected using LumiGLO reagent from Cell Signaling Technology (catalogue number 70035) and visualized in a Fuji LAS-3000 imager.

**RNA structure analyses**

The CHIKV −1 PRF and TCR signals were structurally assayed by SHAPE as described previously with the following modifications (63, 64). DNA templates for in vitro transcription reactions were generated by PCR amplification using Dream Taq DNA polymerase from Thermo Fisher (catalogue number FERK1071PM). Complementary primers for the Renilla and firefly regions were used to amplify the inserted CHIKV sequence and attach a T7 promoter sequence to the 5’ end of the ampiclons. In vitro transcription was carried out with a T7 MEGAscript kit from Life Technologies (catalogue number AM1334). Transcribed RNA was purified with a MEGAclear cleanup kit (Life Technologies, catalogue number 1908), and the quality of the RNA samples was assessed through urea-PAGE. Modification of flexible bases with N-methylisatoic anhydride (NMIA) was carried out as described previously (21). The oligonucleotide 5’-AGGATAGAATGGCGCCGGGCC-3’ was 5’-labeled using γ[³²P]ATP from PerkinElmer Life Sciences (catalogue number BLU502Z250UC) and polynucleotide kinase. The primer was annealed to modified RNA, and subsequent reverse transcription (using Superscript III reverse transcriptase, Thermo Scientific, catalogue number 18080044) was carried out as reported previously (63, 64). Radioactivity of cDNA samples was standardized with a liquid scintillator prior to electrophoresis, though 8% urea-PAGE (Sequagel Urea Gel system, National Diagnostics, catalogue number EC-833). cDNA products were visualized on a Fujifilm phosphorimaging system. Visual clarity (brightness and cropping) of gel images was adjusted with Adobe Photoshop Lightroom 5. All primers were purchased through Integrated DNA Technologies.

**Three-dimensional structural modeling of CHIKV −1 PRF and TCR stimulatory elements**

All-atom models were generated using the MC-Fold and MC-Sym pipeline programs (65). RNA sequences for the CHIKV −1 PRF and TCR signals were imported into the MC-Fold program to generate a series of secondary structures as described previously (21). The highest-scoring model was selected to represent the predicted three-dimensional structure of the viral PRF signals.

**Phylogenetic analyses**

Accession numbers of CHIKV sequences isolated from patients of either African-Asian origin (n = 14) or Caribbean origin (n = 5) were considered for phylogenetic analysis. Translational recoding sequences (PRF and TCR) from these strains were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)⁶ (66).

**Statistical analyses**

Translational recoding assays repeated in triplicate (experimental replicates) were independently repeated a minimum of three times (biological replicates), and mean technical replicate values for each independent biological replicate are shown on graphs along with standard deviation of biological replicates. Statistical analyses were conducted using a Student’s t test or one-way analysis of variance as appropriate using Prism, version 6, software (GraphPad).

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