

1 **Design and use of Chikungunya virus replication templates utilizing mammalian and**  
2 **mosquito RNA polymerase I mediated transcription.**

3  
4 **Running title: RNA polymerase I based CHIKV trans-replicase.**

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28 **ABSTRACT**

29 Chikungunya virus (CHIKV) is a mosquito-borne alphavirus. It has a positive sense RNA  
30 genome that also serves as the mRNA for four non-structural proteins (nsPs) representing  
31 subunits of the viral replicase. Coupling of nsP and RNA synthesis complicates analysis of  
32 viral RNA replication. We developed trans-replication systems, where production of  
33 replication competent RNA and expression of viral replicase are uncoupled. Mammalian and  
34 mosquito RNA polymerase I promoters were used to produce non-capped RNA templates,  
35 which are poorly translated relative to CHIKV replicase generated capped RNAs. It was  
36 found that, in human cells, constructs driven by RNA polymerase I promoters of human and  
37 Chinese hamster origin performed equally well. In contrast, RNA polymerase I promoters  
38 from *Aedes* mosquitoes exhibited strong species specificity. In both mammalian and mosquito  
39 cells, novel trans-replicase assays had exceptional sensitivity, with up to 10<sup>5</sup>-fold higher  
40 reporter expression in the presence of replicase relative to background. Using this highly  
41 sensitive assay to analyse CHIKV nsP1 functionality, several mutations that severely reduced,  
42 but did not completely block, CHIKV replicase activity were identified: (i) tagging the N-  
43 terminus of nsP1 with eGFP; (ii) mutations D63A and Y248A blocking the RNA capping;  
44 (iii) mutation R252E affecting nsP1 membrane anchoring. In contrast, a mutation in the nsP1  
45 palmitoylation site completely inactivated CHIKV replicase in both human and mosquito  
46 cells and was lethal for the virus. Our data confirms that this novel system provides a valuable  
47 tool to study CHIKV replicase, RNA replication and virus-host interactions.

48

49 **IMPORTANCE**

50 Chikungunya virus (CHIKV) is a medically important pathogen responsible for recent large-  
51 scale epidemics. The development of efficient therapies against CHIKV has been hampered  
52 by gaps in our understanding of how non-structural proteins (nsPs) function to form the viral  
53 replicase and replicate virus RNA. Here we describe an extremely sensitive assay to analyse  
54 the effects of mutations on virus RNA synthesis machinery in both cells of mammalian (host)  
55 and mosquito (vector) origin. Using this system several lethal mutations in CHIKV nsP1 were  
56 shown to reduce but not completely block the ability of its replicase to synthesize viral RNAs.  
57 However, in contrast to related alphaviruses, CHIKV replicase was completely inactivated by  
58 mutations preventing palmitoylation of nsP1. These data can be used to develop novel, virus-  
59 specific antiviral treatments.

## 60 INTRODUCTION

61 Genome replication of RNA viruses is carried out by an RNA-dependent-RNA polymerase  
62 complex (replicase) that consists of one or more virus-encoded proteins and may also  
63 incorporate host-cell proteins. For positive-strand RNA viruses the virus-encoded  
64 component(s) of replicase are directly translated from the RNA genome. Its expression  
65 depends on RNA replication and *vice versa*; RNA replication depends on expression and  
66 functional activity of viral replicase. Thus, for these viruses RNA synthesis and replicase  
67 expression are functionally coupled.

68 Alphaviruses (family *Togaviridae*) comprise a group of positive-strand RNA viruses that  
69 includes important human pathogens such as Chikungunya virus (CHIKV) as well as several  
70 well-studied model viruses including Semliki Forest virus (SFV) and Sindbis virus (SINV).  
71 Most alphaviruses are mosquito-vectored (1) and can efficiently replicate in cells of vertebrate  
72 and arthropod origin. Alphavirus infection in vertebrate cells is highly cytotoxic. In contrast,  
73 infection of arthropod cells is non-cytotoxic and results in a persistent low-level infection.  
74 Alphaviruses have RNA genomes that are approximately 12 kb in size with a 5' cap and 3'  
75 poly(A) tail. The genome consists of two open reading frames (ORFs). The 3' ORF encodes  
76 for a structural polyprotein which is translated from a subgenomic (SG) RNA transcribed  
77 under the control of a SG promoter in infected cells (2). The virus-encoded replicase subunits,  
78 termed non-structural proteins (nsPs), are expressed in the form of a non-structural (ns)  
79 polyprotein precursor(s) (P123 and P1234) from the ORF located at the 5' two-thirds of the  
80 virus genome (3). The ns-polyprotein is proteolytically processed by nsP2 which has protease  
81 activity (4) initially resulting in a short-lived negative-strand RNA polymerase (P123 + nsP4)  
82 that is subsequently converted into a stable positive-strand RNA polymerase  
83 (nsP1+nsP2+nsP3+nsP4) (5). All four nsPs as well as most of their processing intermediates  
84 (P1234, P123 and P23) are strictly required for alphavirus RNA replication (3).

85 Replicative functions of alphavirus replicase proteins are relatively well-studied. nsP1 is a  
86 virus-specific methyl- and guanylyltransferase and serves as membrane anchor for virus  
87 replicase (6, 7). nsP2 is a papain-like cysteine protease (8), NTPase (9), RNA triphosphatase  
88 (10) and RNA helicase (11). nsP3 of Old World alphaviruses binds cellular G3BP proteins  
89 that are required for RNA replication (12). It also contains an N-terminal macro-domain with  
90 the ability to bind ADP-ribose and remove it from mono ADP-ribosylated substrates. This is  
91 crucial for viral RNA replication (13). nsP4 is the catalytic subunit of viral RNA polymerase  
92 (14, 15) and also has terminal adenylyltransferase activity (16). In addition, all of these  
93 proteins have a number of essential functions that are not directly linked to viral RNA  
94 replication. Thus, nsP1 has been reported to antagonize an anti-viral protein, tetherin (17),  
95 nsP2 of Old World alphaviruses triggers degradation of host cell RNA polymerase II (18) and  
96 antagonizes type-I interferon signalling (19), nsP3 modulates the phosphatidylinositol-3-  
97 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (20) and is responsible  
98 for translational shutoff in infected cells (21). nsP4 is involved in suppression of the unfolded  
99 protein response (22). While enzymatic functions of nsPs are well conserved for all  
100 alphaviruses their non-replicative functions exhibit a significant variation depending on virus  
101 and its host. For example nsP2 is cytotoxic only in the case of Old World alphaviruses (23)  
102 and only in vertebrate cells (18). The multiple functions of nsPs, their variation between  
103 different alphaviruses and the coupled nature of nsP expression and viral RNA replication  
104 complicates the analysis of functional significance of nsPs, their different mutations as well as  
105 investigating the role of host cell interaction partners in alphavirus RNA replication.

106 De-coupling the viral replicase protein(s) expression from viral RNA synthesis represents a  
107 powerful approach to study viral RNA replication. This has been proven useful to study the  
108 replication of influenza virus (24, 25), flavivirus Kunjin virus (26) and nodavirus flock house  
109 virus (27). In addition, the ability of viral replicase to amplify RNA template provided *in*

110 *trans* has also allowed construction of yeast-based replication systems for bromo-, noda- and  
111 tombusviruses (28–30). In the case of alphaviruses, de-coupled replication systems are  
112 generally designed to amplify truncated RNA templates where the ns- and structural ORFs of  
113 alphavirus genome are replaced by sequences encoding for different reporter proteins; such  
114 systems are often referred to as trans-replication systems. Trans-replication systems have been  
115 developed for SINV, SFV and CHIKV and used to study alphavirus replication complex  
116 biogenesis (31–33), RNA template sequence requirements (34), analysis of the impact of  
117 template length on the size of replication complexes (35), tagging of different replicase  
118 proteins (36) and analysis of the impact of different mutations introduced into replicase  
119 proteins on viral RNA replication in mammalian (8, 37, 38) and mosquito cells (39). While  
120 these studies have universally found the alphavirus trans-replication systems to be efficient  
121 and robust tools they have also revealed certain technical limitations. Namely, to produce  
122 replication competent template RNA and mRNA for expression of replicase polyproteins,  
123 alphavirus trans-replication systems have traditionally used either bacteriophage T7 RNA  
124 polymerase and corresponding promoters (33) or cellular RNA polymerase II promoters such  
125 as the immediate early promoter of human cytomegalovirus (CMV) or *Aedes aegypti*  
126 polyubiquitin promoter (Ubi) (36, 39). The major drawback of the use of bacteriophage T7  
127 RNA polymerase promoters is that use of such trans-replication systems is restricted to cell  
128 lines expressing T7 RNA polymerase, which are generally not available for cell types relevant  
129 for *in vivo* alphavirus infections. The use of promoters for cellular RNA polymerase II allows  
130 the use of a wider range of cell types. However, such systems suffer from reduced sensitivity,  
131 especially with regards to replicase-mediated amplification of reporter activity used to replace  
132 the ns-ORF of virus genome and thus expressed from full-length RNA template. The effect is  
133 due to high background activity of reporter resulting from its efficient translation using RNA  
134 polymerase II generated capped transcripts. This activity is often comparable or even higher

135 than reporter activity produced from much more abundant viral replicase generated full-length  
136 positive-strand RNAs (36, 39).

137 In this study several approaches were applied to overcome the above-mentioned limitations of  
138 alphavirus trans-replicase systems. It was found that inserting the sequence, corresponding to  
139 the 5' end of the CHIKV genome, directly at the start site of human, Chinese hamster, *Aedes*  
140 *aegypti* or *Aedes albopictus* RNA polymerase I promoter allowed production of replication-  
141 competent transcripts. These RNAs are, presumably due to the lack of 5' cap-structure, poorly  
142 translated resulting in significantly reduced background levels of included reporters and  
143 enhanced sensitivity of the trans-replication assay. The template constructs with human or  
144 Chinese hamster RNA polymerase I promoters were active in various primate cell lines.  
145 Higher species specificity was observed for template constructs harboring either *Aedes*  
146 *aegypti* or *Aedes albopictus* RNA polymerase I promoter.

147 The increased sensitivity of the system was used to re-evaluate 13 mutant versions of CHIKV  
148 replicase previously reported to be inactive or to have activities close to background level. A  
149 set of mutations in CHIKV nsP1, including both mutations in methyl- and guanylyltransferase  
150 active site and mutations affecting membrane anchoring of nsP1, were also analyzed using  
151 CHIKV trans-replicase and infectious clone systems. Highly consistent results confirmed  
152 some findings previously reported for SFV; however, it was also found that, unlike SFV,  
153 mutation of the palmitoylation site of nsP1 in CHIKV is lethal in the context of viral genome  
154 and completely abolishes its replicase activity. These findings confirm the improved trans-  
155 replication system as an extremely sensitive and robust system to study alphavirus RNA  
156 replication.

157

## 158 RESULTS

159 Trans-replicases of alphaviruses allow analysis of synthesis of full-length negative- and  
160 positive-strand copies of suitable template RNAs as well as SG RNAs transcribed from SG  
161 promoter(s) included into template RNA. However, directly analysing such RNAs using  
162 northern blotting and/or strand-specific RT-qPCR is time- and resource consuming and not  
163 convenient for large scale experiments such as screening libraries of antiviral compounds.  
164 Incorporation of one or more reporter coding sequences into template RNA constructs allows  
165 the use of the much more convenient measurement of reporter activities as proxy for synthesis  
166 of corresponding positive-strand RNAs, and is also applicable in high-throughput format.  
167 Therefore in the current study, as previously (8, 36, 39), firefly luciferase (Fluc) was used to  
168 substitute most of the ns-ORF and *Gaussia* luciferase (Gluc) to substitute the structural ORF  
169 in template constructs (Fig. 1B, 2B). For simplicity, hereafter the full-length RNA serving as  
170 template for Fluc translation is termed “genomic RNA” (and its synthesis as “replication”),  
171 RNA synthesized from the SG promoter that serves as template for Gluc translation is termed  
172 “SG RNA” (and its synthesis as “transcription”) and all RNAs synthesized by CHIKV trans-  
173 replicase are referred to as “viral RNAs”.

174 It has been observed that alphavirus trans-replication systems, where initial template RNA  
175 subsequently used by virus replicase is produced by cellular RNA polymerase II, can be  
176 efficiently used for analysis of transcription but not of RNA replication (36, 39). The intrinsic  
177 problem is that although large amounts of genomic RNAs are synthesized from such  
178 templates by viral replicase (36) the activity of Fluc reporter from such RNAs is masked by  
179 high background levels originating from the efficient translation of smaller amounts of initial  
180 capped template RNA transcripts. Therefore, the increase of Fluc activity is only observed for  
181 replicase mutants that boost viral RNA synthesis to levels considerably exceeding these  
182 achieved by wild type (wt) replicase (39). Even the amplification of signal for Gluc, that in



183 commonly used cell lines is typically more than 1000-fold for wt replicase (36), can be  
184 insufficient for the analysis of mutant replicases with severely reduced RNA synthesis  
185 activity. Therefore systems with higher sensitivity and with more prominent boost of Gluc  
186 and especially Fluc activities are required.

187 **Design of plasmids for production of non-capped template RNAs.** It has been observed  
188 that use of inefficiently translated template RNA transcripts synthesized by RNA polymerase  
189 of bacteriophage T7 increased the sensitivity of a trans-replication system (36). Importantly,  
190 these data also indicate that replicases of alphaviruses are capable of binding to and initiating  
191 the replication of template RNAs lacking a 5' cap structure. This property was used in four  
192 new templates designed for use in mammalian cells. In CMV-HH-Fluc-Gluc template the  
193 leader sequence corresponding to residues 1-137 of tobacco mosaic virus (TMV) including  
194 first 23 codons of its replicase followed by three terminator codons and a hammer head  
195 ribozyme (HH RZ) was inserted between the start site of CMV promoter and a nucleotide,  
196 corresponding to the 5' end of CHIKV genome (Fig. 1B), so that the primary capped  
197 transcript has 193 non-viral nucleotides (Table 1) upstream of CHIKV-specific sequence.  
198 Upon ribozyme cleavage, non-capped RNAs with the authentic 5' end of CHIKV genome and  
199 therefore suitable for use by CHIKV replicase are generated. Similarly, in HSPolI-HH-Fluc-  
200 Gluc constructs, a full length promoter for human RNA polymerase I was used to drive the  
201 transcription of initial template RNA while HH RZ was used to generate the authentic 5' end  
202 (Fig. 1B, Table 1). In addition, two constructs utilizing the design that has been successfully  
203 used to develop reverse genetics systems of negative-strand RNA viruses (40–44) were made.  
204 These constructs are HSPolI-Fluc-Gluc and CGPolI-Fluc-GLuc, using only non-transcribed  
205 parts of RNA polymerase I promoters from human and Chinese hamster, respectively (Fig.  
206 1B, Table 1).

207 RNA polymerase I promoters and terminators have been mapped for both principal vectors of  
208 CHIKV, *Aedes aegypti* (45) and *Aedes albopictus* (46, 47). Interestingly, in these promoters  
209 the region with highest sequence similarity is located immediately downstream of the  
210 transcription start site of rRNA (45). Three *Aedes*-specific template RNA expressing  
211 constructs were designed. First, in AegPolI-HH-Fluc-Gluc constructs, the full length upstream  
212 promoter of *Aedes aegypti* RNA polymerase I and HH RZ were used (Table 1); the construct  
213 also contained the *Aedes aegypti* RNA polymerase I terminator (Fig. 2B). Next, in the  
214 template construct designated AegPolI-Fluc-Gluc only non-transcribed part of the promoter  
215 was used (Table 1). Finally, in the template construct designated AlbPolI-Fluc-Gluc, the  
216 *Aedes aegypti* RNA polymerase I promoter and terminator were replaced with their  
217 counterparts from *Aedes albopictus* (Table 1). Hepatitis delta virus negative strand ribozyme  
218 (HDV RZ) sequence was added to ensure cleavage of all RNA templates immediately  
219 downstream of poly(A) sequence (Fig. 1B, 2B).

220 **Template RNA encoding plasmids utilizing human and Chinese hamster RNA**  
221 **polymerase I promoters are efficient in primate cells.** A panel of constructs consisting of  
222 CMV-Fluc-Gluc and four new templates (CMV-HH-Fluc-Gluc, HSPolI-Fluc-Gluc, HSPolI-  
223 HH-Fluc-Gluc and CGPolI-Fluc-Gluc) was analyzed in two primate (U2OS (human), Vero  
224 E6 (African green monkey)) and two rodent (BHK-21 (Syrian golden hamster) and CHO  
225 (Chinese hamster)) cell lines. The Fluc and Gluc activities detected in CHO cells were always  
226 close to the background level suggesting that CHIKV replicase works inefficiently, if at all, in  
227 CHO cells; for this reason these cells were excluded from further analysis. Results obtained  
228 for CMV-Fluc-Gluc template were consistent with these previously observed (36); the  
229 expression of Fluc marker occurred at a high level also in cells co-transfected with CMV-  
230 Fluc-Gluc + CMV-P1234<sup>GAA</sup> – a negative control containing a polymerase-inactivating  
231 mutation in nsP4. The increase of Fluc activity in the presence of active (*versus* inactive)

232 replicase was very low (~2 fold) (Fig. 3A). In contrast, strong boost of Gluc activity was  
233 observed in all cell lines, being highest in U2OS and BHK-21 (~20,000-fold) and slightly  
234 lower in Vero E6 cells (~5,000-fold) (Fig. 3A).

235 Adding a leader and HH RZ to the template reduced Fluc background in control cells less  
236 than 10-fold. The high Fluc activity in the presence of inactive replicase indicates that  
237 additional TMV sequences and HH RZ cleavage were not sufficient to block Fluc translation.

238 It is likely that CHIKV replicase cannot efficiently use templates with 193 additional residues  
239 upstream of the native 5' end of the viral genome (Table 1). If so, boosting of expression of  
240 both reporters by active replicase (Fig. 3A) indicates that ribozyme-mediated cleavage did  
241 occur at some level. The low cleavage efficiency/speed apparently led to only low amounts of  
242 templates with correct 5' ends in cells transfected with CMV-HH-Fluc-Gluc which in turn  
243 resulted in reduced Fluc and especially Gluc activity in the presence of CHIKV-P1234.  
244 Consequently, boost of Fluc expression remained low (Fig. 3A). Compared to those achieved  
245 for CMV-Fluc-Gluc, boosts of Gluc activities for CMV-HH-Fluc-Gluc were significantly  
246 lower in all three cell lines, typically less than 1,000-fold (Fig. 3A). Taken together, it was  
247 concluded that while used approach was not efficient enough to prevent Fluc translation from  
248 RNA polymerase II generated transcripts HH RZ was capable to generate at least some  
249 amount of initial transcripts with proper 5' ends that were subsequently utilized by CHIKV  
250 replicase.

251 Use of RNA polymerase I promoters greatly improved system performance. Although the  
252 absolute levels of Fluc and Gluc activities observed in cells transfected with HSPolI-Fluc-  
253 Gluc + CMV-P1234 or CMV-Fluc-Gluc + CMV-P1234 were similar in U2OS cells, the much  
254 lower background activity of both reporters expressed by HSPolI-Fluc-Gluc made the assay  
255 using the latter template generally much more sensitive. First and most importantly, the boost  
256 of Fluc activity was significantly increased in all cell lines used, being highest (>2,000-fold)

257 in U2OS cells (Fig. 3A). Second, the boost of Gluc activity also significantly increased in  
258 U2OS cells where it exceeded 100,000-fold; the effect was less pronounced but still  
259 significant in Vero E6 cells. In contrast, in BHK-21 cells CMV-Fluc-Gluc reached higher  
260 boost levels of Gluc expression compared to HSPolI-Fluc-Gluc (Fig. 3A). As CHIKV  
261 replicates well in both Vero E6 and BHK-21 cells the observed trend most likely reflects a  
262 reduced activity of human RNA polymerase I promoter in monkey and especially in rodent  
263 cells relative to the human-derived U2OS cells. The effect of incorporating HH RZ was  
264 consistent with the observation above - expression of reporters in cells transfected with  
265 HSPolI-HH-Fluc-Gluc template was reduced which resulted in significantly diminished boost  
266 efficiencies of both markers in primate cells (Fig. 3A). Only in BHK-21 cells, boost of Gluc  
267 activity achieved through the use of HSPolI-HH-Fluc-Gluc was similar to that achieved using  
268 HSPolI-Fluc-Gluc and the boost of Fluc activity was even more prominent (Fig. 3A). Most  
269 likely, the presence of the downstream region of human RNA polymerase I promoter allowed  
270 it to be used more efficiently in rodent cells.

271 Finally, construct CGPolI-Fluc-Gluc was tested in all three cell lines. Surprisingly, the  
272 construct performed poorly (~8-fold boost of Fluc and ~380-fold boost of Gluc activities) in  
273 BHK-21 cells. In contrast, it was highly active in U2OS and Vero E6 cells showing boosts  
274 comparable to these observed for HSPolI-Fluc-Gluc construct suggesting this rodent RNA  
275 polymerase I promoter is highly active in primate cells (Fig. 3A). Taken together, these  
276 experiments revealed U2OS cells and HSPolI-Fluc-Gluc construct as the most sensitive  
277 combination; therefore it was selected for subsequent experiments.

278 Measurement of Fluc and Gluc activities is a convenient, but indirect, method for analysis of  
279 CHIKV replicase activity. To demonstrate that Fluc and Gluc activities indeed reflect  
280 synthesis of viral positive-strand RNAs, northern blot analysis was performed using  
281 transfected U2OS and BHK-21 cells. Negative strand synthesis, which is not detectable

282 through the measurement of reporter activities (34), was also assessed. Efficient synthesis of  
283 genomic and SG RNAs was observed in U2OS cells co-transfected with CMV-Fluc-Gluc,  
284 HSPolI-Fluc-Gluc or CGPolI-Fluc-Gluc and CMV-P1234. The presence of HH RZ reduced  
285 positive-strand RNA synthesis for both CMV and HSPolI promoter-based vectors; again,  
286 most likely this indicates that the HH RZ is not efficient enough in generating RNAs with  
287 proper 5' ends. The synthesis of negative-strand RNA was found to follow the same pattern  
288 (Fig. 3B, left panels). In BHK-21 cells, strong replication/transcription was observed only for  
289 CMV-Fluc-Gluc derived template RNAs. The replication of template RNA generated from  
290 CMV-HH-Fluc-Gluc was diminished and no replication products could be detected in cells  
291 transfected with CMV-P1234 and HSPolI-Fluc-Gluc, HSPolI-HH-Fluc-Gluc or CGPolI-Fluc-  
292 Gluc (Fig. 3B, right panels). Taken together these results demonstrated an excellent  
293 correlation between levels of replicase generated RNAs and expression of reporters translated  
294 from viral positive-strand RNAs.

295  
296 **Template RNA encoding plasmids utilizing RNA polymerase I promoters from *Aedes***  
297 **mosquitoes display species specificity.** A panel consisting of Ubi-Fluc-Gluc and three new  
298 constructs (AegPolI-HH-Fluc-Gluc, AegPolI-Fluc-Gluc and AlbPolI-Fluc-Gluc) was  
299 analyzed in *Aedes albopictus* derived C6/36 cells and *Aedes aegypti* derived AF319 cells. As  
300 previously observed (39), background activity of Fluc reporter in cells transfected with Ubi-  
301 Fluc-Gluc limited the boost of its expression by active CHIKV replicase; only ~3-fold or ~6-  
302 fold increase of Fluc activity was observed in C6/36 and AF319 cells, respectively (Fig. 4A).  
303 In contrast, boost of Gluc activity was ~800-fold in C6/36 and ~9,000-fold in AF319 cells  
304 (Fig. 4A).  
305 Both AegPolI-HH-Fluc-Gluc and AegPolI-Fluc-Gluc constructs were highly active in AF319  
306 cells. Again, the use of RNA polymerase I promoter reduced background levels of both

307 reporters and, subsequently, increased the boost of both markers: highly increased (~100-fold)  
308 boost of Fluc activity and more slightly, but still significantly, increased (~20,000-fold) boost  
309 of Gluc activity were observed (Fig. 4A). In contrast to human cells where the construct  
310 lacking hammer head ribozyme significantly outperformed the HH RZ version (Fig. 3A) no  
311 significant difference between AegPolII-HH-Fluc-Gluc and AegPolII-Fluc-Gluc was observed  
312 in AF319 cells (Fig. 4A). This may indicate that in *Aedes aegypti* cells the presence of the  
313 downstream region of RNA polymerase I promoter has bigger impact on the activity of the  
314 RNA polymerase I promoter and thus compensates for the inefficient generation of initial  
315 transcripts with authentic 5' ends by HH RZ cleavage. Alternatively, or in addition, it may  
316 indicate more efficient cleavage of primary transcripts by HH RZ, as lower temperature (28  
317 °C instead of 37 °C) may facilitate the formation of the ribozyme structure.

318 AlbPolII-Fluc-Gluc was also highly active and revealed little cell specificity: Fluc and Gluc  
319 boosts in homologous mosquito cells were only slightly higher (Fig. 4A) and the difference  
320 between mosquito cell lines was not statistically significant ( $p=0.276$  for Fluc and  $p=0.090$  for  
321 Gluc). In contrast, AegPolII-Fluc-Gluc was almost completely inactive in C6/36 cells.  
322 Surprisingly, the activity of AegPolII-HH-Fluc-Gluc in C6/36 cells was rather similar to that  
323 of Ubi-Fluc-Gluc (Fig. 4A). These data indicate that the upstream part of RNA polymerase I  
324 promoter of *Aedes aegypti* is not sufficient to drive transcription in *Aedes albopictus* cells.  
325 However, the presence of highly conserved sequences located downstream of the transcription  
326 start site (45) compensates for this defect. Taken together, it was found that the most efficient  
327 template/mosquito cell combination was AegPolII-Fluc-Gluc and AF319 cells; for C6/36 cells  
328 the best performance was observed for AlbPolII-Fluc-Gluc template (Fig. 4A). These  
329 combinations were therefore selected for subsequent experiments.

330 To confirm that the observed differences in Fluc and Gluc reporter activities indeed reflect  
331 differences in the amount of viral RNAs northern blot analysis was performed. Again, an

332 excellent correlation between the amount of replicase generated RNAs and expression levels  
333 of reporter proteins was observed. Thus, in C6/36 cells the highest level of replicase generated  
334 RNAs was observed for Ubi-Fluc-Gluc followed by AlbPolI-Fluc-Gluc derived templates.  
335 Replicase generated RNAs were also clearly detected in C6/36 cells transfected with AegPolI-  
336 HH-Fluc-Gluc but not with AegPolI-Fluc-Gluc (Fig. 4B, left panel). In AF319 cells, the  
337 highest RNA levels were observed when AegPolI-Fluc-Gluc or AegPolI-HH-Fluc-Gluc were  
338 used with minimal differences between these two. High levels of replicase generated RNAs  
339 were also detected in cells transfected with Ubi-Fluc-Gluc; in cells transfected with AlbPolI-  
340 Fluc-Gluc their levels were much lower (Fig. 4B, right panel). It was also observed that  
341 similar to the reporter activities, the levels of replicase made positive-strand transcripts in  
342 mosquito cells were lower than in mammalian cells; in order to obtain comparable images 5-  
343 fold excess of mosquito cell derived RNAs and longer exposure of northern blot was required.  
344 Consistently, we were easily able to detect negative-strand RNAs in mammalian cells (Fig  
345 3B) while in mosquito cells their levels were below the limit of detection of the assay.

346  
347 **CHIKV infection triggers replication of plasmid-encoded template RNAs.** Template RNA  
348 encoding constructs also have the potential to serve as sensors of virus infection *in vitro* and  
349 *in vivo* (48). For efficient detection, an advanced sensor must possess high sensitivity (high  
350 on/off ratio) and, in induced (infected) state, expression of reporter(s) at a level suitable for  
351 detection. In CHIKV trans-replicase system both of these requirements are clearly met:  
352 expression of Fluc and Gluc markers occur at high level and the on/off ratios are extremely  
353 high, especially for Gluc (>100,000-fold; Fig. 3A). However, a trans-replication system is  
354 different from natural virus infection. Therefore response of selected reporters to virus was  
355 also analysed. For these experiments, U2OS cells and CMV-Fluc-Gluc or HSPolI-Fluc-Gluc



356 templates as well as C6/36 cells and Ubi-Fluc-Gluc or AlbPolI-Fluc-Gluc templates were  
357 used.

358 Infection of U2OS cells transfected with CMV-Fluc-Gluc resulted in significant, (~10-fold)  
359 decrease of Fluc activity (Fig. 5A, left panel). Most likely this effect was caused by inhibition  
360 of expression of Fluc from capped transcripts produced in the nucleus, either due to shutdown  
361 of their synthesis by RNA polymerase II and/or because of virus-induced translational  
362 shutdown. In contrast, expression of the Gluc marker increased ~350-fold indicating efficient  
363 synthesis of Gluc-expressing SG RNAs by incoming virus replicase (Fig. 5A, left panel). In  
364 U2OS cells transfected with HSPolI-Fluc-Gluc CHIKV infection significantly increased  
365 expression of both markers. Expression of Fluc was boosted ~50-fold. The effect, opposite to  
366 that observed in cells transfected with CMV-Fluc-Gluc, presumable derives from the much  
367 lower background level of Fluc activity in non-infected cells. It is also possible that the RNA  
368 polymerase I is less susceptible to CHIKV infection induced degradation, which might then  
369 contribute to the observed effect. Boost of Gluc marker was, as expected, even more  
370 prominent (~30,000-fold increase).

371 Infection of C6/36 cells transfected with Ubi-Fluc-Gluc or AlbPolI-Fluc-Gluc resulted in  
372 minor increase of Fluc activity. Although the effect was statistically significant the increase of  
373 Fluc activity upon infection never exceeded 2-fold. As in mammalian cells the boost of Gluc  
374 activity was more prominent, ~55-fold for both vector types and highly significant (Fig. 5B).  
375 The absolute values of Gluc activity in cells transfected with Ubi-Fluc-Gluc were ~15-fold  
376 higher than these in cells transfected with AlbPolI-Fluc-Gluc (Fig. 5B). This correlates with  
377 the observation that the SG RNA levels in the trans-replication system were also higher when  
378 Ubi-Fluc-Gluc was used as the source of template RNA for CHIKV replicase (Fig 4B, left  
379 panel). It may indicate that in C6/36 cells the polyubiquitin promoter is stronger than the  
380 truncated *Aedes albopictus* RNA polymerase I promoter.



381

382 **Trans-replication systems utilizing RNA polymerase I can be used to distinguish lethal**  
383 **mutations from ones having strong negative impact on CHIKV RNA synthesis.**

384 Many of the mutations introduced into the alphavirus ns-proteins tend to reduce the infectivity  
385 of the mutant viruses by 1000- to 10,000-fold (38, 49). For that reason, the sensitivity of the  
386 systems relying on the use of CMV-Fluc-Gluc template in U2OS cells or T7-Fluc-Gluc  
387 template in BSR cells may not be sufficient to distinguish between truly lethal (completely  
388 blocking RNA replication) and strongly attenuating mutations which often allow virus to be  
389 rescued and undergo reversion/pseudoreversion or compensation during subsequent  
390 replication. Based on this consideration we re-evaluated the phenotypes of twelve CHIKV  
391 replicase mutants previously reported to be inactive or possess strongly reduced replicase  
392 activity.

393 Analysis of mutants harboring eGFP at the C-terminal region of nsP1 confirmed previous  
394 findings. Insertion of eGFP after amino acid residue 516 of nsP1 (P1<sup>E</sup>234-C) was  
395 considerably better tolerated than similar insertions after residues 492, 497 or 525 (P1<sup>E</sup>234-A,  
396 P1<sup>E</sup>234-B and P1<sup>E</sup>234-D, respectively) (Fig. 6A, B). It was also observed that from these  
397 three mutants P1<sup>E</sup>234-D displayed the highest transcription activity in U2OS cells (Fig. 6B).  
398 In contrast, in BSR cells it had the lowest transcription activity (36). Most likely this reflects a  
399 host-cell specific effect of the mutation. Indeed, we have previously observed that in rodent  
400 cells, CHIKV replicase mutations tend to have a more prominent effect on the trans-replicase  
401 activity than in human cells (39). The ability of mutant replicase with eGFP attached to the C-  
402 terminus of nsP4 (P1234<sup>E</sup>) to perform low level (activity ~1% from that of wt replicase)  
403 replication and transcription was also confirmed (Fig 6A, B); this is consistent with the  
404 previous observation that virus harboring a similar insertion is viable but rapidly loses the  
405 inserted marker (36). A newly constructed replicase harboring eGFP at N-terminus of nsP1

406 displayed Fluc activity of only ~2-fold over background (activity at the presence of CMV-  
407 P1234<sup>GAA</sup>), however this difference was statistically significant ( $p<0.01$ ). The transcription  
408 was better detectable (the boost of Gluc activity ~900-fold over background) and highly  
409 significant ( $p<0.0001$ ). Thus, addition of eGFP to the N-terminus of nsP1 strongly inhibits,  
410 but does not completely block, the activity of CHIKV replicase. A virus harboring  
411 corresponding insertion might therefore be viable but likely also highly unstable.

412 Mutation in the active site of capping enzyme (P1<sup>DA</sup>234) allowed both Fluc (Fig. 6A) and  
413 Gluc (Fig. 6B) markers expression to occur at levels significantly above background  
414 ( $p<0.0001$  and  $p<0.001$ , respectively) clearly indicating synthesis of CHIKV positive-strand  
415 RNAs. As for this mutant the replicase-made RNAs are presumably non-capped and therefore  
416 poorly translated their relative amounts were probably even higher than could be deduced  
417 from the increase of reporter expression. This finding clearly supports the hypothesis that  
418 synthesis of alphavirus positive-strand RNAs can occur in the absence of nsP1 capping  
419 activity (37, 39). The effects observed for the mutation in the membrane binding peptide of  
420 nsP1 (P1<sup>WA</sup>234) and for mutations known to reduce cytotoxicity (P12<sup>EK</sup>34, P12<sup>EKPG</sup>34) (Fig  
421 6A, B) were consistent with previous findings (36, 39).

422 A mutation in the active site of nsP2 helicase/NTPase/triphosphatase (P12<sup>KN</sup>34) was clearly  
423 lethal for CHIKV as no activity above background was displayed (Fig 6A, B). Interestingly, a  
424 similar mutation in the context of SFV has been reported to allow reversions to occur (50). If  
425 so, the effect of this mutation is different among different alphaviruses. As the properties of  
426 P1<sup>DA</sup>234 suggest that RNA capping is not an absolute requirement for RNA synthesis it can  
427 be concluded that it was lack of RNA helicase and/or NTPase activity of nsP2 that blocked  
428 synthesis of CHIKV positive-strand RNAs. No Fluc expression above background was  
429 observed for P12<sup>CA</sup>34, P12<sup>WA</sup>34 and P12<sup>CA+SA</sup>34 that have mutations in active site of nsP2  
430 protease. This is in agreement with our previous findings that CHIKV harboring such

431 mutations is not viable (8). All three mutants displayed very weak (~3-fold over background)  
432 but still significant (p-values <0.01; <0.0001 and <0.01 respectively) transcription activities.  
433 It is possible that this small activity may reflect some minor ability of unprocessed P1234 to  
434 perform viral RNA synthesis and/or that the bond between nsP3 and nsP4 in P1234 undergoes  
435 very inefficient spontaneous hydrolysis or cleavage by cellular enzymes. Taken together, the  
436 trans-replication system used here allowed clear separation of lethal replicase mutations from  
437 these that allow virus to be rescued. This conclusion is supported by data obtained using the  
438 RNA polymerase I based trans-replication system for analysis of effects of mutations in the  
439 helicase domain of CHIKV nsP2 (51). Similarly, in difficult to transfect murine neuronal cells  
440 the differences between lethal and viable mutants of nsP3 macro domain were clearly  
441 observed (13).

442

443 **Mutations in membrane binding regions of nsP1 have strong negative effect on CHIKV**  
444 **RNA replication and infectivity.** In order to perform detailed analysis of CHIKV nsP1  
445 functions, the D63A mutation was introduced into Ubi-P1234 and into CMV-ICRES1, an  
446 infectious cDNA (icDNA) clone of CHIKV. In addition, three additional mutations were  
447 introduced into CMV-ICRES1, CMV-P1234 and Ubi-P1234. First, Tyr248 was substituted to  
448 Ala (Y248A). This residue is located in the membrane binding peptide region of nsP1 of  
449 alphaviruses and is important for membrane binding of nsP1 (52, 53) and/or for its activity as  
450 a capping enzyme (37, 54). Second, Arg252, located inside the membrane binding peptide,  
451 was substituted to Glu (R252E). Third, Cys residues 417-419, representing the palmitoylation  
452 site of CHIKV nsP1, were substituted with Ala residues (3C3A). All these mutations have  
453 been previously studied in the context of SFV trans-replicase and, with exception of D64A (in  
454 SFV numeration is based on the amino acid residue numbers in its nsP1), also in the context  
455 of SFV icDNA. Briefly, in the SFV trans-replicase system none of the mutants allowed

456 positive-strand RNA synthesis above background level; however, mutations D64A and  
457 Y249A did allow negative-strand RNA synthesis and spherule formation (37). In the context  
458 of SFV genome 3C3A mutation allowed virus rescue (55) and generation of second-site  
459 adaptive mutations (56) while all three mutations in membrane binding peptide were lethal  
460 (57). As we have already revealed, the phenotypes of SFV<sup>W259A</sup> and CHIKV<sup>W258A</sup> are clearly  
461 different (39). It was also very recently reported that mutation of C417-C419 to A417-A419  
462 in the context of CHIKV 181/25 genome results in drastic reduction of the virus RNA levels  
463 in cells transfected with transcripts from a corresponding mutant icDNA (58). However,  
464 authors of this study did not report the presence or absence of infectious virus progeny.  
465 Therefore it was of interest to compare the effects of nsP1 mutations on the rescue and RNA  
466 replication of SFV and CHIKV.

467 The infectious centre assay (ICA) revealed that only the CMV-ICRES1<sup>DA</sup> was able to form  
468 plaques; however, its infectivity was ~100,000-fold lower than that of wt CHIKV. The lack of  
469 infectivity for CMV-ICRES1<sup>YA</sup>, CMV-ICRES1<sup>RE</sup> and CMV-ICRES1<sup>3C3A</sup> was confirmed by  
470 western blotting that could not reveal the presence of capsid protein in cells transfected with  
471 any of these three plasmids. In contrast, synthesis of capsid protein was observed for  
472 CHIKV<sup>D63A</sup>, consistent with the data on infectious virus rescue (Fig. 7A). Sequencing of the  
473 mutated region in the genome of rescued CHIKV<sup>DA</sup> revealed, however, that the introduced  
474 mutation had reverted. Nevertheless, this data clearly confirms that the lack of capping  
475 activity did not completely prevent positive-strand RNA synthesis which, in turn, allowed  
476 reversion to take place. As no infectious progeny was found for CMV-ICRES1<sup>3C3A</sup> it was also  
477 concluded that, in contrast to SFV, the mutation of the nsP1 palmitoylation site is lethal for  
478 CHIKV.

479 Next, the effects of these mutants were analysed in U2OS cells using HSPolI-Fluc-Gluc  
480 template and CMV-P1234 replicase plasmids. With the exception of nsP1<sup>RE</sup> all nsP1 proteins

481 were found to be expressed at similar levels (Fig. 7B). As R253E mutation has similar effect  
482 on SFV nsP1 (37) it can be hypothesized that nsP1<sup>RE</sup> fails to bind to membranes and this  
483 results in destabilization of the protein. The CMV-P1<sup>WA</sup>234 was able to boost both Gluc and  
484 Fluc expression (~60,000-fold and ~150-fold respectively). In both cases, the difference in  
485 levels achieved using wt CMV-P1234 was ~5-fold (Fig. 7B). At 39°C, however, only ~800-  
486 fold boost of Gluc and ~4-fold boost of Fluc expression was observed for CMV-P1<sup>WA</sup>234; a  
487 difference from wt CMV-P1234 >300-fold and highly significant (p<0.0001) for both  
488 replication and transcription markers. Thus, the temperature sensitive phenotype resulting  
489 from W258A mutation, formerly revealed only for transcription (39), is clearly applicable for  
490 replication as well. Consistent with data from ICA and capsid protein expression, no replicase  
491 activity above background level was observed for CMV-P1<sup>3C3A</sup>234 and CMV-P1<sup>RE</sup>234 (Fig.  
492 7B). When the assay was performed at 28°C the CMV-P1<sup>3C3A</sup>234 remained completely  
493 inactive. In contrast, at reduced temperature Fluc and Gluc activities were boosted by CMV-  
494 P1<sup>RE</sup>234 ~3.3-fold and ~350-fold, respectively, and were both significantly above background  
495 level (p<0.01 for Fluc and p<0.05 for Gluc). Thus, in the context of CHIKV replicase, the  
496 R252E mutation results in a strong temperature sensitive phenotype. The substitution  
497 introduced to CMV-ICRES<sup>RE</sup> (CGC codon to GAG) requires two nucleotide substitutions for  
498 pseudoreversion (GAG to CGG or AGG Arg codons). Based on our experience such  
499 mutations do not revert when introduced to alphavirus icDNAs; therefore rescue of CMV-  
500 ICRES1<sup>RE</sup> at a permissive temperature (28 °C) was not attempted. CMV-P1<sup>DA</sup>234 was, again,  
501 able to boost expression of both Fluc and Gluc (Fig. 7A) indicating the presence of sufficient  
502 replicase activity for virus rescue to occur. These activities were, apparently, reduced too  
503 strongly (compared to wt CMV-P1234 ~130-fold for replication and ~230-fold for  
504 transcription) to allow preservation of introduced mutation that could be reverted by change  
505 of a single nucleotide residue (GCT for Ala to GAT for Asp). CMV-P1<sup>Y248A</sup>234 displayed

506 even stronger attenuation yet both Fluc and Gluc activities were clearly and significantly  
507 ( $p<0.05$ ) above background levels (Fig. 7B). The failure to rescue CMV-ICRES1<sup>YA</sup> (Fig. 7A)  
508 is most likely due to the nature of the introduced substitution (TAC codon to GCC) as two  
509 nucleotide substitutions are required for reversion. Taken together, in U2OS cells only  
510 palmitoylation site mutation resulted in replicase that was completely inactive in all  
511 conditions used. D63A, Y248A and R252E substitutions all displayed some, albeit strongly  
512 reduced, replicase activity at least at some of the tested conditions.

513 Finally, the same set of mutations was analysed in *Aedes aegypti*-derived AF319 cells using  
514 AegPolII-Fluc-Gluc template and Ubi-P1234 replicase plasmids. As in mammalian cells, all  
515 nsP1 proteins except nsP1<sup>RE</sup> were found to be expressed at similar levels (Fig 7C). Again,  
516 only the W258A mutant generated a replication signal similar to that of wt. This data does  
517 not, however, exclude synthesis of positive-strand genomic RNAs by the D63A mutant (and  
518 possibly Y248A mutant) as for this mutant the non-capped replicase-made RNAs would not  
519 have a translation advantage over the initial RNA polymerase I made transcripts. Indeed, the  
520 analysis of transcription signal, which offers higher sensitivity, revealed Gluc activities,  
521 significantly above background for D63A ( $p<0.05$ ) and Y248A ( $p<0.05$ ) mutants indicating  
522 synthesis and translation of SG RNAs (Fig. 7C). The Ubi-P1<sup>RE</sup>234 also displayed ability to  
523 increase Gluc levels; however, at this case boost was minor (~3.3-fold) and not statistically  
524 significant ( $p=0.167$ ). Finally, no replication or transcription was detected for replicase  
525 encoded by Ubi-P1<sup>3C3A</sup>234. The correlation of data from mammalian and mosquito cells  
526 indicates that defects caused by nsP1 mutations were not host cell specific. Our data also  
527 confirmed that the phenotypes caused by the D63A and Y248A mutations in CHIKV and  
528 their counterparts in SFV are similar. This might also be the case for R252E mutation, but the  
529 tools used to study it in the context of SFV had inadequate sensitivity. In contrast, the  
530 phenotype caused by 3C3A mutation in the context of SFV and CHIKV was clearly different:

531 in the former it must allow replicase activity necessary for virus rescue while in the latter no  
532 evidence of RNA replication could be revealed by any of the highly sensitive assays.

533 **DISCUSSION.**

534 Use of promoters and terminators of cellular RNA polymerase I resulted in a replicase system  
535 of unprecedented sensitivity. The increased sensitivity was mostly due to the fact that  
536 background level of reporter expression was considerably reduced with little (mosquito cells)  
537 or no (mammalian cells) loss of marker expression at the presence of viral replicase.  
538 Similarly, it may also be advantageous that transcripts made by RNA polymerase I do not  
539 undergo splicing. This may be important for sequences derived from the genomes of positive-  
540 strand RNA viruses that often contain cryptic splicing signals. Use of an RNA polymerase I  
541 based system may also improve stability of RNA transcripts. RNAs made from alphavirus  
542 template constructs (Fig. 1B, 2B) have a bicistronic structure. If made by RNA polymerase II  
543 these RNAs behave as mRNAs with abnormally long 3' UTR that contains non-translated  
544 Gluc ORF and may therefore be recognized by cellular mRNA degradation machinery.  
545 Finally, compared to promoters of RNA polymerase II the RNA polymerase I promoter, that  
546 is normally used for synthesis of ribosomal RNAs, is less likely to have strongly differential  
547 expression or silencing in different tissues.

548 A caveat for the construction of efficient RNA polymerase I mediated template-RNA  
549 expression constructs is species-specificity, which was observed for both mammalian (Fig. 3)  
550 and *Aedes* mosquito (Fig. 4) derived promoters. Interestingly, no clear correlation between  
551 promoter origin and the efficiency of the corresponding system in cells from different  
552 organisms was observed. Thus, a human RNA polymerase I promoter had no obvious  
553 advantage over that of Chinese hamster in primate cells (Fig. 3) and was also found to work in  
554 murine cells (13). In contrast, none of these promoters worked efficiently in BHK-21 cells.  
555 The reasons for this remained unknown though it can be speculated that the RNA polymerase  
556 I of Syrian golden hamster cannot recognize the promoter fragments used. This species  
557 specificity represents a significant problem for design of template-RNA expressing constructs



for non-model species where the RNA polymerase I promoters/terminators are not well characterized. Given the low conservation of the sequences of these elements an analysis of RNA polymerase I based transcription of these organisms may be required before efficient template-RNA producing constructs can be designed. An obvious way to alleviate at least some of these problems would be the use of ribozymes to trim the 5' and 3' ends of RNA transcripts. In this regard it should be noticed that while we experienced no difficulties with trimming of the 3' ends of transcripts with an HDV RZ, the use of HH RZ to generate authentic 5' ends essentially failed in mammalian cells. However, it worked reasonably well in the case of mosquito cells alleviating problems related to the observed species specificity (Fig. 4). Therefore, it can be considered as a potential approach for species where the information about RNA polymerase I promoter sequence and function is limited or absent. Consistent with our previous findings (36) relatively little transcript was generated from CMV-Fluc-Gluc by RNA polymerase II and these RNAs were poorly detectable by northern blot analysis (Fig.3B). This was also the case for Ubi-Fluc-Gluc transcripts in mosquito cells (Fig. 4B). In contrast, genomic RNAs synthesized by wt CHIKV replicase were abundant (Fig. 3B, 4B). However, the increase of RNA copy number resulted in disproportionate boost of Fluc expression (Fig. 3A, 4A). Most likely this lack of correlation between RNA and Fluc reporter levels is a consequence of a basic property of alphavirus infection: ns-proteins are synthesized only at the early stages of infection while the synthesis of their mRNAs (virus genomes) remains active until cell death (59). Hence, only a small amount of RNA genomes are used as mRNAs and in the late infection the ratio of ns-proteins to RNA genomes drops dramatically. Our trans-replication system appears to capture this important property of alphavirus infection, but this effect limits the use of genomic (Fluc) reporter activity for the analysis of genomic RNA synthesis. For the RNA polymerase II based system the nucleus-made capped RNA transcripts are the first and most efficiently translated mRNAs for Fluc.

583 The increase in replicase-generated genomic RNAs, observed by northern blotting, occurs at a  
584 later stage when they are poorly translated, if at all. Therefore in the RNA polymerase II  
585 based system, translation of genomic reporter (Fluc) from initial transcripts can mask that  
586 from replicase-generated RNAs. Use of non-capped transcripts made by RNA polymerase I  
587 avoids this effect.

588 It was also observed that in contrast to replication in mammalian cells, replication in mosquito  
589 cells resulted in abnormally high SG to genomic RNA ratio (Fig. 3B and 4B). This may relate  
590 to the use of a truncated 3' UTR in the template RNAs. The native 3' UTR of CHIKV  
591 LR2006OPY1 strain comprises 498 nucleotide residues. In contrast, template RNAs used in  
592 this study contain only the last 110 nucleotides (36). The missing part of CHIKV 3' UTR  
593 contains repeated sequence motifs which have only a minimal effect on CHIKV replication in  
594 mammalian cells (60). In contrast, however, a deletion of this region caused a prominent  
595 reduction of CHIKV replication in both *Aedes aegypti* and *Aedes albopictus* cells (61). To the  
596 best of our knowledge, the molecular basis of this defect has not been reported. Thus, it is  
597 plausible that the defect may include a shift of SG to genomic RNA ratio into the favor of the  
598 former. This shift might reflected an altered ratio of synthesis or instability of bicistronic  
599 genomic RNA with truncated 3' UTR. If so, this trans-replication system may serve as useful  
600 tool for studies on the role of the upstream part of 3' UTR in RNA synthesis and/or stability.  
601 Such an analysis was, however, outside the scope of current study.

602 As shown here, alphavirus replicase can use RNA polymerase I generated RNAs as templates;  
603 this emphasizes that the 5' cap-structure is not required for RNA replication. Even the  
604 ribozyme-generated 5' ends that lack 5' phosphate can still be used by alphavirus replicase.  
605 At the same time it is well known that *in vitro* generated non-capped transcripts from  
606 alphavirus icDNAs are not infectious. Our data indicate that this is most likely a consequence  
607 of low level of replicase proteins synthesis rather than a lack in the ability of the replicase to

608 use such transcripts as templates. If so, the role of the cap-structure of viral RNA is to ensure  
609 the correct level of ns-protein expression, stabilize the RNA in the cellular environment (62)  
610 and prevent its recognition by cellular pattern recognition receptors. It has been recently  
611 demonstrated that alphaviruses synthesise non-capped RNAs in normal infection and that  
612 these RNAs are packed into virions (62). Furthermore, it was subsequently shown that an  
613 artificial increase of capping efficiency leads to overproduction of viral nsPs and decrease of  
614 virion formation (63). Authors of these studies did also reveal that virions made in  
615 mammalian cells contain mostly non-capped RNAs while virions made in mosquito cells  
616 contain mostly capped RNAs and have therefore lower particle per PFU ratios (62). These  
617 findings clearly correlate with the behavior of our trans-replication templates. In mammalian  
618 cells, the replicase of an incoming virus was capable to use both capped and non-capped  
619 template RNAs equally well resulting in similar final levels of marker expression (Fig. 5A).  
620 In C6/36 mosquito-derived cells, capped transcripts performed clearly better reaching higher  
621 expression levels (Fig. 5B). This may indicate that in C6/36 cells the non-capped RNAs are  
622 used by viral replicase less efficiently than capped ones. The lack of cap applies, however,  
623 only for the initial replication event as CHIKV replicase facilitates the capping of the RNAs  
624 made by the formed replication complexes; hence thereafter the replication process in the cell  
625 should proceed identically regardless the capping status of initial RNA transcripts. If so, the  
626 lower efficiency of replication (Fig. 5) could indicate that replication is initiated in relatively  
627 few C6/36 cells. A similar phenomenon has previously been observed in C6/36 cells  
628 transfected with a construct expressing SINV template; upon infection with SINV the  
629 replication of the RNA template was evident in only ~5% of the infected cells (48). In line  
630 with these observations we have identified several replicase mutations that boosted replication  
631 of the CHIKV template RNA in mosquito cells between 10-30-fold (39). In contrast, a  
632 mutation in P1234 that is capable of boosting replication of template RNA in mammalian cell

633 considerably above the level achieved by wt P1234 has never been identified. All these  
634 findings indicate that initiation of template-RNA replication in mosquito cells by virus-  
635 encoded replicase is relatively inefficient. Most likely it is a consequence of the different  
636 environment in mammalian and mosquito cells. The initiation of template RNA replication is  
637 a complex process which includes RNA recognition by virus and host encoded replicase  
638 components, their interaction with host cell membranes, formation and maturation of  
639 replication complexes and so on; analysis of this multi-step process is the topic of on-going  
640 research in our laboratories and different template-RNA expression constructs represent  
641 valuable tools for such studies.

642 As shown in previous studies, alphavirus trans-replicase is an excellent tool for the analysis of  
643 nsPs functions. Here we took advantage of the increased sensitivity of the RNA polymerase I  
644 based system and analysed the effects of different mutations on the ability of CHIKV nsP1 to  
645 support viral RNA synthesis. The analysis not only confirmed our previous findings that the  
646 C-terminus of nsP1 tolerates insertion of eGFP tag (36) but revealed also that such a tag can  
647 be incorporated into the N-terminus of nsP1. Though the replicase harboring such a tag had  
648 severely diminished activity the finding still clearly demonstrates that N-terminally tagged  
649 nsP1 is a functional protein. It is unclear why an insertion of eGFP to the N-terminus of nsP1  
650 has higher negative impact on its function compared to a similar insertion in the C-terminal  
651 region. It may be a consequence of the N-terminus proximity to important functional motifs  
652 such as the catalytic His residue of guanylyltransferase and the catalytic Asp residue of  
653 methyltransferase, being located only 37 and 63 amino acid residues downstream,  
654 respectively (64). At this point it is not clear whether the similarity of properties of P<sup>E</sup>1234  
655 and P1<sup>DA</sup>234 (Fig. 5) is coincidental or reflects the similar nature of the defect (presumably  
656 lack of RNA capping) caused by these mutations. It was also found that mutations Y248A and  
657 R252E, counterparts of which have been shown to abolish ability of SFV replicase to

658 synthesize positive-strand RNAs (37), severely reduced but did not completely blocked  
659 CHIKV replicase activity. In these cases, the observed difference between SFV and CHIKV  
660 could most likely be explained by the more sensitive assay used in this study and the  
661 temperature sensitive nature of the defect(s) caused by R252E mutation. More likely than not,  
662 both of these mutations affect SFV and CHIKV in the same way. In contrast, the effect of  
663 mutation in the palmitoylation site of CHIKV nsP1 was clearly different from that reported in  
664 SFV. No activity of this mutant CHIKV genome or its replicase was evident in our studies  
665 indicating the lethal nature of the mutation. Interestingly, another defect of similar type has  
666 also been described. Inability of nsP3 of SFV to bind cellular G3BP proteins results in  
667 attenuated phenotype (65); in contrast, binding of G3BPs by nsP3 is crucial for CHIKV  
668 replication (12). nsP1 palmitoylation is required for correct interactions of alphavirus  
669 replicase proteins and their association with membranes (56). It has also been suggested that  
670 G3BPs participate in the formation of replicase complexes (12). Therefore it can be  
671 speculated that both of these differences between CHIKV and SFV may have a common  
672 cause.

673 The replication and transcription of reporter-encoding template RNA has been used for  
674 detection of SINV infection in mosquitoes. *In vivo*, up to ten-fold activation of reporter  
675 expression upon infection by homologous virus was observed. Interestingly, when the same  
676 experiment was performed using transiently transfected C6/36 cells, the boost of marker  
677 expression was more modest, ~2-fold (48). In a comparable setup, both Ubi-Fluc-Gluc and  
678 AlbPoli-Fluc-Gluc derived template RNAs showed ~55-fold boost of reporter expression  
679 (Fig. 5B) clearly outperforming the previously analysed SINV template construct. Therefore  
680 it is likely that the constructs reported in this study would have superior properties that would  
681 make them suitable for the generation of stable insect cell lines and transgenic mosquitoes.  
682 The same applies, likely to much bigger extent, to mammalian cells and HSPoli-Fluc-Gluc

683 template expression construct. In a transient setup, nearly 30,000-fold activation of Gluc  
684 expression was observed upon virus infection (Fig 5A). As the background of the Gluc  
685 expression was negligible and the on/off ratio as good or superior to any reported inducible  
686 expression system to-date, the design may have several potential uses. This includes *in vitro*  
687 applications such as inducible expression of toxic proteins in cell culture or construction of  
688 cell lines for easy detection and quantification of alphavirus infection. In addition, a potential  
689 *in vivo* application might be the generation, through transgenesis, of animals carrying such an  
690 inducible reporter. This could in principle be used to monitor and trace virus infection with  
691 unprecedented sensitivity and accuracy.

692

## 693 MATERIALS AND METHODS

### 694 Cells.

695 All mammalian cell lines were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.  
696 U2OS human bone osteosarcoma cells (ATCC HTB-96) were maintained in Iscove's  
697 modified Dulbecco's medium (Gibco) containing 10% fetal bovine serum (FBS; GE  
698 Healthcare) and 2 mM L-glutamine. Vero E6 African green monkey kidney cells (ATCC  
699 CCL-81) were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS  
700 and 2 mM L-glutamine. BHK-21 baby hamster kidney cells (ATCC CCL-10) were grown in  
701 Glasgow's minimal essential medium (Gibco) containing 10% FBS, 2% tryptose phosphate  
702 broth (TPB) and 200 mM HEPES pH 7.2. All mosquito-derived cell lines were maintained at  
703 28°C with no additional CO<sub>2</sub>. *Aedes albopictus* C6/36 cells were maintained in Leibowitz's L-  
704 15 medium (Corning) containing 10% FBS. *Aedes aegypti* Aag2 cell-derived Dicer2 knockout  
705 cell line AF319 (66) was maintained in Leibowitz's L-15 medium (Corning) containing 20%  
706 FBS, 10% TPB and 1X non-essential amino acids. All media were supplemented with 100  
707 U/mL penicillin and 0.1 mg/mL streptomycin.

### 708 Construction of plasmids for production of RNA templates in mammalian cells.

709 CMV-Fluc-Gluc, a vector designed for expression of replication-competent template RNA of  
710 CHIKV using cellular RNA polymerase II in mammalian cells, has been previously described  
711 (36). A 193 bp long sequence, corresponding to 137 5' residues of TMV including 23 first  
712 codons of its replicase ORF followed by three in-frame stop codons and a HH RZ designed to  
713 cleave RNA transcript immediately upstream of the residue corresponding to the 5' end of  
714 CHIKV genome, was inserted between the start site of CMV promoter and the beginning of  
715 CHIKV-specific sequence in CMV-Fluc-Gluc using synthetic DNA fragments (Genscript,  
716 USA) and subcloning procedures; the generated plasmid was designated CMV-HH-Fluc-  
717 Gluc. Similarly, a synthetic DNA fragment containing sequences corresponding to human

718 RNA polymerase I promoter (residues from -211 to +20 with respect to transcription start  
719 site) and HH RZ was used to replace T7 RNA promoter in T7-Fluc-Gluc (36); a 100 bp long  
720 sequence, corresponding to mouse RNA polymerase I terminator, was inserted downstream of  
721 the sequence corresponding to HDV RZ. The generated plasmid was designated HSPolI-HH-  
722 Fluc-Gluc. The deletion of sequence corresponding to the downstream region of human RNA  
723 polymerase I promoter (residues +1 to +20) and HH RZ was performed using PCR-based  
724 mutagenesis, the generated plasmid was designated HSPolI-Fluc-Gluc. Finally, the human  
725 RNA polymerase I promoter in HSPolI-Fluc-Gluc was replaced by the corresponding  
726 sequence (positions -227 to -1 with respect to transcription start site) of Chinese hamster  
727 (*Cricetulus griseus*) resulting in a plasmid designated CGPolI-Fluc-Gluc (Fig. 1B). Sequence  
728 of all plasmids was verified using Sanger sequencing. Sequences from residue -10 of  
729 promoter to residue 10 of CHIKV are shown in Table 1; full sequences are available from  
730 authors upon request.

731 **Construction of plasmids for production of RNA templates in mosquito cells.**

732 Ubi-Fluc-Gluc, a vector designed for the expression of replication-competent template RNA  
733 of CHIKV using cellular RNA polymerase II in mosquito cells, has been previously described  
734 (39). To obtain mosquito RNA polymerase I based constructs, an intron, present in Ubi-Fluc-  
735 Gluc, was removed. As the RNA polymerase I promoters of *Aedes aegypti* and *Aedes*  
736 *albopictus* share little similarity (45, 46) separate vectors for template RNA production were  
737 constructed for cells derived from these two mosquito species. To obtain a vector for *Aedes*  
738 *albopictus* cells, the polyubiquitin promoter and Simian virus 40 terminators of Ubi-Fluc-  
739 Gluc were replaced with *Aedes albopictus* RNA polymerase I promoter and putative  
740 terminator (100 bp), respectively. A 250 bp promoter fragment (residues -250 to -1) was used,  
741 such that the first nucleotide of the CHIKV genome corresponds to the transcription start. The  
742 generated plasmid was designated AlbPolI-Fluc-Gluc. Similar substitutions were made using



743 the RNA polymerase I promoter (residues -250 to -1) and putative terminator (100 bp) of  
744 *Aedes aegypti*. This resulted in a plasmid designated AegPolI-Fluc-Gluc. Finally, the  
745 sequence corresponding to residues +1 to +50 of RNA polymerase I promoter of *Aedes*  
746 *aegypti* followed by the sequence corresponding to a HH RZ, was inserted between start site  
747 of promoter and the residue corresponding to the 5' end of CHIKV genome in AegPolI-Fluc-  
748 Gluc; the resulting plasmid was designated AegPolI-HH-Fluc-Gluc (Fig. 2B). Sequence of all  
749 plasmids was verified using Sanger sequencing. Sequences from residue -10 of promoter to  
750 residue 10 of CHIKV are shown in Table 1; full sequences are available from authors upon  
751 request.

752 **Construction of plasmids for expression of mutant replicase and mutant icDNA**  
753 **constructs.**

754 Construction of CMV-P1234, CMV-P1234<sup>GAA</sup>, CMV-P1<sup>E</sup>234-A, CMV-P1<sup>E</sup>234-B, CMV-  
755 P1<sup>E</sup>234-C, CMV-P1<sup>E</sup>234-D, CMV-P1234<sup>E</sup>, CMV-P12<sup>EK</sup>34, CMV-P12<sup>EKPG</sup>34, CMV-  
756 P12<sup>KN</sup>34, CMV-P1<sup>DA</sup>234 and CMV-P1<sup>WA</sup>234 has been previously described (36, 39). In order  
757 to generate constructs designated CMV-P12<sup>CA</sup>34, CMV-P12<sup>WA</sup>34 and CMV-P12<sup>CASA</sup>34 the  
758 region corresponding to mutated nsP2 protease was transferred from T7-P12<sup>CA</sup>34, T7-  
759 P12<sup>WA</sup>34 and T7-P12<sup>CA+SA</sup>34 plasmids (8) to the CMV-P1234 plasmid. To fuse eGFP to the  
760 N-terminus of nsP1 a flexible Gly-Gly-Ser-Gly-Gly-Ser linker was added to the C-terminus of  
761 eGFP. Using site-directed PCR mutagenesis and subcloning, a plasmid designated CMV-  
762 P<sup>E</sup>1234 was generated. Additional point mutations were incorporated in CMV-P1234 using  
763 site-directed PCR mutagenesis: Y248A (CMV-P1<sup>YA</sup>234), R252E (CMV-P1<sup>RE</sup>234) and  
764 substitution of cysteine residues 417-419 of nsP1 to alanine residues (CMV-P1<sup>3C3A</sup>234). The  
765 latter three mutations as well as D63A substitution were also incorporated into CMV-ICRES1  
766 (also called DREP-ICRES1), an icDNA clone of CHIKV LR2006OPY1 isolate (67) using  
767 site-directed PCR mutagenesis and subcloning procedures. The resulting clones were

768 designated CMV-ICRES1<sup>DA</sup>, CMV-ICRES1<sup>YA</sup>, CMV-ICRES1<sup>RE</sup> and CMV-ICRES1<sup>3C3A</sup>.

769 Sequence of all plasmids was verified using Sanger sequencing.

770 **Trans-replication assay.**

771 The trans-replication assay was performed as previously described (36). Briefly, U2OS, Vero  
772 E6 and BHK-21 cells grown in 12-well plates were co-transfected with 1 µg of template-  
773 expressing vector (CMV-Fluc-Gluc, CMV-HH-Fluc-Gluc, HSPolI-HH-Fluc-Gluc, HSPolI-  
774 Fluc-Gluc or CGPolI-Fluc-Gluc) and 1 µg of CMV-P1234 (or its mutant variants) using  
775 Lipofectamine LTX (Thermo Fisher Scientific) reagent according to the manufacturer's  
776 instructions. Transfected cells were incubated at 37°C for 18 h. C6/36 and AF319 cells grown  
777 in 12-well plates were co-transfected with 0.5 µg of template expressing vector (Ubi-Fluc-  
778 Gluc, AegPolI-HH-Fluc-Gluc, AegPolI-Fluc-Gluc or AlbPolI-Fluc-Gluc) and 0.5 µg of Ubi-  
779 P1234 (or its mutant variants) using Lipofectamine LTX and incubated at 28°C for 48 h. After  
780 incubation, cells were lysed and Fluc and Gluc activities were measured using the Dual-  
781 Luciferase-Reporter assay on a Glomax SIS luminometer (Promega). All Fluc and Gluc  
782 activities were normalized to these obtained for cells co-transfected with plasmids expressing  
783 corresponding template RNA and CMV-P1234<sup>GAA</sup> or Ubi-P1234<sup>GAA</sup> (for mammalian and  
784 mosquito cell experiments, respectively) controls. All assays were repeated at least three  
785 times.

786 **Northern blotting.**

787 U2OS, BHK-21, C6/36 and AF319 cells were co-transfected with plasmids coding for  
788 CHIKV replicase and RNA template as described above. At 18 h (U2OS, BHK-21) or 48 h  
789 (C6/36, AF319) post-transfection total RNA was extracted using TRIzol® reagent (Life  
790 Technologies). Equal amounts of total RNA (for mammalian cells: 2 µg for positive and 10  
791 µg for negative strand analysis; for mosquito cells: 10 µg for positive and 10 µg for negative  
792 strand analysis) were denatured for 10 min at 70°C in 2X RNA loading dye (Thermo

Scientific), cooled on ice and separated on a denaturing gel (1% agarose/6% formaldehyde) using 1X MOPS buffer. RNA was transferred to a Hybond-N+ filter (GE Healthcare) and fixed using a UV Stratalinker 1800 (Stratagene). Digoxigenin (DIG)-labelled RNA probe complementary to residues 42-390 of the sequence encoding for Gluc marker was used to detect positive-strand RNAs; probe corresponding to residues 51-376 of the sequence encoding for Fluc marker was used to detect negative-strand RNAs. Filters were hybridized overnight; blots were washed and developed according to the manufacturer's (Roche) protocols.

#### **Activation of template replication by CHIKV infection.**

U2OS cells grown in 12-well plates were transfected with CMV-Fluc-Gluc or HSPoIII-Fluc-Gluc plasmids. C6/36 cells grown in 12-well plates were transfected with Ubi-Fluc-Gluc or AlbPolII-Fluc-Gluc plasmids. At 18 h (U2OS) or 36 h (C6/36) post transfection cells were either infected with CHIKV at an MOI 10 or mock-infected. At 24 h (U2OS) or 72 h (C6/36) post-infection (h.p.i) cells were collected, lysed and Fluc and Gluc activities were measured as described above.

#### **Virus rescue and infectious centre assay.**

Virus rescue in BHK-21 cells was performed as previously described (68). ICA was performed essentially as previously described (49) except that cells were transfected with 5 µg of endotoxin-free plasmids CMV-ICRES1, CMV-ICRES1<sup>DA</sup>, CMV-ICRES1<sup>YA</sup>, CMV-ICRES1<sup>RE</sup> or CMV-ICRES1<sup>3C3A</sup>. Virus stocks were collected at 24 h (wt CHIKV) or at 48 h (mutant CHIKV variants) post transfection. Obtained stocks were clarified by centrifugation at 3000xg for 10 minutes and virus titers were determined using standard plaque assay on BHK-21 cells.

The transfected cells were collected at the same time as corresponding stocks. Cells were lysed by boiling in SDS gel-loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20%

glycerol, 200 mM DTT, and 0.2% bromophenol blue). Lysate corresponding to 50,000 transfected cells was loaded on a 10% polyacrylamide gel. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected using antibodies against CHIKV capsid protein (in-house);  $\beta$ -actin (sc-47778; Santa Cruz Biotechnology) was used as a loading control. The membranes were then incubated with appropriate secondary antibodies conjugated to fluorescent labels (LI-COR) and proteins were visualized using a LI-COR Odyssey Fc imaging system.

#### **Statistical analysis.**

Statistical analysis was done using GraphPad Prism software. Data were analyzed using Student's unpaired one tailed t-test.

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#### **CONFLICTS OF INTEREST**

Authors declare no conflict of interest

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- 1039

1040 **FIGURES.**

1041 **Figure 1.**

1042 **Schematic representation of CMV-P1234 plasmid for CHIKV replicase expression and**  
1043 **plasmids for expression of RNA templates used in mammalian cells.** (A) Expression

1044 construct for CHIKV ns-proteins. CMV - CMV IE promoter; LI - leader region of herpes  
1045 simplex virus thymidine kinase gene with an artificial intron; SV40Ter - SV40 late  
1046 polyadenylation region. Red arrow highlights the position of the inactivating mutation in the  
1047 nsP4 catalytic site. (B) Constructs expressing template RNAs. CMV\* - CMV IE promoter  
1048 followed by TMV-derived leader; HSPolI\* - full-length (-211 to +20) promoter for human  
1049 RNA polymerase I; HSPolI - truncated (-211 to -1) promoter for human RNA polymerase I;  
1050 CGPolI - truncated (-227 to -1) promoter for Chinese hamster RNA polymerase I; HH RZ-  
1051 hammer head ribozyme. The 5' and 3' UTRs are from CHIKV; N77 - region encoding for the  
1052 77 N-terminal amino acid residues of nsP1; SG - CHIKV SG promoter; HDV RZ - antisense  
1053 strand ribozyme of hepatitis delta virus, MmTer - terminator for RNA polymerase I from  
1054 mouse (*Mus musculus*). The position of the second intron of the human beta globin gene  
1055 (hBG) in CMV-Fluc-Gluc and CMV-HH-Fluc-Gluc is marked. The vector backbone is not  
1056 shown, drawings are not in scale.

1057

1058 **Figure 2.**

1059 **Schematic representation of Ubi-P1234 plasmid for CHIKV replicase expression and**  
1060 **plasmids for expression of RNA templates used in mosquito cells.** (A) Expression

1061 construct for CHIKV ns-polypotein. Ubi\* - full-length *Aedes aegypti* polyubiquitin  
1062 promoter; UL - transcribed leader of polyubiquitine gene containing naturally occurring  
1063 intron; SV40Ter - SV40 late polyadenylation region. Red arrow highlights the position of the  
1064 inactivating mutation in the nsP4 catalytic site. (B) Constructs expressing template RNAs.

1065 Ubi – truncated polyubiquitine promoter; AegPolI\* – full-length (-250 to +50) promoter for  
 1066 *Aedes aegypti* RNA polymerase I; AegPolI – truncated (-250 to -1) promoter for *Aedes*  
 1067 *aegypti* RNA polymerase I; AlbPolI – truncated (-250 to -1) promoter for *Aedes albopictus*  
 1068 RNA polymerase I; HH RZ – hammer head ribozyme; AegTer – tentative terminator for  
 1069 *Aedes aegypti* RNA polymerase I; AlbTer – tentative terminator for *Aedes albopictus* RNA  
 1070 polymerase I. The 5' and 3' UTRs are from CHIKV; N77 - region encoding for the 77 N-  
 1071 terminal amino acid residues of nsP1; SG - CHIKV SG promoter; HDV RZ - antisense strand  
 1072 ribozyme of hepatitis delta virus. In Ubi-Fluc-Gluc, the position of the second intron of the  
 1073 *Drosophila melanogaster* alcohol dehydrogenase gene (int) is marked. The vector backbone is  
 1074 not shown, drawings are not in scale.

1075

### 1076 **Figure 3.**

1077 **Comparison of template constructs in mammalian cells.** (A) U2OS, Vero E6 and BHK-21  
 1078 cells were all co-transfected with CMV-P1234 and one of CMV-Fluc-Gluc (CMV), CMV-  
 1079 HH-Fluc-Gluc (CMV HH), HSPolI-Fluc-Gluc (HSPolI), HSPolI-HH-Fluc-Gluc (HSPolI  
 1080 HH), or CGPolI-Fluc-Gluc (CGPolI). Control cells were all co-transfected with CMV-  
 1081 P1234<sup>GAA</sup> and the same template-expressing plasmids. Cells were lysed at 18 h post  
 1082 transfection. Fluc (replication, left panel) and Gluc (transcription, right panel) activities  
 1083 generated by the active replicase were normalized to controls. Each column represents an  
 1084 average of three independent experiments; error bars represent standard deviation. \*  
 1085 designates  $p < 0.05$ , \*\* designates  $p < 0.01$ , \*\*\* designates  $p < 0.001$  and \*\*\*\*\* designates  
 1086  $p < 0.0001$ , ns – not significant (Student's unpaired t-test). (B) U2OS and BHK-21 cells were  
 1087 all co-transfected with CMV-P1234 and one of CMV-Fluc-Gluc, CMV-HH-Fluc-Gluc,  
 1088 HSPolI-HH-Fluc-Gluc, HSPolI-Fluc-Gluc or CGPolI-Fluc-Gluc; control cells were all co-  
 1089 transfected with CMV-P1234<sup>GAA</sup> and the same template-expressing plasmids or were mock-



transfected. Samples were collected at 18 h post transfection. RNA was analyzed by northern blotting using a probe corresponding to the Fluc reporter gene to detect negative strands (lower panel) or probe complementary to Gluc reporter gene to detect positive strands (upper panel). “Genomic RNA” designates the full-length template RNA; note that an RNA of same size is also synthesized by cellular RNA polymerases I and II and is therefore at the cases of some promoters also detectable at the presence of inactive replicases (GAA). “Subgenomic RNA” designates RNA synthesized by CHIKV replicase using the SG promoter. The experiment was repeated two times with similar results; data from one experiment is shown.

1098

1099 **Figure 4.**

1100 **Comparison of template constructs in mosquito cells.** (A) C6/36 and AF319 cells were all  
1101 co-transfected with Ubi-P1234 and one of Ubi-Fluc-Gluc (Ubi), AegPolII-Fluc-Gluc  
1102 (AegPolII), AegPolII-HH-Fluc-Gluc (AegPolII HH) or AlbPolII-Fluc-Gluc (AlbPolII). Control  
1103 cells were all co-transfected with Ubi-P1234<sup>GAA</sup> and the same template-expressing plasmids.  
1104 Cells were lysed at 48 h post transfection. Fluc (replication, left panel) and Gluc  
1105 (transcription, right panel) activities generated by the active replicase were normalized to  
1106 controls. Each column represents an average of three independent experiments; error bars  
1107 represent standard deviation. \* designates  $p < 0.05$ , \*\* designates  $p < 0.01$ , ns – not significant  
1108 (Student’s unpaired t-test). (B) C6/36 and AF319 cells were all co-transfected with Ubi-  
1109 P1234 and one of Ubi-Fluc-Gluc, AlbPolII-Fluc-Gluc, AegPolII-Fluc-Gluc or AegPolII-HH-  
1110 Fluc-Gluc; control cells were all co-transfected with Ubi-P1234<sup>GAA</sup> and the same template-  
1111 expressing plasmids or were mock-transfected. Samples were collected at 48 h post  
1112 transfection. Positive-strand RNAs were revealed and the data is presented as described for  
1113 Fig. 3B except that 5-fold more total RNA and longer exposure were used to obtain the

1114 image. The experiment was repeated two times with similar results; data from one experiment  
1115 is shown.

1116 **Figure 5.**

1117 **CHIKV infection triggers replication and transcription of template RNAs produced**  
1118 **from CMV-Fluc-Gluc, HSPolI-Fluc-Gluc, Ubi-Fluc-Gluc and AlbPolI-Fluc-Gluc**  
1119 **plasmids.** (A) U2OS cells were transfected with CMV-Fluc-Gluc or HSPolI-Fluc-Gluc  
1120 plasmids. At 18 h post transfection cells were either infected with CHIKV at an MOI 10 or  
1121 mock-infected. Cells were collected at 24 h p.i., lysed and Fluc (replication, left panel) and  
1122 Gluc (transcription, right panel) activities measured and normalized to the number of  
1123 transfected cells. (B) C6/36 cells were transfected with Ubi-Fluc-Gluc or AlbPolI-Fluc-Gluc  
1124 plasmids. At 36 h post transfection cells were either infected with CHIKV at an MOI 10 or  
1125 mock-infected. Cells were collected at 72 h p.i., lysed and Gluc activities measured and  
1126 normalized to the number of transfected cells. Each column represents an average of at least  
1127 three independent experiments; error bars represent standard deviation. \*\*\*\* designates  
1128  $p < 0.0001$  (Student's unpaired t-test).

1129

1130 **Figure 6.**

1131 **Re-evaluation of activities of replicase mutants possessing no or strongly reduced RNA**  
1132 **synthesis abilities.** U2OS cells were all co-transfected with HSPolI-Fluc-Gluc and one of  
1133 CMV-P<sup>E</sup>1234, CMV-P1<sup>E</sup>234-A, CMV-P1<sup>E</sup>234-B, CMV-P1<sup>E</sup>234-C, CMV-P1<sup>E</sup>234-D, CMV-  
1134 P1234<sup>E</sup>, CMV-P1<sup>DA</sup>234, CMV-P1<sup>WA</sup>234, CMV-P12<sup>EK</sup>34, CMV-P12<sup>EKPG</sup>34, CMV-P12<sup>KN</sup>34,  
1135 CMV-P12<sup>CA</sup>34, CMV-P12<sup>WA</sup>34 or CMV-P12<sup>CA+SA</sup>34. Control cells were co-transfected with  
1136 HSPolI-Fluc-Gluc and CMV-P1234 or CMV-P1234<sup>GAA</sup>. Cells were lysed at 18 h post  
1137 transfection. (A) Fluc (replication) and (B) Gluc (transcription) activities generated by the  
1138 active replicase were normalized to controls. Each column represents an average of three

1139 independent experiments; error bars represent standard deviation. Names of mutant  
1140 polyproteins expressed by replicase expression plasmids are indicated below the graphs. With  
1141 exceptions of these marked „ns“ (not significant) all other mutants showed activity  
1142 significantly higher compared to negative control (P1234<sup>GAA</sup>) (Student's unpaired t-test).

1143

1144 **Figure 7.**

1145 **Effects of mutations in nsP1 of CHIKV for infectious virus rescue and trans-replicase**

1146 **activities in mammalian and mosquito cells.** (A) BHK-21 cells were transfected with one of

1147 the following plasmids: CMV-ICRES1, CMV-ICRES1<sup>DA</sup>, CMV-ICRES1<sup>YA</sup>, CMV-ICRES1<sup>RE</sup>

1148 or CMV-ICRES1<sup>3C3A</sup>. Left: results of ICA. Right: western blot of lysates from transfected

1149 cells collected at 24 h p.t. CHIKV capsid protein was revealed by corresponding rabbit

1150 polyclonal antiserum;  $\beta$ -actin was used as loading control. Data from one reproducible

1151 experiment out of two independent experiments is shown. (B) U2OS cells were all co-

1152 transfected with HSPolII-Fluc-Gluc and one of CMV-P1234, CMV-P1<sup>DA</sup>234, CMV-P1<sup>YA</sup>234,

1153 CMV-P1<sup>RE</sup>234, CMV-P1<sup>WA</sup>234, CMV-P1<sup>3C3A</sup>234 or CMV-P1234<sup>GAA</sup>. Samples were

1154 collected at 18 h post transfection. Production of positive-strand RNAs was estimated

1155 measuring activities of Fluc (left panel) and Gluc (right panel) as described in Fig 3A. Each

1156 column represents an average of three independent experiments; error bars represent standard

1157 deviation. Viral protein expression was verified by western blotting using anti-nsP1

1158 antiserum. (C) AF319 cells were all co-transfected with AegPolII-Fluc-Gluc and one of Ubi-

1159 P1234, Ubi-P1<sup>DA</sup>234, Ubi-P1<sup>YA</sup>234, Ubi-P1<sup>RE</sup>234, Ubi-P1<sup>WA</sup>234, Ubi-P1<sup>3C3A</sup>234 or Ubi-

1160 P1234<sup>GAA</sup>. Samples were collected at 48 h post transfection. Production of positive-strand

1161 RNAs was estimated measuring activities of Fluc (left panel) and Gluc (right panel) as

1162 described in Fig 3A. Each column represents an average of three independent experiments;

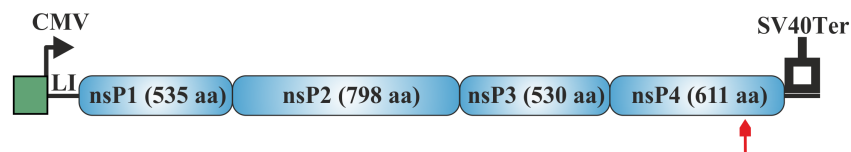
1163 error bars represent standard deviation. Viral protein expression was verified by western  
1164 blotting as described in panel B.

1165

1166

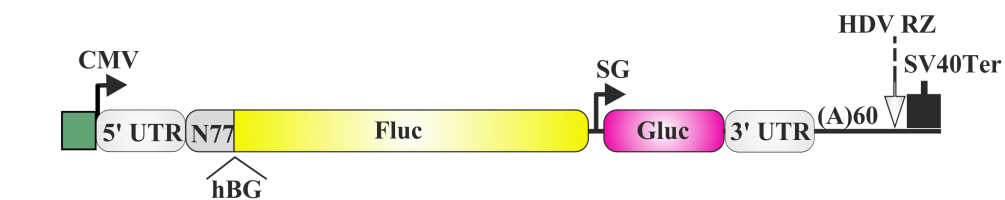
**A**

CMV-P1234

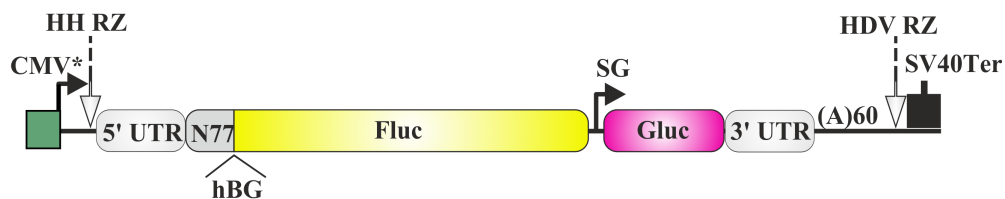


**B**

CMV-Fluc-Gluc



CMV-HH-Fluc-Gluc



HSPolI-HH-Fluc-Gluc



HSPolI-Fluc-Gluc

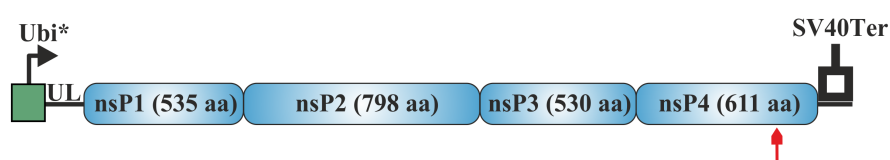


CGPolI-Fluc-Gluc

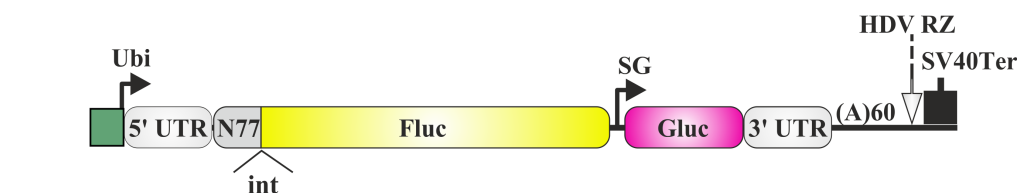


**A**

Ubi-P1234

**B**

Ubi-Fluc-Gluc



AegPoll-HH-Fluc-Gluc

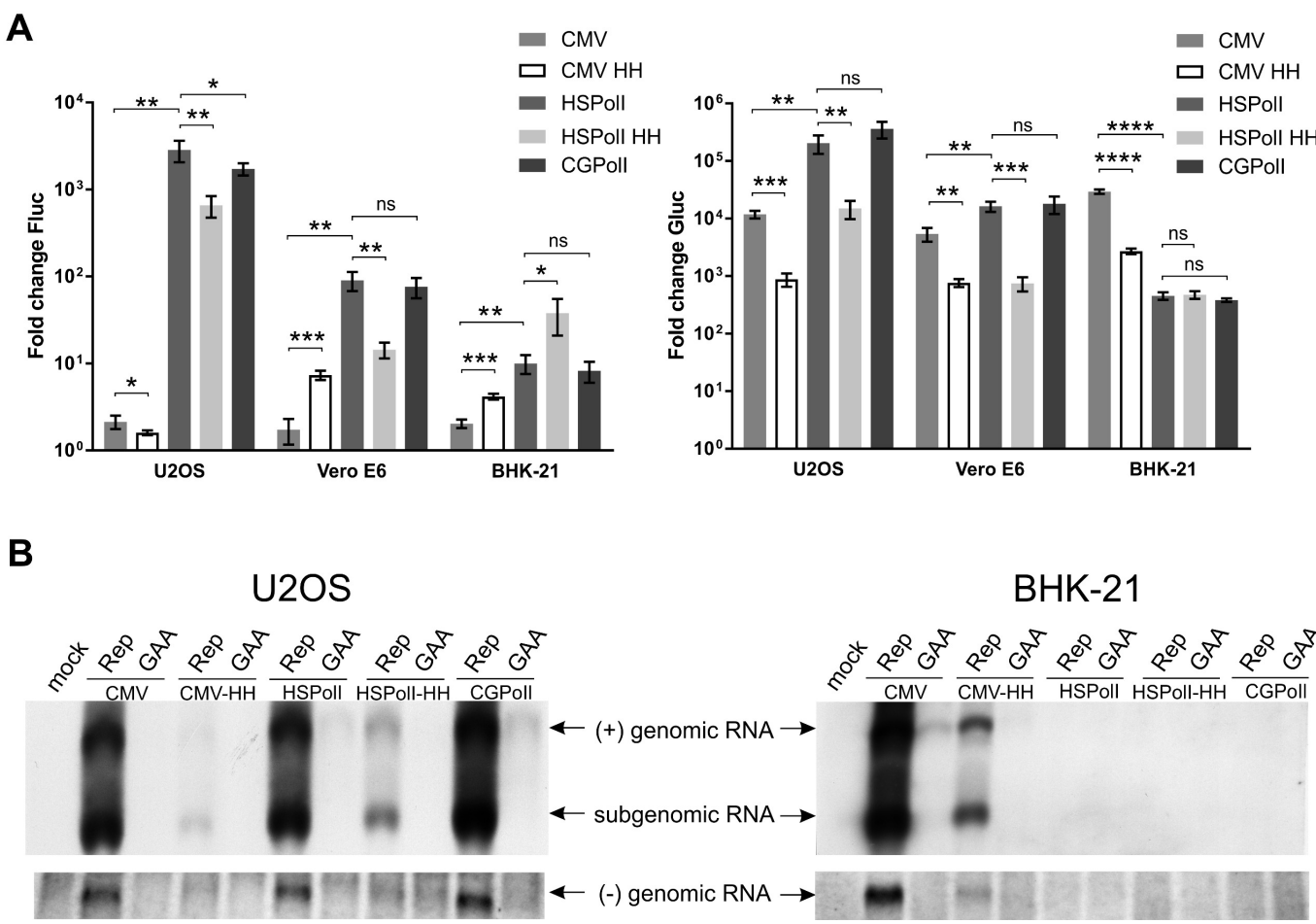


AegPoll-Fluc-Gluc

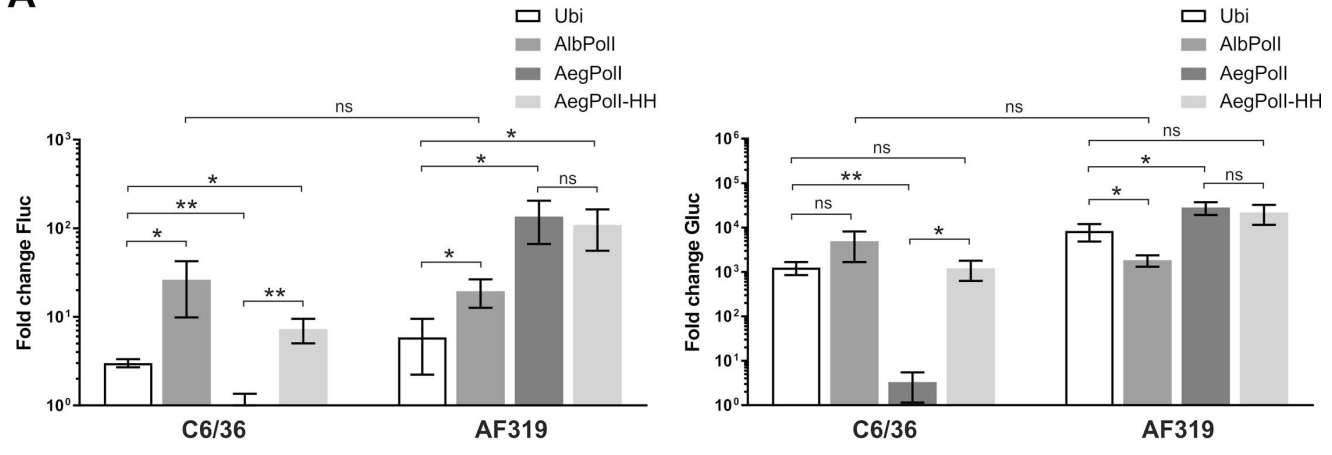


AlbPoll-Fluc-Gluc

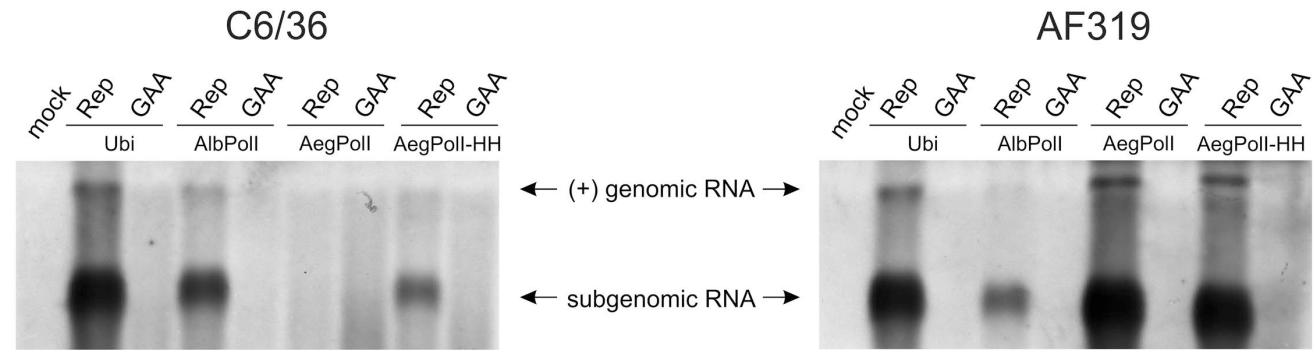




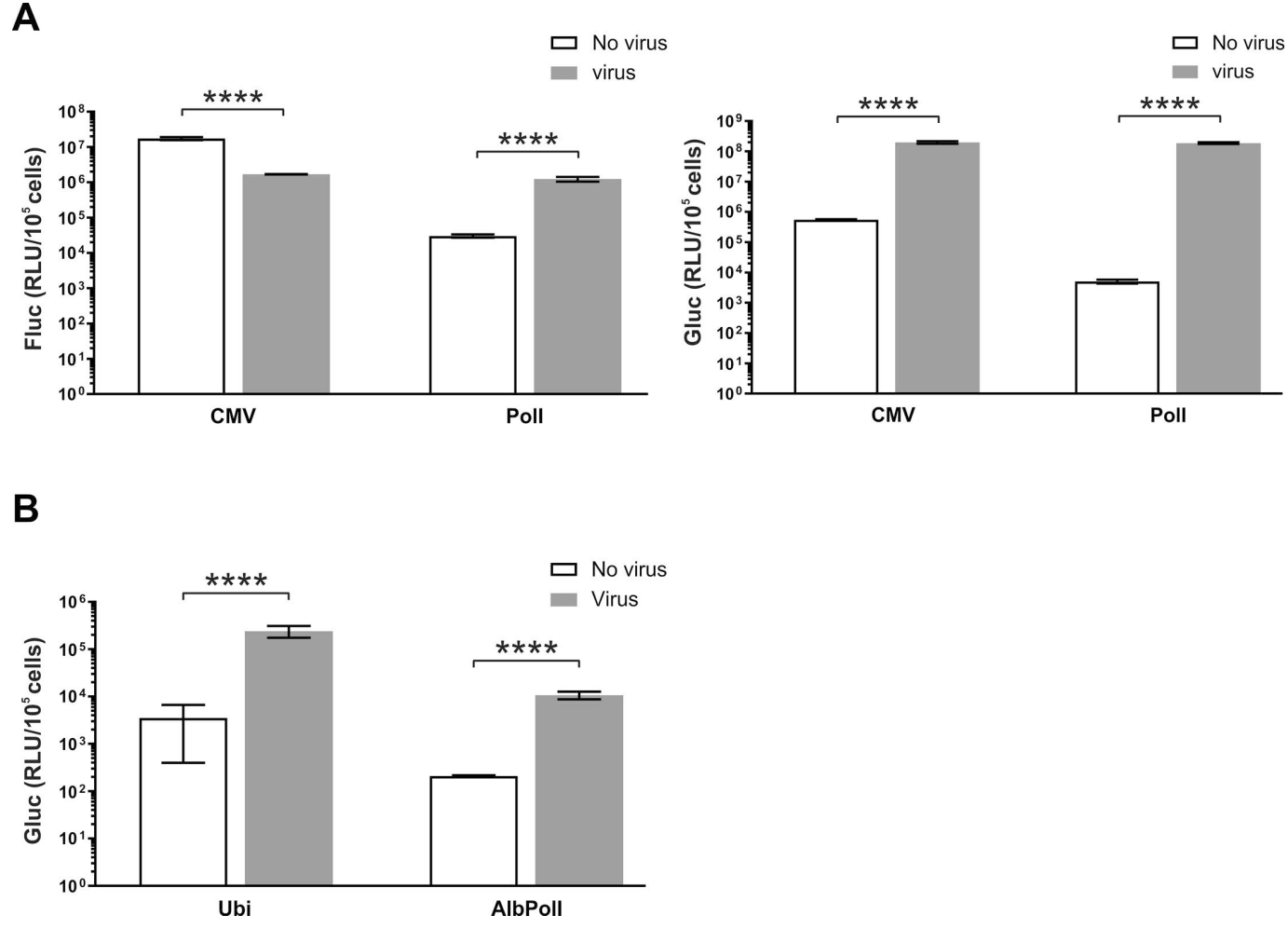
**A**

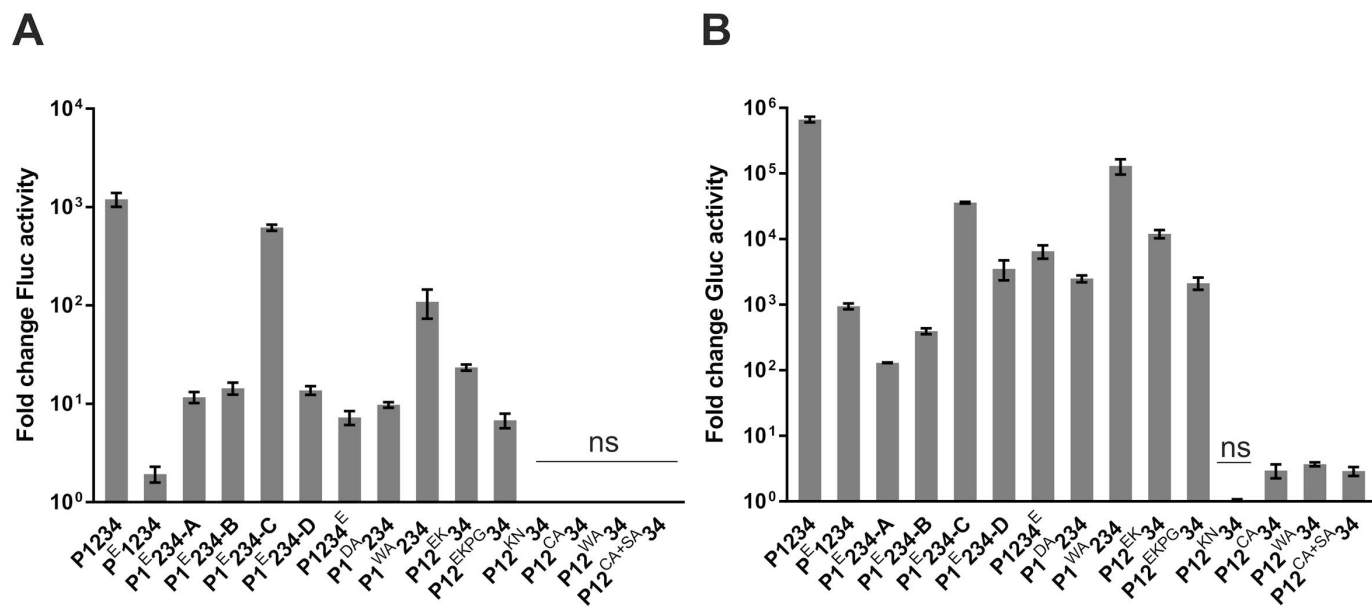
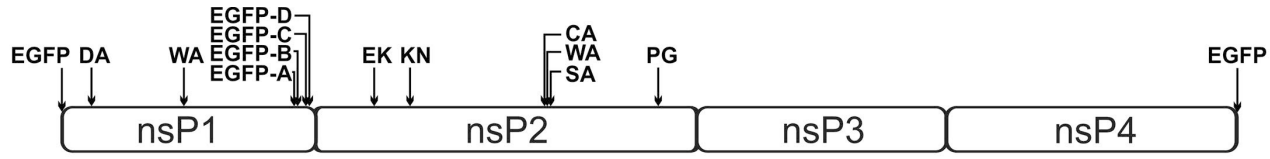


**B**









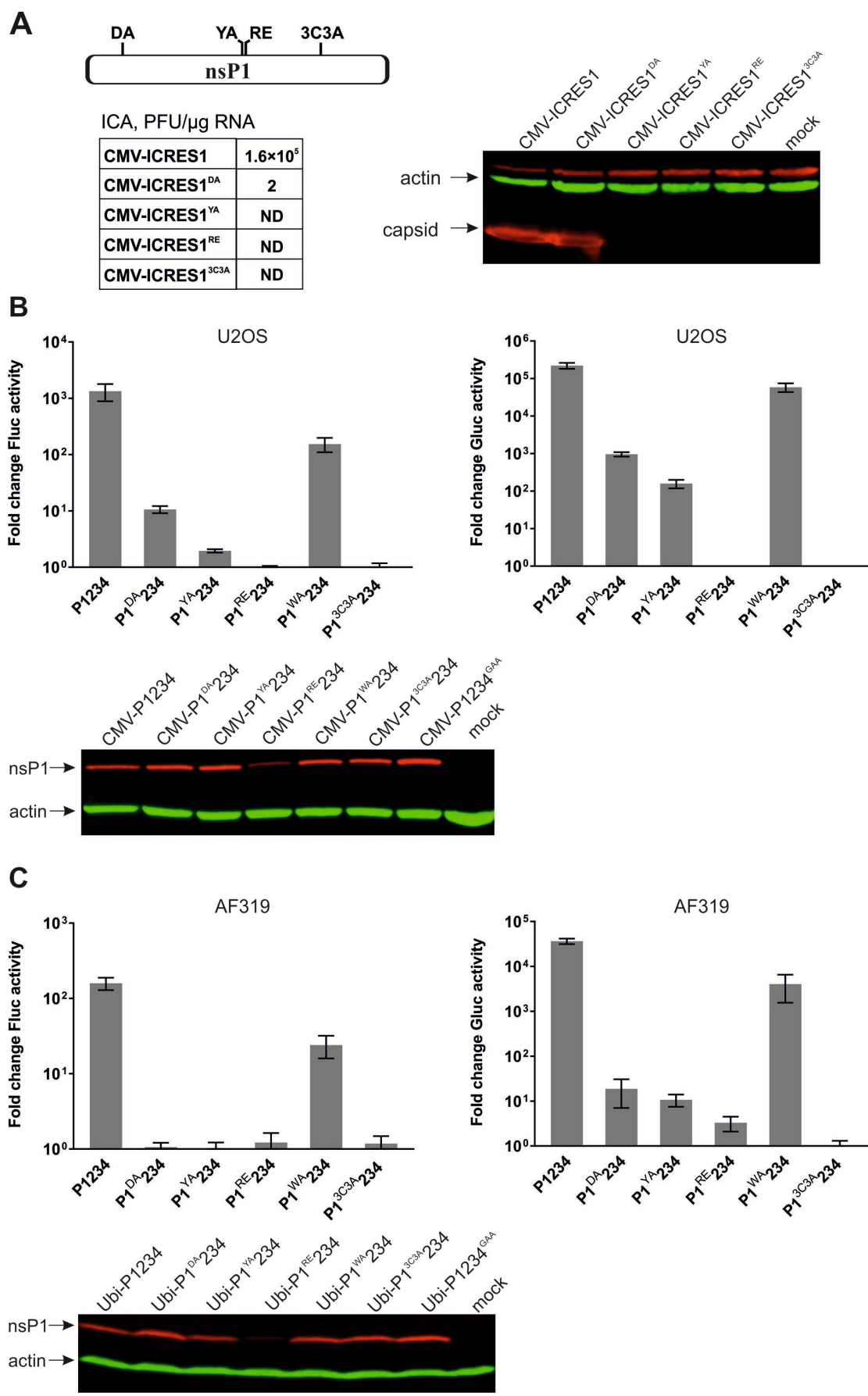


Table 1. Sequences of promoter – CHIKV 5' UTR junctions for plasmids expressing template RNAs

Plasmid	Promoter residues -10 to -1	Downstream part of promoter	Leader +HH RZ (bold)	CHIKV residues 1-10
CMV-Fluc-Gluc	AGTGAACCGT	–	–	ATGGCTGCGT
CMV-HH-Fluc-Gluc	AGTGAACCGT	–	GTATTTTACAACAATTACCAACAACAACAACAACAACAACATTAC AATTACTATTTACAATTACAATGGCATAACACAGACAGCTACCACAT CAGCTTTGCTGGACACTGTCCGAGGAAACAACCTCTTGGTCTAATAAT <b>AAAGCCATCTGATGAGAGCGAAAGCTCGAACTGGAGGAACTCCAGTC</b>	ATGGCTGCGT
HSPoll-HH-Fluc-Gluc	CCGGGTATT	GCTGACACGCTGTCTCTGG	<b>AGCCATCTGATGAGAGCGAAAGCTCGAACTGGAGGAACTCCAGTC</b>	ATGGCTGCGT
HSPoll-Fluc-Gluc	CCGGGTATT	–	–	ATGGCTGCGT
CGPoll-Fluc-Gluc	TGACACGCTT	–	–	ATGGCTGCGT
Ubi-Fluc-Gluc	AAACCAGCTC	–	–	ATGGCTGCGT
AegPoll-HH-Fluc-Gluc	AAAACCCTTC	AGGGAGGAAGGCAGTGT GCGTGGACCGGCAGGAA AATGTTCCGAAAGCAA	<b>AGCCATCTGATGAGAGCGAAAGCTCGAACTGGAGGAACTCCAGTC</b>	ATGGCTGCGT
AegPoll-Fluc-Gluc	AAAACCCTTC	–	–	ATGGCTGCGT
AlbPoll-Fluc-Gluc	AAAACCCTAT	–	–	ATGGCTGCGT