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

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

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Words in Abstract: 242

Words in Importance: 125

Words in main text: 9745 words (excluding figure legends and references)

Figures: 7

Tables: 1

ABSTRACT

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

Journal of Virology JVI Journal of Virology

IMPORTANCE

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

INTRODUCTION

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

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

Replicative functions of alphavirus replicase proteins are relatively well-studied. nsP1 is a virus-specific methyl- and guanylyltransferase and serves as membrane anchor for virus replicase (6, 7). nsP2 is a papain-like cysteine protease (8), NTPase (9), RNA triphosphatase (10) and RNA helicase (11). NsP3 of Old World alphaviruses binds cellular G3BP proteins that are required for RNA replication (12). It also contains an N-terminal macro-domain with the ability to bind ADP-ribose and remove it from mono ADP-ribosylated substrates. This is crucial for viral RNA replication (13). nsP4 is the catalytic subunit of viral RNA polymerase (14, 15) and also has terminal adenylyltransferase activity (16). In addition, all of these proteins have a number of essential functions that are not directly linked to viral RNA replication. Thus, nsP1 has been reported to antagonize an anti-viral protein, tetherin (17), nsP2 of Old World alphaviruses triggers degradation of host cell RNA polymerase II (18) and antagonizes type-I interferon signalling (19), nsP3 modulates the phosphatidylinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (20) and is responsible for translational shutoff in infected cells (21). nsP4 is involved in suppression of the unfolded protein response (22). While enzymatic functions of nsPs are well conserved for all alphaviruses their non-replicative functions exhibit a significant variation depending on virus and its host. For example nsP2 is cytotoxic only in the case of Old World alphaviruses (23) and only in vertebrate cells (18). The multiple functions of nsPs, their variation between different alphaviruses and the coupled nature of nsP expression and viral RNA replication complicates the analysis of functional significance of nsPs, their different mutations as well as investigating the role of host cell interaction partners in alphavirus RNA replication.

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

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

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

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

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

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

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

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

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

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

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

Adding a leader and HH RZ to the template reduced Fluc background in control cells less than 10-fold. The high Fluc activity in the presence of inactive replicase indicates that additional TMV sequences and HH RZ cleavage were not sufficient to block Fluc translation. It is likely that CHIKV replicase cannot efficiently use templates with 193 additional residues upstream of the native 5' end of the viral genome (Table 1). If so, boosting of expression of both reporters by active replicase (Fig. 3A) indicates that ribozyme-mediated cleavage did occur at some level. The low cleavage efficiency/speed apparently led to only low amounts of templates with correct 5' ends in cells transfected with CMV-HH-Fluc-Gluc which in turn resulted in reduced Fluc and especially Gluc activity in the presence of CHIKV-P1234. Consequently, boost of Fluc expression remained low (Fig. 3A). Compared to those achieved for CMV-Fluc-Gluc, boosts of Gluc activities for CMV-HH-Fluc-Gluc were significantly lower in all three cell lines, typically less than 1,000-fold (Fig. 3A). Taken together, it was concluded that while used approach was not efficient enough to prevent Fluc translation from RNA polymerase II generated transcripts HH RZ was capable to generate at least some amount of initial transcripts with proper 5' ends that were subsequently utilized by CHIKV replicase.

Use of RNA polymerse I promoters greatly improved system performance. Although the absolute levels of Fluc and Gluc activities observed in cells transfected with HSPolI-Fluc-Gluc + CMV-P1234 or CMV-Fluc-Gluc + CMV-P1234 were similar in U2OS cells, the much lower background activity of both reporters expressed by HSPolI-Fluc-Gluc made the assay using the latter template generally much more sensitive. First and most importantly, the boost 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 AegPolI-HH-Fluc-Gluc and AegPolI-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 ^{o}C instead of 37 ^{o}C) may facilitate the formation of the ribozyme structure.
318 AlbPolI-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, AegPolI-Fluc-Gluc was almost completely inactive in C6/36 cells.
322 Surprisingly, the activity of AegPolI-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 AegPolI-Fluc-Gluc and AF319 cells; for C6/36 cells
328 the best performance was observed for AlbPolI-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

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

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

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

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

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

Analysis of mutants harboring eGFP at the C-terminal region of nsP1 confirmed previous findings. Insertion of eGFP after amino acid residue 516 of nsP1 ($P1^{E}234$-C) was considerably better tolerated than similar insertions after residues 492, 497 or 525 ($P1^{E}234$-A, $P1^{E}234$-B and $P1^{E}234$-D, respectively) (Fig. 6A, B). It was also observed that from these three mutants $P1^{E}234$-D displayed the highest transcription activity in U2OS cells (Fig. 6B). In contrast, in BSR cells it had the lowest transcription activity (36). Most likely this reflects a host-cell specific effect of the mutation. Indeed, we have previously observed that in rodent cells, CHIKV replicase mutations tend to have a more prominent effect on the trans-replicase activity than in human cells (39). The ability of mutant replicase with eGFP attached to the C-terminus of nsP4 ($P1234^{E}$) to perform low level (activity ~1% from that of wt replicase) replication and transcription was also confirmed (Fig 6A, B); this is consistent with the previous observation that virus harboring a similar insertion is viable but rapidly loses the 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^{GAA}), 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^{DA}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 absentse of nsP1 capping
419 activity (37, 39). The effects observed for the mutation in the membrane binding peptide of
420 nsP1 (P1^{WA}234) and for mutations known to reduce cytotoxicity (P12^{EK}34, P12^{EKPG}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^{KN}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^{DA}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^{CA}34, P12^{WA}34 and P12^{CA+SA}34 that have mutations in active site of nsP2
430 protease. This is in agreement with our previous findings that CHIKV harboring such

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

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

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

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

Next, the effects of these mutants were analysed in U2OS cells using HSPolI-Fluc-Gluc template and CMV-P1234 replicase plasmids. With the exception of nsP1^{RE} all nsP1 proteins

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

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

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

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

DISCUSSION.

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

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

use such transcripts as templates. If so, the role of the cap-structure of viral RNA is to ensure the correct level of ns-protein expression, stabilize the RNA in the cellular environment (62) and prevent its recognition by cellular pattern recognition receptors. It has been recently demonstrated that alphaviruses synthesise non-capped RNAs in normal infection and that these RNAs are packed into virions (62). Furthermore, it was subsequently shown that an artificial increase of capping efficiency leads to overproduction of viral nsPs and decrease of virion formation (63). Authors of these studies did also reveal that virions made in mammalian cells contain mostly non-capped RNAs while virions made in mosquito cells contain mostly capped RNAs and have therefore lower particle per PFU ratios (62). These findings clearly correlate with the behavior of our trans-replication templates. In mammalian cells, the replicase of an incoming virus was capable to use both capped and non-capped template RNAs equally well resulting in similar final levels of marker expression (Fig. 5A). In C6/36 mosquito-derived cells, capped transcripts performed clearly better reaching higher expression levels (Fig. 5B). This may indicate that in C6/36 cells the non-capped RNAs are used by viral replicase less efficiently than capped ones. The lack of cap applies, however, only for the initial replication event as CHIKV replicase facilitates the capping of the RNAs made by the formed replication complexes; hence thereafter the replication process in the cell should proceed identically regardless the capping status of initial RNA transcripts. If so, the lower efficiency of replication (Fig. 5) could indicate that replication is initiated in relatively few C6/36 cells. A similar phenomenon has previously been observed in C6/36 cells transfected with a construct expressing SINV template; upon infection with SINV the replication of the RNA template was evident in only ~5% of the infected cells (48). In line with these observations we have identified several replicase mutations that boosted replication of the CHIKV template RNA in mosquito cells between 10-30-fold (39). In contrast, a 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 constucts 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 $P^{E}1234$
655 and $P1^{DA}234$ (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

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

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

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

MATERIALS AND METHODS

Cells.

All mammalian cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2. U2OS human bone osteosarcoma cells (ATCC HTB-96) were maintained in Iscove's modified Dulbecco's medium (Gibco) containing 10% fetal bovine serum (FBS; GE Healthcare) and 2 mM L-glutamine. Vero E6 African green monkey kidney cells (ATCC CCL-81) were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS and 2 mM L-glutamine. BHK-21 baby hamster kidney cells (ATCC CCL-10) were grown in Glasgow's minimal essential medium (Gibco) containing 10% FBS, 2% tryptose phosphate broth (TPB) and 200 mM HEPES pH 7.2. All mosquito-derived cell lines were maintained at 28°C with no additional CO_2. *Aedes albopictus* C6/36 cells were maintained in Leibowitz's L-15 medium (Corning) containing 10% FBS. *Aedes aegypti* Aag2 cell-derived Dicer2 knockout cell line AF319 (66) was maintained in Leibowitz's L-15 medium (Corning) containing 20% FBS, 10% TPB and 1X non-essential amino acids. All media were supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin.

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

CMV-Fluc-Gluc, a vector designed for expression of replication-competent template RNA of CHIKV using cellular RNA polymerase II in mammalian cells, has been previously described (36). A 193 bp long sequence, corresponding to 137 5' residues of TMV including 23 first codons of its replicase ORF followed by three in-frame stop codons and a HH RZ designed to cleave RNA transcript immediately upstream of the residue corresponding to the 5' end of CHIKV genome, was inserted between the start site of CMV promoter and the beginning of CHIKV-specific sequence in CMV-Fluc-Gluc using synthetic DNA fragments (Genscript, USA) and subcloning procedures; the generated plasmid was designated CMV-HH-Fluc-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^{GAA}, CMV-P1^{E}234-A, CMV-P1^{E}234-B, CMV-
755 P1^{E}234-C, CMV-P1^{E}234-D, CMV-P1234^{E}, CMV-P12^{EK}34, CMV-P12^{EKPG}34, CMV-
756 P12^{KN}34, CMV-P1^{DA}234 and CMV-P1^{WA}234 has been previously described (36, 39). In order
757 to generate constructs designated CMV-P12^{CA}34, CMV-P12^{WA}34 and CMV-P12^{CASA}34 the
758 region corresponding to mutated nsP2 protease was transferred from T7-P12^{CA}34, T7-
759 P12^{WA}34 and T7-P12^{CA+SA}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^{E}1234 was generated. Additional point mutations were incorporated in CMV-P1234 using
763 site-directed PCR mutagenesis: Y248A (CMV-P1^{YA}234), R252E (CMV-P1^{RE}234) and
764 substitution of cysteine residues 417-419 of nsP1 to alanine residues (CMV-P1^{3C3A}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

designated CMV-ICRES1^{DA}, CMV-ICRES1^{YA}, CMV-ICRES1^{RE} and CMV-ICRES1^{3C3A}. Sequence of all plasmids was verified using Sanger sequencing.

Trans-replication assay.

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

Northern blotting.

U2OS, BHK-21, C6/36 and AF319 cells were co-transfected with plasmids coding for CHIKV replicase and RNA template as described above. At 18 h (U2OS, BHK-21) or 48 h (C6/36, AF319) post-transfection total RNA was extracted using TRIzol® reagent (Life Technologies). Equal amounts of total RNA (for mammalian cells: 2 µg for positive and 10 µg for negative strand analysis; for mosquito cells: 10 µg for positive and 10 µg for negative 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 HSPolII-Fluc-Gluc plasmids. C6/36 cells grown in 12-well plates were transfected with Ubi-Fluc-Gluc or AlbPolI-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^{DA}, CMV-ICRES1^{YA}, CMV-ICRES1^{RE} or CMV-ICRES1^{3C3A}. 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); β-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.

ACKNOWLEDGMENTS

The authors are grateful to Mark A. Kay for the pMC.BESPX vector and Alain Kohl for AF319 cells. This work was supported by European Regional Development Fund through the Centre of Excellence in Molecular Cell Engineering, Estonia, 2014-2020.4.01.15-013 (AM), institutional research funding (IUT20-27) from Estonian Research Council (AM) and by The Wellcome Trust (200171/Z/15/Z) (AM, RF and LA). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

CONFLICTS OF INTEREST

Authors declare no conflict of interest

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1039

FIGURES.

Figure 1.

Schematic representation of CMV-P1234 plasmid for CHIKV replicase expression and plasmids for expression of RNA templates used in mammalian cells. (A) Expression construct for CHIKV ns-proteins. CMV - CMV IE promoter; LI – leader region of herpes simplex virus thymidine kinase gene with an artificial intron; SV40Ter - SV40 late polyadenylation region. Red arrow highlights the position of the inactivating mutation in the nsP4 catalytic site. (B) Constructs expressing template RNAs. CMV* - CMV IE promoter followed by TMV-derived leader; HSPolI* – full-length (-211 to +20) promoter for human RNA polymerase I; HSPolI – truncated (-211 to -1) promoter for human RNA polymerase I; CGPolI – truncated (-227 to -1) promoter for Chinese hamster RNA polymerase I; HH RZ– hammer head ribozyme. The 5′ and 3′ UTRs are from CHIKV; N77 - region encoding for the 77 N-terminal amino acid residues of nsP1; SG - CHIKV SG promoter; HDV RZ - antisense strand ribozyme of hepatitis delta virus, MmTer – terminator for RNA polymerase I from mouse (*Mus musculus*). The position of the second intron of the human beta globin gene (hBG) in CMV-Fluc-Gluc and CMV-HH-Fluc-Gluc is marked. The vector backbone is not shown, drawings are not in scale.

Figure 2.

Schematic representation of Ubi-P1234 plasmid for CHIKV replicase expression and plasmids for expression of RNA templates used in mosquito cells. (A) Expression construct for CHIKV ns-polyprotein. Ubi* - full-length *Aedes aegypti* polyubiquitin promoter*;* UL - transcribed leader of polyubiquitine gene containing naturally occurring intron; SV40Ter - SV40 late polyadenylation region. Red arrow highlights the position of the inactivating mutation in the nsP4 catalytic site. (B) Constructs expressing template RNAs.

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

Figure 3.

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

Figure 4.

Comparison of template constructs in mosquito cells. (A) C6/36 and AF319 cells were all co-transfected with Ubi-P1234 and one of Ubi-Fluc-Gluc (Ubi), AegPolI-Fluc-Gluc (AegPolI), AegPolI-HH-Fluc-Gluc (AegPolI HH) or AlbPolI-Fluc-Gluc (AlbPolI). Control cells were all co-transfected with Ubi-P1234^{GAA} and the same template-expressing plasmids. Cells were lysed at 48 h post transfection. Fluc (replication, left panel) and Gluc (transcription, right panel) activities generated by the active replicase were normalized to controls. Each column represents an average of three independent experiments; error bars represent standard deviation. * designates $p<0.05$, ** designates $p<0.01$, ns – not significant (Student's unpaired t-test). (B) C6/36 and AF319 cells were all co-transfected with Ubi-P1234 and one of Ubi-Fluc-Gluc, AlbPolI-Fluc-Gluc, AegPolI-Fluc-Gluc or AegPolI-HH-Fluc-Gluc; control cells were all co-transfected with Ubi-P1234^{GAA} and the same template-expressing plasmids or were mock-transfected. Samples were collected at 48 h post transfection. Positive-strand RNAs were revealed and the data is presented as described for 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^{E}1234, CMV-$P1^{E}$234-A, CMV-$P1^{E}$234-B, CMV-$P1^{E}$234-C, CMV-$P1^{E}$234-D, CMV-
1134 $P1234^{E}$, CMV-$P1^{DA}$234, CMV-$P1^{WA}$234, CMV-$P12^{EK}$34, CMV-$P12^{EKPG}$34, CMV-$P12^{KN}$34,
1135 CMV-$P12^{CA}$34, CMV-$P12^{WA}$34 or CMV-$P12^{CA+SA}$34. Control cells were co-transfected with
1136 HSPolI-Fluc-Gluc and CMV-P1234 or CMV-$P1234^{GAA}$. 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

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

Figure 7.

Effects of mutations in nsP1 of CHIKV for infectious virus rescue and trans-replicase activities in mammalian and mosquito cells. (A) BHK-21 cells were transfected with one of the following plasmids: CMV-ICRES1, CMV-ICRES1^{DA}, CMV-ICRES1^{YA}, CMV-ICRES1^{RE} or CMV-ICRES1^{3C3A}. Left: results of ICA. Right: western blot of lysates from transfected cells collected at 24 h p.t. CHIKV capsid protein was revealed by corresponding rabbit polyclonal antiserum; β-actin was used as loading control. Data from one reproducible experiment out of two independent expreiments is shown. (B) U2OS cells were all co-transfected with HSPolI-Fluc-Gluc and one of CMV-P1234, CMV-P1^{DA}234, CMV-P1^{YA}234, CMV-P1^{RE}234, CMV-P1^{WA}234, CMV-P1^{3C3A}234 or CMV-P1234^{GAA}. Samples were collected at 18 h post transfection. Production of positive-strand RNAs was estimated measuring activities of Fluc (left panel) and Gluc (right panel) as described in Fig 3A. Each column represents an average of three independent experiments; error bars represent standard deviation. Viral protein expression was verified by western blotting using anti-nsP1 antiserum. (C) AF319 cells were all co-transfected with AegPolI-Fluc-Gluc and one of Ubi-P1234, Ubi-P1^{DA}234, Ubi-P1^{YA}234, Ubi-P1^{RE}234, Ubi-P1^{WA}234, Ubi-P1^{3C3A}234 or Ubi-P1234^{GAA}. Samples were collected at 48 h post transfection. Production of positive-strand RNAs was estimated measuring activities of Fluc (left panel) and Gluc (right panel) as 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.
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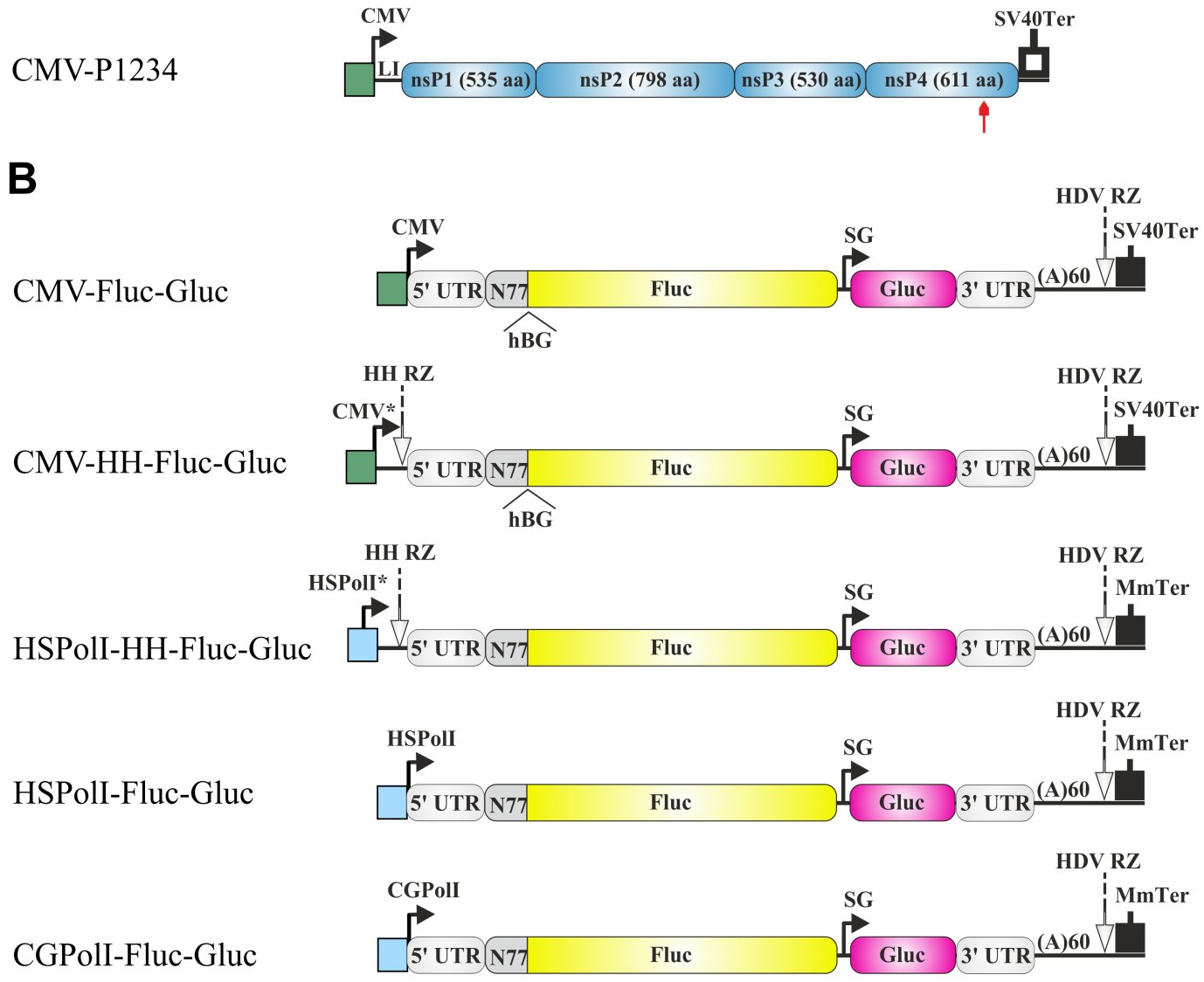
1166



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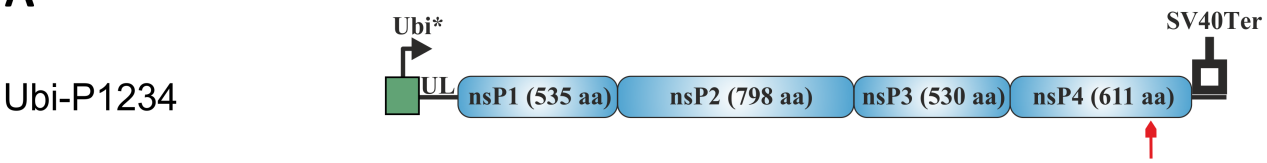

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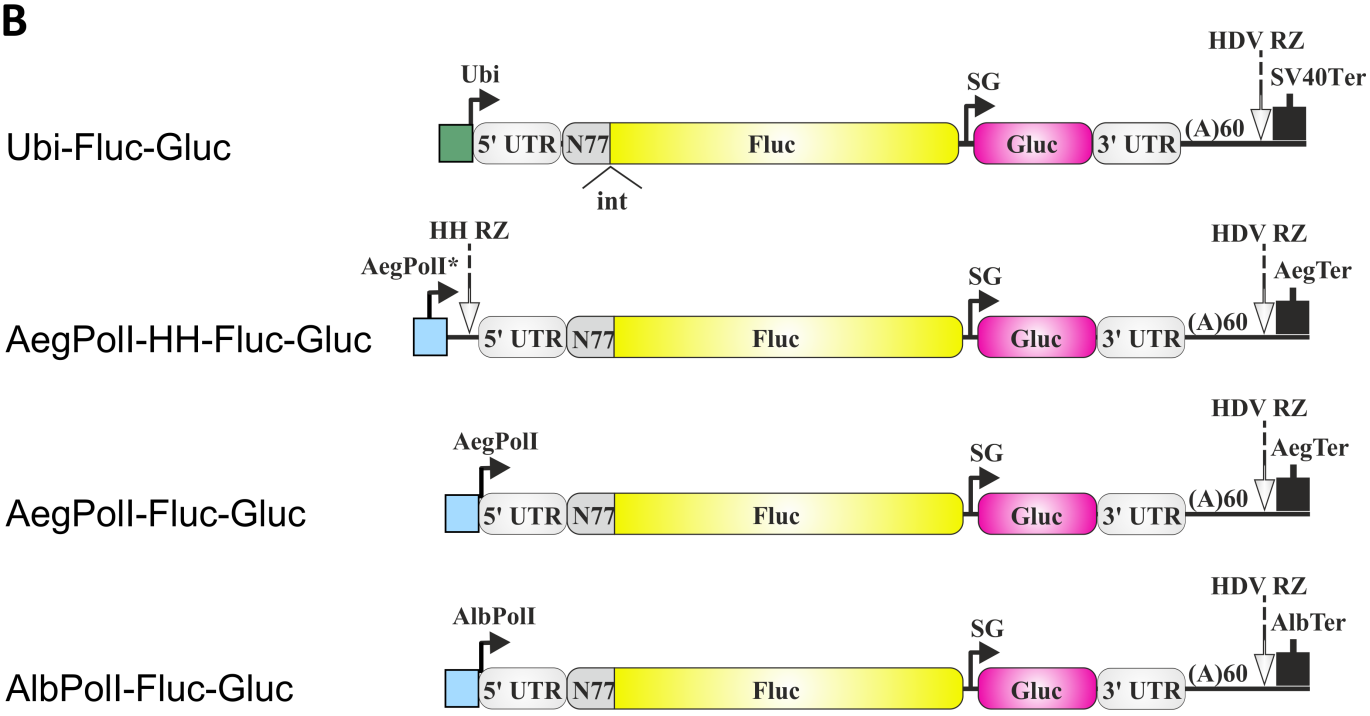

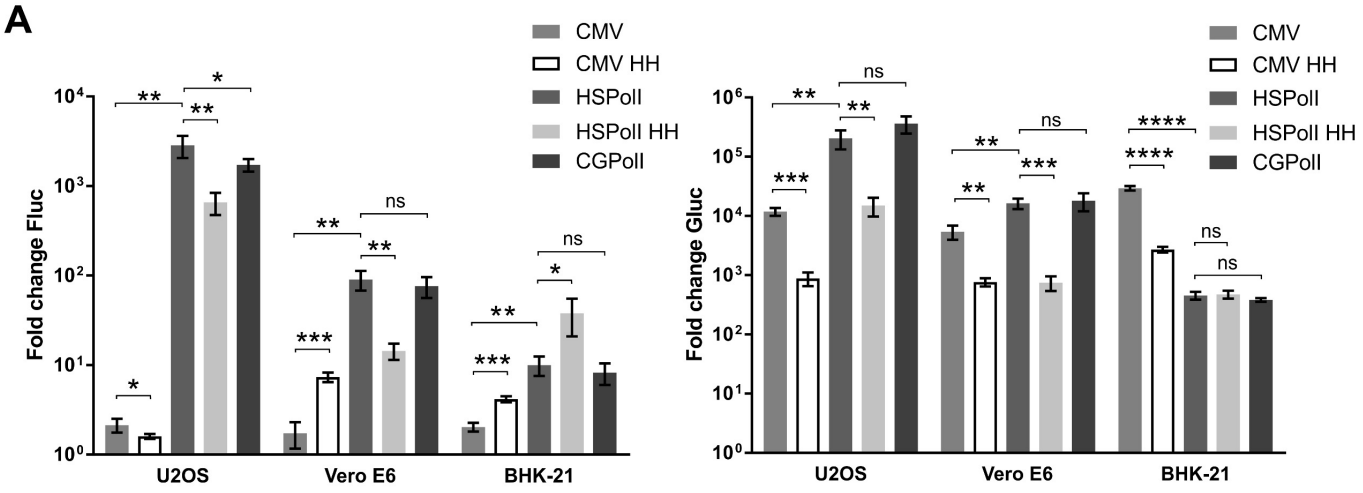

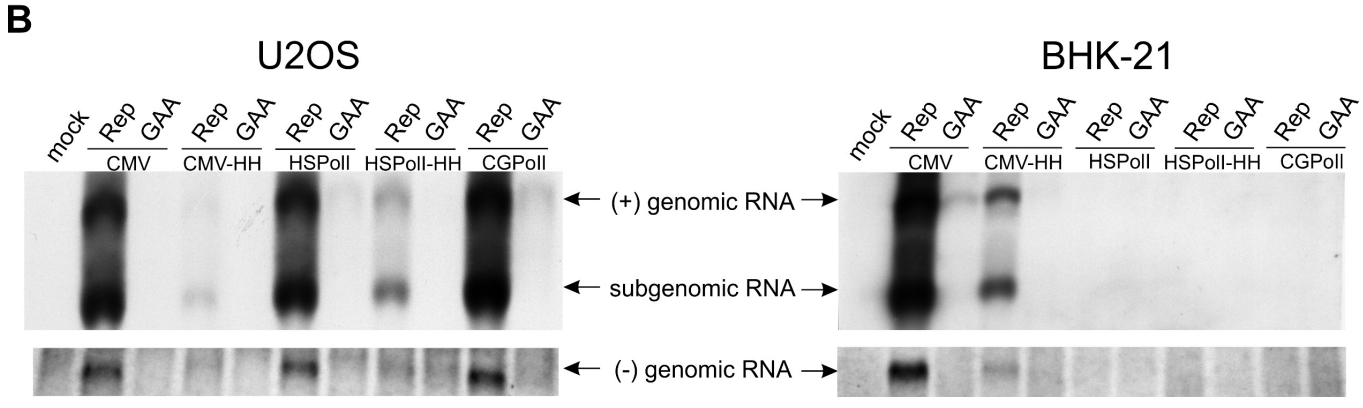

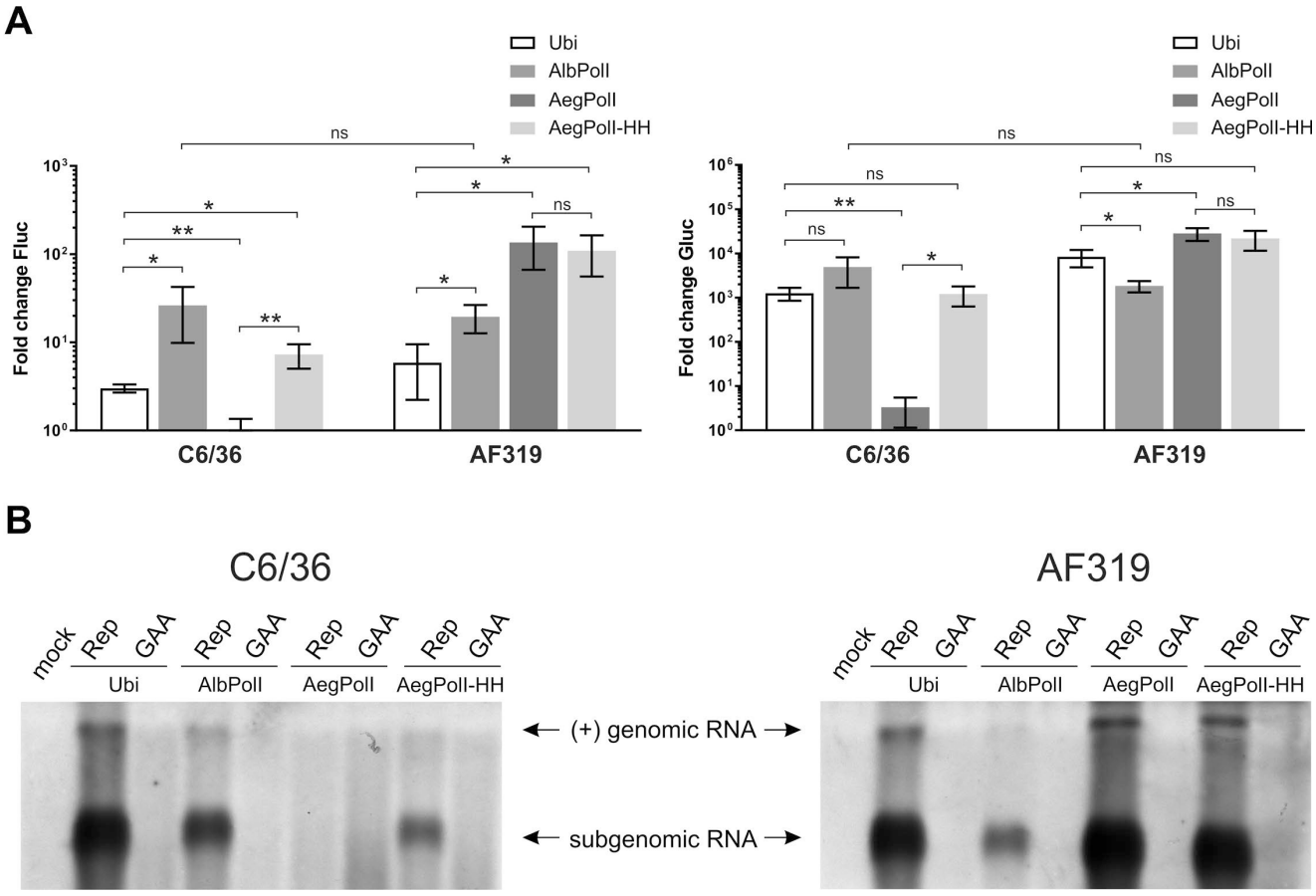

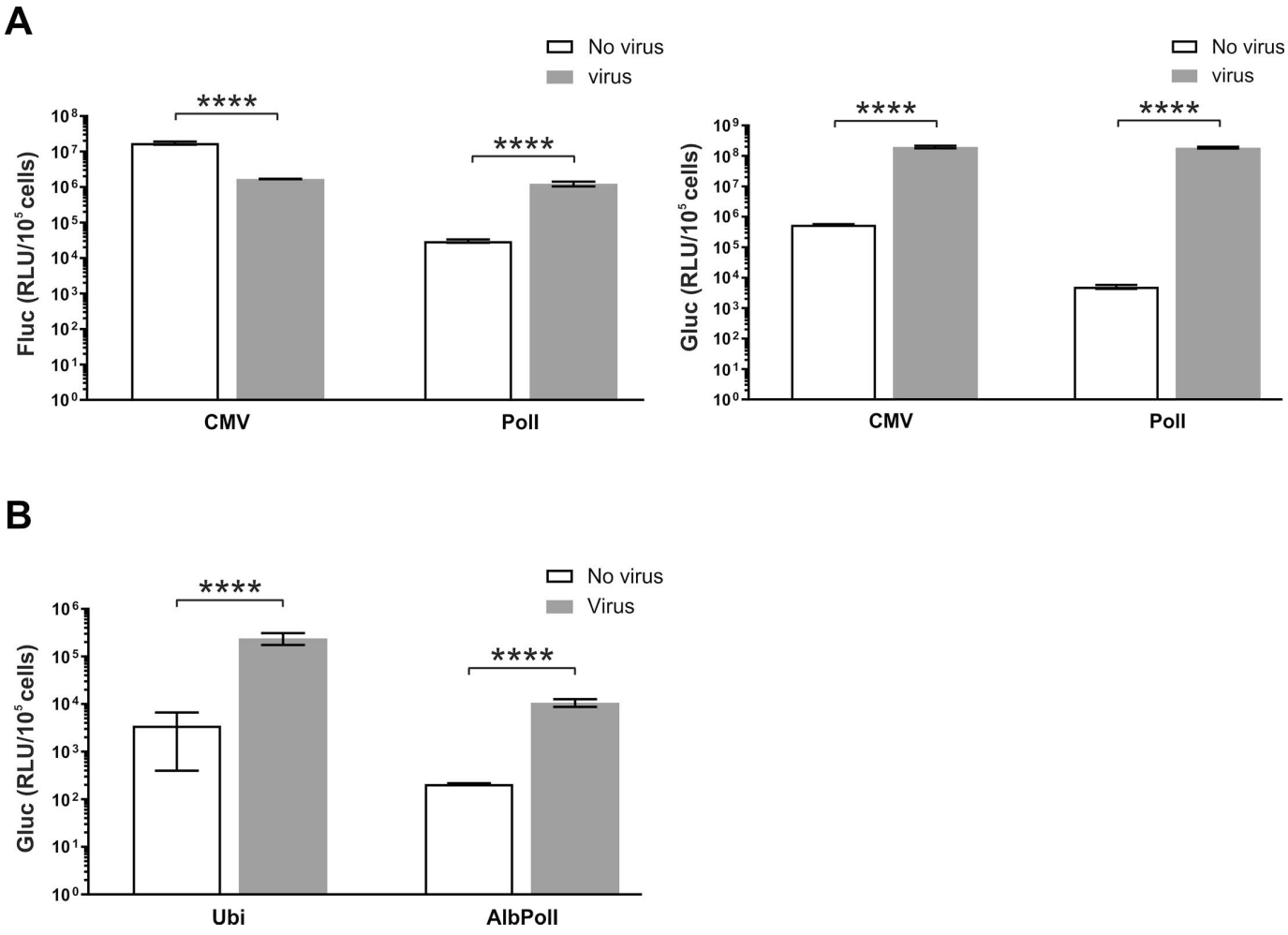

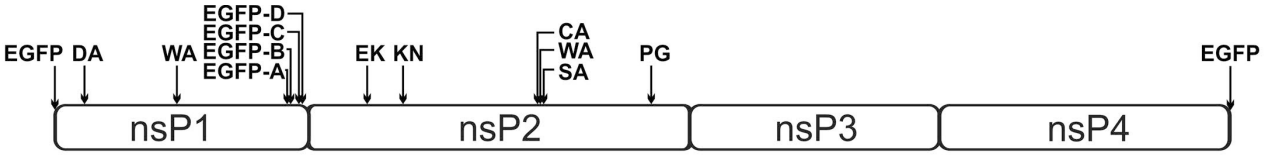

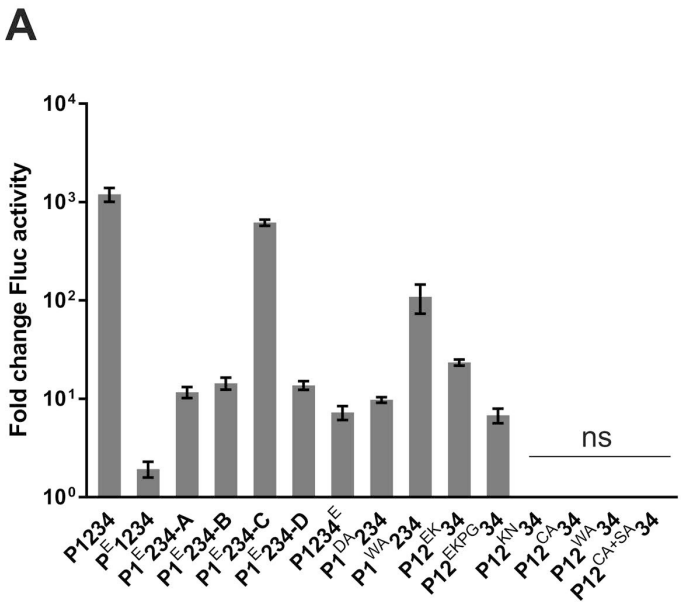

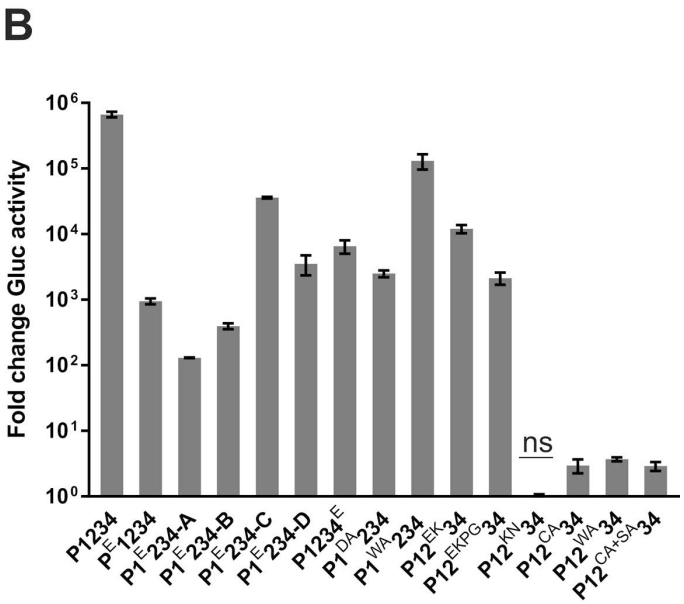

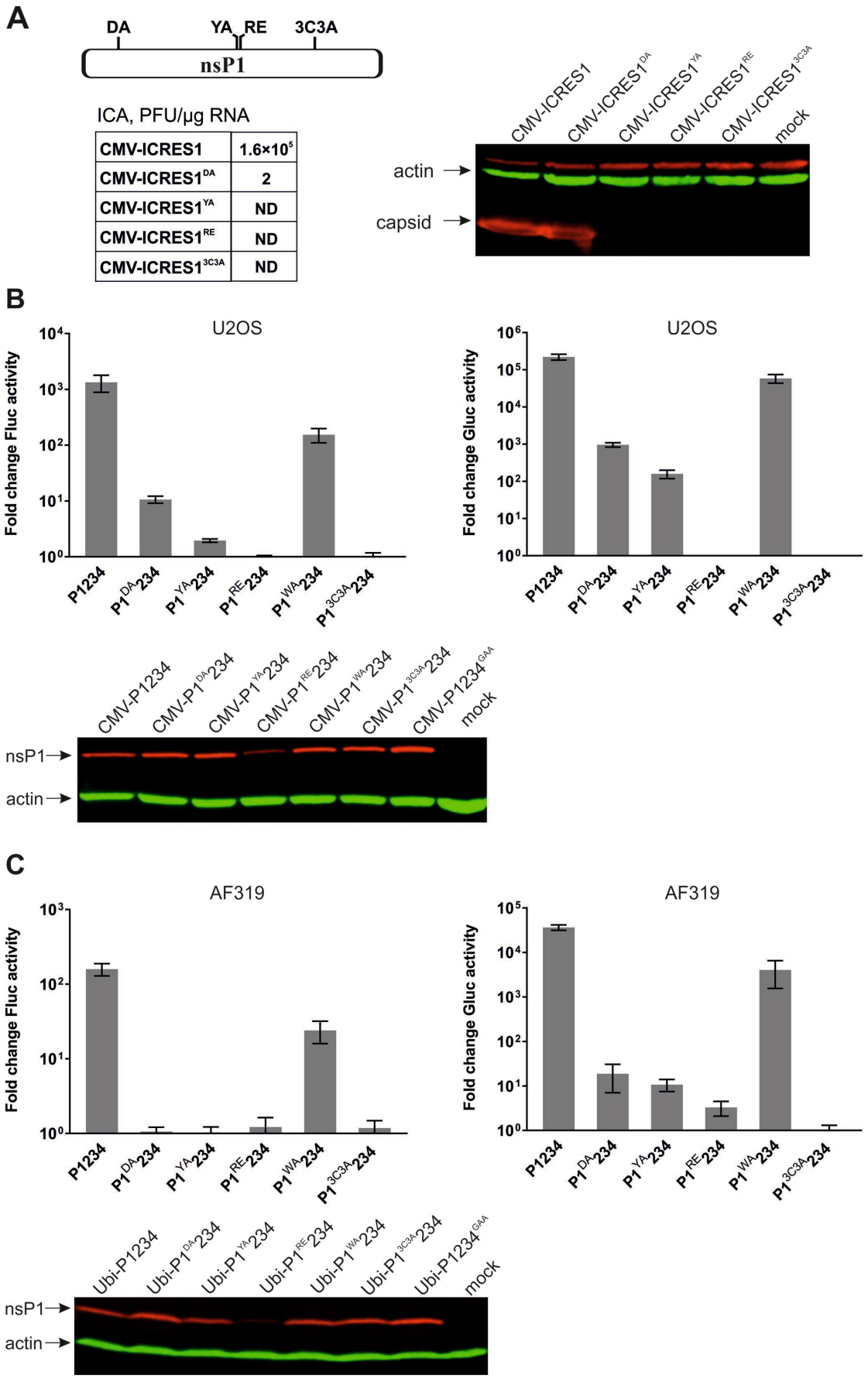

Table 1. Sequences of promoter – CHIKV 5' UTR junctions for plasmids expressing template RNAs

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	–	GTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTAC AATTACTATTTACAATTACAATGGCATACACACAGACAGCTACCACAT CAGCTTTGCTGGACACTGTCCGAGGAAACAACTCCTTGGTCTAATAAT AA**AGCCATCTGATGAGAGCGAAAGCTCGAAACTGGAGGAAACTCCAGTC**	ATGGCTGCGT
HSPolI-HH-Fluc-Gluc	CCGGGTTATT	GCTGACACGCTGTCCTCTGG	**AGCCATCTGATGAGAGCGAAAGCTCGAAACTGGAGGAAACTCCAGTC**	ATGGCTGCGT
HSPolI-Fluc-Gluc	CCGGGTTATT	–	–	ATGGCTGCGT
CGPolI-Fluc-Gluc	TGACACGCTT	–	–	ATGGCTGCGT
Ubi-Fluc-Gluc	AAACCAGCTC	–	–	ATGGCTGCGT
AegPolI-HH-Fluc-Gluc	AAAACCCTTC	AGGGAGGAAGGCAGTGT GCGTGGACCGGCAGGAA AATGTTCCGAAAGCAA	**AGCCATCTGATGAGAGCGAAAGCTCGAAACTGGAGGAAACTCCAGTC**	ATGGCTGCGT
AegPolI-Fluc-Gluc	AAAACCCTTC	–	–	ATGGCTGCGT
AlbPolI-Fluc-Gluc	AAAACCCTAT	–	–	ATGGCTGCGT