An *in vitro* assay to study chikungunya virus RNA synthesis and the mode of action of inhibitors

Irina C. Albulescu,† Ali Tas,† Florine E. M. Scholte, Eric J. Snijder and Martijn J. van Hemert

Molecular Virology Laboratory, Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne alphavirus that causes severe persistent arthralgia. To better understand the molecular details of CHIKV RNA synthesis and the mode of action of inhibitors, we have developed an *in vitro* assay to study CHIKV replication/transcription complexes isolated from infected cells. In this assay 32P-CTP was incorporated into the CHIKV genome, subgenomic (sg) RNA and into a ~7.5 kb positive-stranded RNA, termed RNA II. We mapped RNA II, which was also found in CHIKV-infected cells, to the 5' end of the genome up to the start of the sgRNA promoter region. Most of the RNA-synthesizing activity, negative-stranded RNA and a relatively large proportion of nsP1 and nsP4 were recovered from a crude membrane fraction obtained by pelleting at 15,000 g. Positive-stranded RNA was mainly found in the cytosolic S15 fraction, suggesting it was released from the membrane-associated replication/transcription complexes (RTCs). The newly synthesized RNA was relatively stable and remained protected from cellular nucleases, possibly by encapsidation. A set of compounds that inhibit CHIKV replication in cell culture was tested in the *in vitro* RTC assay. In contrast to 3’dNTPs, chain terminators that acted as potent inhibitors of RTC activity, ribavirin triphosphate and 6-aza-UTP did not affect the RNA-synthesizing activity *in vitro*. In conclusion, this *in vitro* assay for CHIKV RNA synthesis is a useful tool for mechanistic studies on the RTC and mode of action studies on compounds with anti-CHIKV activity.

INTRODUCTION

Chikungunya virus (CHIKV) is an alphavirus that is mainly transmitted by *Aedes* mosquitoes and in general causes a severe persistent arthralgia. The virus re-emerged in 2005 in an epidemic form that has affected millions of people mainly in Asia (Burt *et al.*, 2012). Hundreds of infected travellers have arrived in the USA (Gibney *et al.*, 2011) and Europe, which even led to local transmission in Italy in 2007 and France in 2010 (Grandadam *et al.*, 2011; Rezza *et al.*, 2007). A massive CHIKV outbreak that started in October 2013 on the Caribbean island of Saint Martin marked the arrival of CHIKV in the Americas (Leparc-Goffart *et al.*, 2014; Weaver, 2014) and by July 2014 over 100,000 cases were already reported (Fischer & Staples, 2014). These outbreaks illustrate the increasing disease burden of CHIKV, for which there is still no registered vaccine or specific antiviral therapy.

The CHIKV replication cycle involves the early synthesis of negative-stranded RNA (−RNA), which serves as template for the subsequent production of the genome and a subgenomic (sg) mRNA. Genomic RNA and sgRNA are capped, and polyadenylated and these are translated into polyproteins comprising the nonstructural proteins (nsPs) 1–4 and structural proteins C, E3, E2, 6K and E1, respectively. CHIKV RNA synthesis presumably takes place on the cytoplasmic side of the plasma membrane and/or modified endosomal membranes, as described for other alphaviruses (Frolova *et al.*, 2010; Froshauer *et al.*, 2010). *In vitro* activity studies on replication complexes from a variety of positive-stranded RNA (+RNA) viruses, like poliovirus (Baltimore *et al.*, 1963), hepatitis C virus (Ali *et al.*, 2002; Hardy *et al.*, 2003; Lai *et al.*, 2003; Yang & Huang, 2009), West Nile virus (Chu & Westaway, 1985; Gruen & Brinton, 1986), dengue virus (You & Padmanabhan, 1999) and severe acute respiratory syndrome coronavirus (SARS-CoV; van Hemert *et al.*, 2008b) have contributed to our understanding of +RNA virus replication. For the alphaviruses Semliki forest virus (SFV) and Sindbis virus (SINV) such *in vitro* assays have been developed as well (Barton *et al.*, 1991).

We have now established an *in vitro* assay to characterize the activity and composition of CHIKV replication/transcription complexes (RTCs) isolated from infected cells. Besides developing a useful tool to screen for inhibitors of CHIKV RNA synthesis, our study provides...
more insight into the molecular details of CHIKV RNA synthesis and the mode of action of several inhibitors. Our in vitro studies also revealed the synthesis of a previously unrecognized 7.5 kb CHIKV RNA (RNA II) that is collinear with the 5' end of the genome up to the start of the sgRNA promoter region. This RNA species was also detected in CHIKV-infected cells and resembles a product in SINV-infected cells that was previously coined 'RNA II' (Wielgosz & Huang, 1997).

RESULTS

Isolation of active RTCs from CHIKV-infected cells

We set out to isolate CHIKV RTCs with the highest possible RNA-synthesizing activity from infected Vero E6 cells. We therefore first analysed CHIKV RNA synthesis in vivo by metabolic labelling with 3H-uridine at various time points post-infection (p.i.; Fig. 1). The rate of CHIKV RNA synthesis increased up to 8 h p.i., after which a modest decline was observed. Based on these results, the isolation procedure of CHIKV RTCs was started at 6 h p.i. to ensure that a good amount of activity would remain after the relatively lengthy procedure (1–2 h).

Approximately $1 \times 10^8$ CHIKV-infected Vero E6 cells were harvested at 6 h p.i. and homogenized in hypotonic buffer. Nuclei and debris were pelleted and the post-nuclear supernatant (PNS) was fractionated by centrifugation, yielding a 15,000 g pellet (P15) and supernatant (S15; Fig. 2a). The PNS, P15 and S15 fractions were assayed for CHIKV RNA-synthesizing activity by measuring the incorporation of $^{32}$P-CTP using the protocol described in Methods.

Reactions performed with PNS prepared from CHIKV-infected cells yielded two major $^{32}$P-labelled reaction products with sizes corresponding to the CHIKV genomic RNA and sgRNA (Fig. 2b). RNA II, a minor product running between genomic RNA and sgRNA, is discussed below. No radiolabelled RNA was detected when the assay was performed using PNS prepared from mock-infected cells (Fig. 2b). Approximately 58% of the RTC activity present in the PNS was recovered in the P15 fraction, while 6% remained in the S15 fraction. The addition of S15 material to the P15 fraction did not enhance the activity of the latter fraction, suggesting that the RTCs in the P15 fraction did not require (host) factors from S15 for their activity. The ~35% overall activity loss compared to the PNS was likely due to damage caused by pelleting and resuspending the RTC.

CHIKV RTCs synthesize RNA II in addition to genome and sgRNA

During the analysis of in vitro synthesized RNA we noticed that besides products with the size of genomic RNA and sgRNA, a ~7.5 kb product was synthesized (Figs 2b and 3a), which we termed RNA II by analogy with a similar RNA previously observed for SINV (Wielgosz & Huang, 1997). Approximately 4% of the incorporated $^{32}$P-CTP was present in RNA II, which was only observed when using reaction conditions that allowed sgRNA synthesis. RNA II was not an artefact of the in vitro system as it was also detected in RNA isolated from CHIKV-infected cells metabolically labelled with $^{3}$H-uridine (Figs 1a and 3a). At the peak of RNA synthesis in vivo, about 2% of the $^{3}$H-label was found in RNA II, while 27 and 71% of the radioactivity was found in genomic RNA and sgRNA, respectively. Based on its size and the correlation between the appearance of RNA II and sgRNA synthesis, we hypothesized it represents a ~7.5 kb fragment located at the 5'-proximal end of the genome, up to the sgRNA promoter region (starting at 7480 nt). To map the 3' end of RNA II, RNA from CHIKV-infected cells was hybridized...
with probes that bind to +RNA between nucleotides 4572–4601 (IA1), or immediately upstream (IA2) or ∼100 nt downstream (IA3) of the subgenomic promoter region (Fig. 3c). As anticipated, probes IA1 and IA2 hybridized to the genome and RNA II, while probe IA3 recognized sgRNA but not RNA II (Figs 3b, c). This result confirmed our hypothesis that RNA II corresponds to the 5′-proximal 7.5 kb of the CHIKV genome up to the subgenomic promoter region.

**Optimization of reaction conditions for in vitro CHIKV RNA synthesis**

A number of assay parameters were varied to determine the optimal conditions for CHIKV RNA synthesis in vitro. Activity remained relatively constant between 20 and 37 °C (Fig. 4a), probably reflecting that CHIKV replicates at low temperatures in its mosquito vectors and at higher temperatures in vertebrate hosts. Synthesis of sgRNA decreased at higher temperatures, while the 32P-incorporation into genomic RNA increased. In further experiments, 30 °C was used as the standard assay temperature, as this allowed the analysis of both genome replication and sgRNA synthesis. Magnesium was required for RTC activity, with 3 mM magnesium being the optimal concentration (Fig. 4b). An ATP-regenerating system was essential as no radiolabelled products were observed when creatine phosphate and creatine phosphokinase were omitted (data not shown). The incorporation of 32P-CTP into CHIKV RNA was readily detectable after a 5 min incubation at 30 °C, and the signal rapidly increased up to 90 min, after which it hardly increased (Figs 4c, e). The addition of pyrophosphatase did not increase the yield suggesting that the decreasing activity was not due to inhibition by pyrophosphate released upon NTP incorporation (data not shown). The incorporation of 32P-CTP into CHIKV RNA was readily detectable after a 5 min incubation at 30 °C, and the signal rapidly increased up to 90 min, after which it hardly increased (Figs 4c, e). The addition of pyrophosphatase did not increase the yield suggesting that the decreasing activity was not due to inhibition by pyrophosphate released upon NTP incorporation (data not shown). The addition of fresh NTPs, creatine phosphate and creatine phosphokinase after a 90 min reaction, followed by an additional 60 min incubation, led to an almost threefold increase in sgRNA labelling compared to a reaction that was incubated for an additional 60 min without replenishing these components (Fig. 4d). The incorporation of radioactivity into genomic RNA was only 1.2-fold higher. This suggests that the reaction rate dropped after 90 min due to exhaustion of one or more reaction components, such as NTPs, and not due to RTC instability. Indeed, RTCs retained most of their activity when they were first kept at 30 °C for 1 h before a reaction was started (data not shown). To assess the stability of in vitro-synthesized RNAs, a reaction was terminated after...
90 min by adding the obligate chain terminator 3’dUTP, after which RNA samples were taken every 20 min during a 60 min chase period. In untreated control samples the amount of radioactive CHIKV RNA increased ~1.2-fold over the chase period (Fig. 4f, squares). The level of radioactive CHIKV RNA in samples in which RNA synthesis was blocked slowly decreased (half-life >60 min) over the chase period (Fig. 4f, circles). The half-life of a (naked) control RNA was less than 5 min (Fig. 4f, triangles), suggesting that the newly synthesized CHIKV RNA was somehow protected from cellular nucleases.

While optimizing the NTP concentration, we discovered that even in the absence of added CTP, GTP and UTP, a substantial amount of mainly genomic RNA was synthesized (Fig. 3g); this was likely due to the pool of endogenous NTPs in PNS (Traut, 1994). The addition of 10 μM NTPs hardly had an effect, probably because the endogenous NTP concentration was already at least 10-fold higher. The addition of 1 mM ATP increased the reaction rate and especially resulted in increased sgRNA transcription and generation of RNA II. The activity in the P15 fraction (expected to contain only very little endogenous NTPs) was strongly dependent on supplied NTPs, as no 32P-CTP incorporation was observed in their absence (Fig. 3g). Final concentrations of 10 μM CTP, UTP and GTP and 1 mM ATP were optimal. Higher NTP concentrations did not substantially increase the activity (not shown).

**CHIKV RTCs incorporate 32P-CTP into single-stranded +RNA**

We next assessed whether *in vitro* synthesized radioactive CHIKV RNAs resulted from genuine RdRp-mediated incorporation of 32P-label or whether they merely resulted from end-labelling of existing RNA molecules by a viral or host cell activity. This was done by hybridizing a DNA probe to the viral RNA, ~2.2 kb from the 3’ end of both genomic RNA and sgRNA, and subsequently cleaving the resulting DNA–RNA duplex using RNase H (Fig. 5a). After this targeted cleavage, the ~9.6 kb fragment located at the 5’-terminal end of the genome was radioactively labelled, thus confirming that 32P-CTP had been incorporated internally and not just at the 3’ end of the CHIKV RNAs (Fig. 5b). The predicted ~2.1 kb fragment located at the 5’ end of the sgRNA was also radioactively labelled and migrated very close to the ~2.2 kb 3’-terminal fragment.

To determine the polarity of the newly synthesized RNA, radioactive reaction products were denatured and hybridized to a membrane containing specific capture probes for positive- and negative-stranded CHIKV RNA and a non-specific control RNA; Fig. 5(c) shows that mainly +RNA was synthesized *in vitro*.

Treatment with ssRNA-specific RNase A/T1 resulted in the degradation of more than 90% of the *in vitro* synthesized RNA, along with the 18S rRNA that was used as internal control (Fig. 5d). Treatment with dsRNA-specific RNase III led to a 10–20% decrease of radioactive CHIKV RNA, suggesting that radioactivity had mainly accumulated in ssRNA. Analysis of the reaction products in a non-denaturing agarose gel revealed that ~35% of this ssRNA was present (as nascent strands) in replicative intermediates (RI), while ~65% had been released as full-length genomic RNA or sgRNA (Fig. 5e, lane 2). R1a and R1b, which are thought to be involved in genomic RNA and sgRNA synthesis, respectively, could not be resolved in this gel system. After denaturation (Fig. 5e, lanes 7–9), R1s were no longer visible, while more ssRNA was detected. When additional NTPs were not added to the reaction, ~85% of the radioactivity accumulated in the RIs and only a small amount of ssRNA appeared to be released (Fig. 5e, lane 3).

We next assessed whether the newly synthesized CHIKV RNA was somehow protected from cellular nucleases. This was done by hybridizing a DNA probe to the viral RNA, ~2.2 kb from the 3’ end of both genomic RNA and sgRNA, and subsequently cleaving the resulting DNA–RNA duplex using RNase H (Fig. 5a). After this targeted cleavage, the ~9.6 kb fragment located at the 5’-terminal end of the genome was radioactively labelled, thus confirming that 32P-CTP had been incorporated internally and not just at the 3’ end of the CHIKV RNAs (Fig. 5b). The predicted ~2.1 kb fragment located at the 5’ end of the sgRNA was also radioactively labelled and migrated very close to the ~2.2 kb 3’-terminal fragment.

To determine the polarity of the newly synthesized RNA, radioactive reaction products were denatured and hybridized to a membrane containing specific capture probes for positive- and negative-stranded CHIKV RNA and a non-specific control RNA; Fig. 5(c) shows that mainly +RNA was synthesized *in vitro*.

Treatment with ssRNA-specific RNase A/T1 resulted in the degradation of more than 90% of the *in vitro* synthesized RNA, along with the 18S rRNA that was used as internal control (Fig. 5d). Treatment with dsRNA-specific RNase III led to a 10–20% decrease of radioactive CHIKV RNA, suggesting that radioactivity had mainly accumulated in ssRNA. Analysis of the reaction products in a non-denaturing agarose gel revealed that ~35% of this ssRNA was present (as nascent strands) in replicative intermediates (RI), while ~65% had been released as full-length genomic RNA or sgRNA (Fig. 5e, lane 2). R1a and R1b, which are thought to be involved in genomic RNA and sgRNA synthesis, respectively, could not be resolved in this gel system. After denaturation (Fig. 5e, lanes 7–9), R1s were no longer visible, while more ssRNA was detected. When additional NTPs were not added to the reaction, ~85% of the radioactivity accumulated in the RIs and only a small amount of ssRNA appeared to be released (Fig. 5e, lane 3).

**Distribution of CHIKV RNA and proteins between the P15 and S15 fractions**

To gain more insight into the composition of the P15 fraction that contains most of the RTC activity (Fig. 2b), we studied the distribution of CHIKV RNA, viral proteins and several cellular marker proteins between P15 and S15. Approximately 90% of −RNA was found in the P15 fraction, where it likely serves as template for +RNA synthesis (Fig. 6a). In contrast, the P15 fraction contained about six times less +RNA than the cytoplasmic S15 fraction (Fig. 6a). The P15 fraction contained 20 or 16% of the total amount of nsP2 and nsP3, respectively. In contrast, 30% of nsP4 and 50% of the nsP1 were found in P15 (Fig. 6b). The membrane fraction (P15) was also enriched in the E2 envelope protein and also contained about 15% of the capsid protein (Fig. 6b). The absence of fibrillarin indicated that the P15 and S15 fractions (and PNS) were not notably contaminated with nuclear material. The P15 fraction did not contain detectable amounts of the cytosolic markers cyclophilin A or actin, while it contained most of the endoplasmic reticulum marker cyclophilin B and the bulk of the plasma membrane marker Na/K ATPase (Fig. 6b, right). The early endosome marker, Rab5, was predominantly found in S15, whereas Rab7, a late endosome marker, was present in both P15 and S15. These results confirmed that P15 was a rather crude fraction that contained membranes of various cellular origins.

Downloaded from www.microbiologyresearch.org by
IP:  54.70.40.11
On: Thu, 03 Jan 2019 05:21:07
Optimization of reaction conditions for the \textit{in vitro} RNA-synthesizing activity of CHIKV RTCs. The temperature (a), \( \text{Mg}^{2+} \) concentration (b) or incubation time (c) was varied and \( ^{32}\text{P}-\text{CTP} \)-labelled RNA products were analysed. The percentages depicted in (d) were added to the reaction and ATP was also regenerated during the reaction (1 mM ATP was added to the reaction and ATP was also regenerated during the reaction). In line with this notion, the inhibitory effect of \( ^{3}\text{dUTP} \) compared to that of the other \( ^{3}\text{dNTPs} \) was likely due to an excess of ATP in the reaction (1 mM ATP was added to the reaction and ATP was also regenerated during the reaction). In line with this notion, the inhibitory effect of \( ^{3}\text{dUTP} \), which was already very strong at a concentration of 10 \( \mu\text{M} \) (Fig. 7b, lane 7), could be reversed by adding an excess of UTP. For example a 20-fold molar excess of UTP over 50 \( \mu\text{M} \) \( ^{3}\text{dUTP} \) restored the RNA-synthesizing activity to a level comparable to that observed in the presence of 1 \( \mu\text{M} \) of the inhibitor (Fig. 7b, compare lanes 6 and 11). Finally, we assayed whether 500 \( \mu\text{M} \) ribavirin triphosphate or 100 \( \mu\text{M} \) 6-aza-UTP affected the kinetics of CHIKV RNA synthesis \textit{in vitro}; this was found not to be the case as shown in Fig. 7(c).

**The \textit{in vitro} RTC assay as a tool for mode of action studies on inhibitors of CHIKV replication**

To determine whether our \textit{in vitro} RTC assay is a suitable tool for mode of action studies on inhibitors of CHIKV replication, we studied the effect of a variety of compounds.

Fig. 4. Optimization of reaction conditions for the \textit{in vitro} RNA-synthesizing activity of CHIKV RTCs. The temperature (a), \( \text{Mg}^{2+} \) concentration (b) or incubation time (c) was varied and \( ^{32}\text{P}-\text{CTP} \)-labelled RNA products were analysed. The percentages depicted under the lanes indicate \( ^{32}\text{P} \) incorporation normalized to the highest observed activity in a given series (100\%), after correction for variations in RNA recovery based on quantification of 18S rRNA (not shown). (d) After a 90 min standard reaction (left lane) fresh NTPs, creatine phosphate and creatine phosphokinase (+) or an equal volume of dilution buffer (−) were added and the reaction was continued for an additional hour. (e) Kinetics of the incorporation of \( ^{32}\text{P}-\text{CTP} \) into CHIKV genomic and sgRNA. (f) Stability of newly synthesized CHIKV RNA in PNS. The incorporation of \( ^{32}\text{P}-\text{CTP} \) into CHIKV RNA was allowed to proceed for 90 min, after which it was blocked by the addition of 0.1 mM \( ^{3}\text{dUTP} \), followed by the quantification of the remaining radioactive CHIKV RNA at the indicated time points of the chase (circles). A reaction that was chased in the absence of \( ^{3}\text{dUTP} \) was included as a control (squares). The triangles show the decay of an \textit{in vitro} transcribed control RNA that was incubated with PNS, after which its integrity was analysed by gel electrophoresis. (g) Assays performed with either PNS or the P15 fraction in the presence of various concentrations of (added) ATP and the other NTPs. G, Genomic RNA; II, RNA II; sg, sgRNA.

**DISCUSSION**

We have successfully developed an \textit{in vitro} system to study CHIKV RNA synthesis, in which \( ^{32}\text{P}-\text{CTP} \) is incorporated into CHIKV genomic RNA and sgRNA. About a third of the radioactivity was recovered in RIs, in addition to which the radioactivity was recovered in RIs, in addition to which mainly single-stranded +RNA accumulated. This was not unexpected considering that lysates were prepared at 6 h
Characterization of \textit{in vitro} synthesized CHIKV RNAs. (a) Schematic representation of the two possible mechanisms that could lead to generation of $^{32}$P-labelled CHIKV RNAs and the targeted RNA cleavage assay with DNA probe and RNase H that was performed to distinguish between these two mechanisms. Genuine RdRp activity would incorporate $^{32}$P-CTP throughout newly synthesized RNA and would result in four radioactive fragments (sizes in italic) after cleavage. Terminal transferase activity would lead to radioactive labelling of only the 2.2 kb 3' terminal fragment of genomic RNA and sgRNA. (b) Result of the targeted cleavage assay on RNA produced in the \textit{in vitro} RTC assay. RNA II was not cleaved since it was not recognized by the DNA probe. (c) Binding of radioactive \textit{in vitro} synthesized CHIKV RNA to a membrane with capture probes specific for CHIKV RNA of positive or negative polarity or a non-specific control probe. (d) Treatment of \textit{in vitro} synthesized CHIKV RNA with ssRNA-specific RNase A/T1 or the dsRNA-specific RNase III, followed by denaturing agarose gel electrophoresis. Ribosomal 18S RNA was included as a control. (e) Non-denaturing agarose gel electrophoresis of reaction products from standard assays (+NTPs) or those without supplied NTPs (~NTPs). \textit{In vitro} transcribed CHIKV genomic RNA was included as control (ctrl). Some samples were treated with RNase A/T1 as indicated above the lanes and the samples in lanes 7–12 were heat denatured in a buffer containing formaldehyde and formamide as used for denaturing gel electrophoresis. The positions of the replicative intermediates Rla and Rlb, replicative forms (RF), genomic RNA, RNA II and sgRNA are indicated.

A crude membrane fraction (P15) had an approximately 10-fold higher RNA synthesizing activity than the cytosolic S15 fraction. P15 contained most of the $-$RNA template for $+$RNA synthesis, while the bulk of $+$RNA was found in S15, suggesting it was released from the membrane-associated RTCs. In lysates, \textit{in vitro}-synthesized CHIKV RNA was relatively stable and appeared to be somehow protected from cellular nucleases. This might be due to its structure, membrane association, presence within poly-somes, or encapsidation (capsid protein was also detected in P15). The S15 fraction contained about 80 % of nsP2 and nsP3, proteins that besides their role in the RTC are known to have several other functions, like inducing a shut-off of host transcription and translation and interacting with a variety of host proteins. Compared to nsP2 and nsP3, the P15 fraction was enriched in the RdRp nsP4 and especially in nsP1. The latter protein is presumably involved in the anchoring of RTCs to membranes.

We discovered that a previously unrecognized CHIKV RNA, which we termed RNA II, was produced besides genomic RNA and sgRNA. RNA II was synthesized both \textit{in vitro} and \textit{in vivo}, and we demonstrated that it represents the first 7.5 kb of the genome. Earlier metabolic labelling studies suggested the presence of similar molecules in both SINV- and SFV-infected cells (Bruton & Kennedy, 1975; Levin & Friedman, 1971). RNA II was first mapped in SINV (Wielgosz & Huang, 1997) and believed to be unique to this virus, but the work herein shows that RNA II is also present in CHIKV-infected cells. The molecule probably results from premature termination (near the sgRNA promoter) of RNA synthesis that initiates from the 3' end of a $-$RNA template that is also engaged in transcription (Rib). This is supported by the observation that RNA II and RF II were only found under conditions that favoured sgRNA synthesis. The reduced levels of
reflecting equal cell numbers of PNS, P15 and S15 of CHIKV- and/or loading of RNA. (b) Western blot analysis of samples spiked with a control RNA to correct for variations in the recovery were detected by hybridization with specific probes. Samples were spiked with a control RNA to correct for variations in the recovery of RNA synthesis, e.g. by analysing mutants, to screen for inhibitors and to study the mode of action of antiviral compounds originating from other (cell-based) screens.

**Fig. 6.** Distribution of CHIKV RNA and viral and cellular marker proteins between the P15 and S15 fractions. (a). Total RNA was isolated from PNS, P15 and S15 fractions (volumes reflecting equal cell numbers) obtained from CHIKV-infected Vero E6 cells. After denaturing agarose gel electrophoresis, −RNA and +RNA were detected by hybridization with specific probes. Samples were spiked with a control RNA to correct for variations in the recovery and/or loading of RNA. (b) Western blot analysis of samples reflecting equal cell numbers of PNS, P15 and S15 of CHIKV-infected cells or a whole-cell lysate of uninfected cells using antibodies specific for the protein indicated next to each panel.

sgRNA and RNA II, and the increased amount of RF I at the expense of ssRNA, in reactions performed at low NTP concentrations make it tempting to speculate on the role of (the ATPase/helicase activity of) nsP2 in transcription. We are currently investigating whether RNA II is capped and polyadenylated, and whether it is merely a by-product of genome replication or has a function in the infected cell.

We have tested several compounds in the *in vitro* assay for CHIKV RNA synthesis to demonstrate it is a useful addition to the toolbox for mode of action studies. As expected chloroquine showed no effect, because *in vivo* it blocks CHIKV entry. Ribavirin and 6-azauridine had no effect as their conversion to an active (triphosphorylated) form by cellular enzymes probably does not occur (to a sufficient level) *in vitro*. These compounds may also act as antimetabolites that *in vivo* affect the cellular NTP pool, like 5-fluorouracil and mycophenolic acid, and hence have no effect in the *in vitro* assay, as NTPs are supplied in the reaction buffer. In cell culture 3-deaza-adenosine inhibits CHIKV replication and it was hypothesized that this might be due to its effect on CHIKV RNA capping (Scholte *et al.*, 2013). In line with this, 3-deaza-adenosine had no direct effect on RNA synthesis *in vitro*. The obligate chain terminators 3’dATP, 3’dGTP and 3’dUTP inhibited CHIKV RNA synthesis *in vitro* at low micromolar concentrations. Their inhibitory effect could be reversed by addition of NTPs, which stresses the importance of not supplying a large excess of NTPs in *in vitro* assays to avoid missing or underestimating the inhibitory effect of (novel) compounds. Using this newly developed assay, we found that ribavirin triphosphate and 6-aza-UTP had no measurable effect on the kinetics of CHIKV RNA synthesis. This may indicate that these compounds were either not incorporated by the RTC or incorporated without affecting the reaction rate. In addition, these compounds could also indirectly affect RNA synthesis *in vivo* through their effect on cellular NTP pools. Obviously, this would have no effect in the *in vitro* system as NTPs are supplied. This is further supported by the fact that the inosine monophosphate dehydrogenase inhibitor mycophenolic acid had no effect *in vitro*, while it inhibits CHIKV in cell culture. Since ribavirin and 6-azauridine increase the viral mutation frequency (Beauchot & Vignuzzi, 2014; Crotty *et al.*, 2002), at least part of their mode of action can probably be attributed to their incorporation into viral RNA.

The *in vitro* assay described here may be used to enhance our understanding of the molecular details of CHIKV RNA synthesis, e.g. by analysing mutants, to screen for inhibitors and to study the mode of action of antiviral compounds originating from other (cell-based) screens.

**METHODS**

**Cells, virus, antiserum and compounds.** Vero E6 cells were infected with CHIKV strain LS3 at an m.o.i. of 5 p.f.u. per cell as previously described (Scholte *et al.*, 2013). Antisera against CHIKV nsP1, nsP2, nsP3, nsP4, and capsid protein were a generous gift from Professor Andres Merits (Institute of Technology, University of Tartu, Tartu, Estonia). The E2 antiserum (Metz *et al.*, 2013) was obtained from Dr Gorben Pijlman (Laboratory of Virology, Wageningen University, Wageningen, The Netherlands). Antisera against cellular markers used were actin (Sigma), fibrillarin, cyclophilins A and B (Santa Cruz), Rab5, Rab7 and Na/K ATPase (Cell Signalling). Ribavirin triphosphate, 3’dATP, 3’dGTP and 3’dUTP were from TriLink BioTechnologies and 6-aza-UTP from Jena Bioscience. Stock solutions of ribavirin, 6-azauridine, 5-fluorouracil, 3’-deaza-adenosine, chloroquine and mycophenolic acid were prepared as previously described (Scholte *et al.*, 2013).

**RNA isolation, denaturing agarose gel electrophoresis, in gel hybridization and detection of 32P-labelled RNA.** RNA isolation (van Hemert *et al.*, 2008a), non-denaturing (Barton *et al.*, 1991) and denaturing formaldehyde agarose gel electrophoresis (Scholte *et al.*, 2013) were performed as previously described. For detection of 32P-labelled RNA, Phosphor Imager screens were exposed to dried gels and scanned with a Typhoon 9410 imager (GE Healthcare). CHIKV RNA and +RNA were specifically detected by hybridization with 32P-labelled probe Hyb2 and Hyb4 (Scholte *et al.*, 2013). Probes IAI (5'-CAATATCGACTGCTATGA-GATGTGCTCAT-3'), IA2 (5'-GTATAGGCGGGTCCCTGAGTT-CTTCGAG-3') and IA3 (5'-TTCCTATTGAAAAAGCTTTTG-3') are complementary to the genome sequences encompassed
within nucleotides 4572–4601, 7452–7479 and 7575–7605, respectively. The 18S rRNA, detected with probe 5′-ATGCCCGCGGC-CCGTCCCTCT-3′, was used as isolation efficiency and loading control for RNA isolated from cells or PNS. P15 and S15 fractions were spiked with an in vitro transcribed RNA containing the 3′ end of the CHIKV genome that was detected with probe Hyb4, to correct for variations in isolation and/or loading.

Metabolic labelling of CHIKV-infected cells. Metabolic labelling of CHIKV-infected cells with 40 Ci 3H-uridine was performed as previously described (Scholte et al., 2013) with the following modifications: Vero E6 cells were seeded in 12-well clusters at a density of 105 cells per well and were infected at an m.o.i. of 5 p.f.u. per cell.

Isolation of RTCs from CHIKV-infected cells. Approximately 1 × 106 CHIKV-infected Vero E6 cells were harvested by trypsinization at 6 h p.i. Cells were resuspended in 4 ml of hypotonic buffer containing 20 mM HEPES (pH 7.5), 10 mM KCl, 1 mM DTT, 0.2 U ml−1 RiboLock (Thermo Scientific) and 2 µg ml−1 ActD. After incubation at 4 °C for 15 min, the cells were disrupted using a Dounce homogenizer. To increase the osmotic value sucrose was added to a final concentration of 250 mM. HEPES and DTT were added to a final concentration of 35 mM and 2.5 mM, respectively. The lysate was cleared by centrifugation at 10 000 g for 10 min to remove unlysed cells, nuclei and cellular debris, yielding a PNS. Part of the PNS was separated by centrifugation at 15 000 g for 15 min into a pellet fraction (P15) and a supernatant fraction S15. The P15 pellet was resuspended in dilution buffer (35 mM HEPES, pH 7.5, 250 mM sucrose, 2.7 mM DTT, 7 mM KCl, 2 µg ActD ml−1 and 0.2 U RiboLock µl−1) using 1/5 of the volume of the PNS from which the pellet originated (now S15). This 5 × concentrated P15 fraction and all other fractions were aliquoted and stored at −80 °C.

**In vitro RNA synthesis assay.** These assays were performed inside a Biosafety Level 3 facility, since infectious CHIKV remained present in the lysates. Standard 30 µl reactions contained 25 µl PNS, 25 µl S15 or P15 (5 µl P15 5 × concentrated mixed with 20 µl dilution buffer), 30 mM HEPES, pH 7.5, 220 mM sucrose, 7 mM KCl, 2.5 mM DTT, 3 mM magnesium acetate, 2 µg ActD ml−1, 0.2 U RiboLock µl−1, 20 mM creatine phosphate, 10 µM creatine phosphokinase ml−1, 10 µCi (370 kBq; 0.12 µM) of α-32P-CTP (Perkin Elmer), 1 mM ATP and either 0.01 or 0.2 mM of GTP, UTP and CTP. Unless otherwise stated, reactions were performed for 60 min at 30 °C and terminated as described (van Hemert et al., 2008b).

**Detection of 32P-labelled reaction products with capture probes.** In vitro transcribed RNAs (1 µg) containing nt 1–1348 of CHIKV – RNA, the 1100 3′-terminal nucleotides of the CHIKV genome, or nt 1–2042 of the unrelated equine arteritis virus (negative control), were immobilized to Hybond-N+ membrane (GE

---

**Fig. 7.** Effect of various compounds on the *in vitro* RNA synthesizing activity of CHIKV RTCs. (a) Standard assays were performed in the presence of 0.5% DMSO, 50 µM 6-azaauridine (6-azaU), 100 µM chlorouracil (CHO), 60 µM 3′-deazaadenosine (3′ deazaA), 150 µM ribavirin, 500 µM fluorouracil (5-FU), or 6 µM mycophenolic acid (MPA). (b) Standard reactions performed in the presence of the obligate chain terminators 3′dATP, 3′dGTP and 3′dUTP at the concentrations indicated above the lanes. The rightmost lane contains RNA synthesized in the presence of 50 µM 3′dUTP and 1 mM UTP. (c) Reaction kinetics of *in vitro* RNA synthesis in the presence of ribavirin triphosphate or 6-azaUTP or in the absence of inhibitor (control) analysed over a time-course of 5–90 min. (d) Quantified *in vitro* 32P-CTP incorporation into CHIKV RNA in the presence of ribavirin triphosphate (squares) or 6-azaUTP (triangles) represented as the percentage of the maximum incorporation in the control reaction (circles).
Healthcare) as previously described (van Hemert et al., 2008b). The membrane was then incubated with half of the 32P-labelled RNA from a 30 μl in vitro reaction. Washing and detection of bound material was performed as previously described (van Hemert et al., 2008b). A 32P-labelled in vitro transcript that contained the 5’ and 3’ UTR of CHIKV or a transcript with the complementary sequence was used both to act as a positive control and to assess the specificity of the immobilized probes.

**RNase treatments.** RNA was treated with a RNase A/T1 mixture under high salt conditions to degrade ssRNA, or with RNase III to degrade dsRNA as previously described (van Hemert et al., 2008a).

**Targeted RNA cleavage assay.** RNA from in vitro reactions was heated to 96 °C for 4 min, followed by hybridization overnight at 45 °C in 20 μl of 40 mM PIPES (pH 6.5), 0.4 M NaCl, 1 mM EDTA and 80% deionized formamide with DNA probe AS25 (5’-GAT- AACTGCGGCCAATACTTAT-3’) that is complementary to nt 9548–9569 of CHIKV. RNase H (7.5 U) was added in a 150 μl mixture. After a 60 min incubation at 37 °C, 225 μl of RNase inactivation solution (Ambion RPA kit) was added, followed by a 30 min incubation at −20 °C. RNA was then precipitated in the presence of GlycoBlue (Ambion) by centrifugation for 30 min at 15,000 g and 4 °C, and analysed by denaturing agarose gel electrophoresis.

**SDS-PAGE and Western blot analysis.** SDS-PAGE and Western blotting using a Trans-Blot Turbo instrument and fluorescent detection of antibodies with a Typhoon 9410 scanner were performed as previously described (Scholte et al., 2013).

**ACKNOWLEDGEMENTS**

This work was supported by the European Union Seventh Framework Programme (FP7/2007–2013) under SILVER grant agreement no. 264286 and the Marie Curie Initial Training Network EUVIRNA Programme (FP7/2007–2013) under SILVER grant agreement no. 260644. We thank Professor Andres Merits and Dr Gorben Pijlman for generously sharing their CHIKV antisera.

**REFERENCES**


