Targeting chikungunya virus replication : insights into chikungunya virus replication and the antiviral activity of suramin in vitro

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Chapter 2

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

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Summary

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 ^{32}P-CTP was incorporated into CHIKV genome and subgenomic (sg) RNA, as well as 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 x g. Positive-stranded RNA was mainly found in the cytosolic S15 fraction, suggesting it was released from the membrane-associated 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, which act as chain terminators and were 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 CHIKV 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 arthralgia that may persist for months. The virus re-emerged in 2005 in an epidemic form and has since affected millions of people mainly in Asia [1]. Hundreds of infected travelers have arrived in the USA [2] and Europe, which even led to local transmission in Italy in 2007 and France in 2010 [3, 4]. A massive, CHIKV outbreak that started in October 2013 on the Caribbean island of Saint Martin marked [5] the arrival of CHIKV in the Americas [6, 7] and up to July 2014 over 100,000 cases were already reported [8]. 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 synthesis of negative-stranded RNA (-RNA) early in infection, which serves as a template for the subsequent production of the genome and a subgenomic (sg) mRNA. Genome and sgRNA are capped and polyadenylated and serve to express 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 [5, 9-11].

In vitro activity studies on replication complexes from a variety of positive-stranded RNA (+RNA) viruses, like poliovirus [12] hepatitis C virus [13-16], West Nile virus [17, 18], Dengue virus [19], and SARS-coronavirus [20], 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 [21].

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 provided 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 found in CHIKV-infected cells and resembles the RNA II that has been observed in SINV-infected cells [22].

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 analyzed CHIKV RNA synthesis *in vivo* by metabolic labeling with ^{3}H-uridine at various time points post infection. Quantification of ^{3}H-labeled CHIKV RNA following denaturing agarose gel electrophoresis and fluorography (Fig. 1) showed that the rate of RNA synthesis increased up to 8 h p.i., after which a modest

decline was observed. Based on these results, we begun the isolation procedure of CHIKV RTCs at 6 h p.i. to ensure that a good amount of activity would remain after the relatively lengthy procedure (1-2 h).

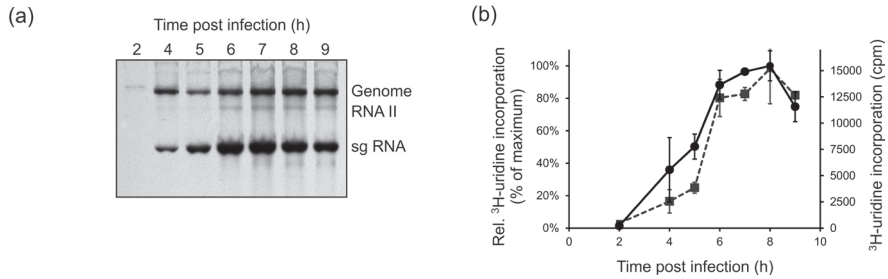

Figure 1. Kinetics of CHIKV RNA synthesis in Vero E6 cells. (a) CHIKV-infected cells were pulse labeled for 1 h with ^{3}H-uridine starting 30 min before the time points indicated above the gel, followed by isolation of RNA, denaturing agarose gel electrophoresis and visualization by fluorography. The positions of genomic and sgRNA (determined by hybridization of CHIKV RNA with a specific probe) and an additional CHIKV-specific ~7.5 kb product (RNA II) are indicated. (b) The rate of CHIKV RNA synthesis (replication and transcription combined) at each time point post infection was quantified by densitometry of the bands shown in panel a (circles) or by direct liquid scintillation counting of isolated RNA (squares). Ribosomal 18S RNA levels were used to correct for variations in isolation. The graph shows ^{3}H-uridine incorporation (relative to the highest observed value) and represents the average ±SD of two experiments.

Approximately 1 x 10^{8} CHIKV-infected Vero E6 cells were harvested by trypsinization at 6 h p.i., followed by homogenization in hypotonic buffer. Nuclei and debris were pelleted and the resulting post-nuclear supernatant (PNS) was fractionated by centrifugation yielding a 15,000 x 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 the Materials section.

Reactions performed with PNS prepared from CHIKV-infected cells yielded two major ^{32}P-labeled reaction products with sizes corresponding the CHIKV genome and sgRNA (Fig. 2b). RNA II, a minor product running between genome and sgRNA will be discussed below. No radiolabeled 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 could be recovered in the P15 fraction, while 6% remained in the S15 fraction. Combining the P15 and S15 fractions yielded an activity that was comparable to the sum of their individual activities, suggesting that the RTCs in the P15 fraction did not require (host) factors from the S15 fraction for their activity. The ~35% overall activity loss compared to the PNS, was likely due to damage caused by RTC pelleting and resuspending.

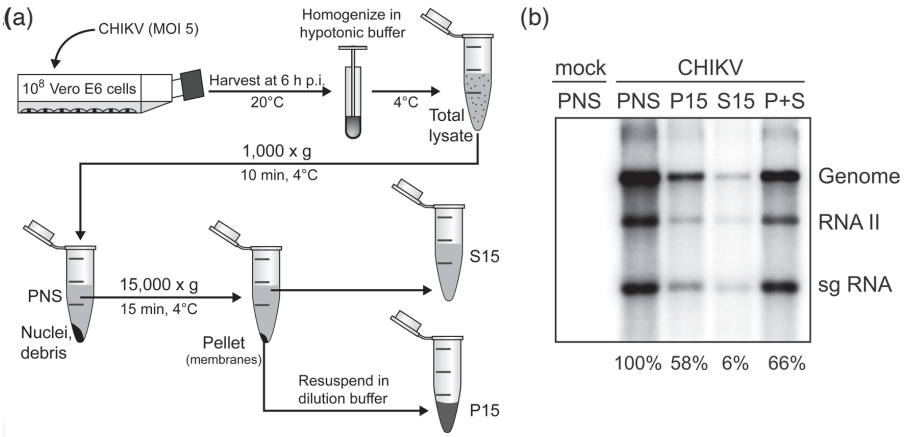

Figure 2. Isolation of active CHIKV RTCs from infected Vero E6 cells. (a) Overview of the isolation procedure. (b) *In vitro* incorporation of ^{32}P-CTP into RNA in PNS prepared from mock-infected cells, or PNS, P15 and S15 fractions obtained from CHIKV-infected cells. RNA was extracted, separated in denaturing agarose gels and visualized using a phosphor imager. The amount of ^{32}P-CTP incorporated into CHIKV genome and sgRNA is indicated under each lane, expressed as % of the radioactivity incorporated by the PNS fraction.

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 genome and sgRNA, a ~7.5-kb product was synthesized, which we termed RNA II (Fig. 2b and 3a). Approximately 4% of the incorporated ^{32}P-CTP was present in RNA II, which was only observed under reaction conditions that allowed sgRNA synthesis. RNA II was not an artifact of the *in vitro* system as it was also detected in RNA isolated from CHIKV-infected cells metabolically labeled with ^{3}H-uridine (see Fig. 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 genome and sgRNA, respectively. Based on its size and the correlation between RNA II and sgRNA synthesis, we hypothesized it represents the 5'-proximal ~7.5 kb of the genome, up to the sgRNA promoter region (starting at nt 7480). We have termed this molecule RNA II, since this name was used for a similar RNA product described for SINV [22]. To map the 3' end of CHIKV 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 nucleotides 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 (Fig. 3b and c). This result confirmed our hypothesis that RNA II corresponds to the 5'-proximal 7.5 kb of the CHIKV genome up to subgenomic promoter region.

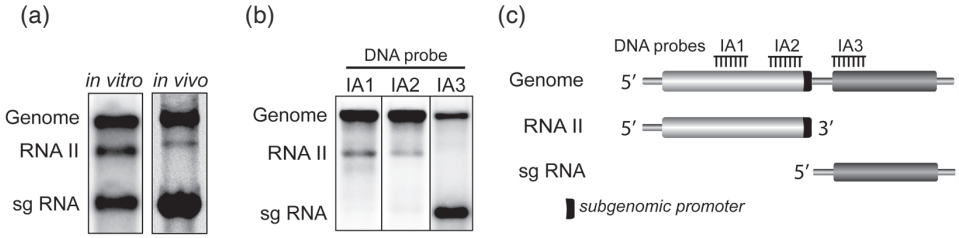

Figure 3. Analysis of the origin of CHIKV RNA II. (a) CHIKV RNA was metabolically labeled with ^{3}H-uridine *in vivo* or with ^{32}P-CTP *in vitro*. The positions of the genome, sgRNA and the minor ~7.5 kb CHIKV-specific product RNA II are indicated. (b) Hybridization analysis of CHIKV RNA using probes that specifically bind the regions of CHIKV+RNA indicated at (c). (c) Schematic representation of the 3 +RNAs synthesized by CHIKV and the locations of the probes IA1, IA2 and IA3 that were used to map the origin of RNA II.

Optimization of reaction conditions for in vitro CHIKV RNA synthesis

A number of assay parameters were tested to determine the optimal conditions for CHIKV RNA synthesis *in vitro*. Activity remained relatively constant over a range of temperatures from 20 to 37°C (Fig. 4a), probably reflecting that CHIKV replicates at low temperatures in mosquito vectors and at higher temperatures in vertebrate hosts. Synthesis of sgRNA decreased at higher temperatures, while the ^{32}P-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 and a concentration of 3 mM was optimal (Fig. 4b). An ATP-regenerating system was essential as no radiolabeled products were observed when creatine phosphate and creatine phosphokinase were omitted (data not shown). The incorporation of ^{32}P-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 further (Fig. 4c and e). Addition of pyrophosphatase did not increase the yield suggesting that the decreasing activity was not due to inhibition by the pyrophosphate that is released during 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 3-fold increase in sgRNA labeling compared to a reaction that was incubated for an additional 60 min without replenishing 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, like 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 the *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 a reaction to which no CTP, GTP and UTP was added, a substantial amount of mainly genomic RNA was synthesized (Fig. 3g). This was likely due to the pool of endogenous NTPs in PNS [23]. Addition of 10 μM of NTPs to the reaction hardly had an effect, probably because the endogenous NTP concentration was already at least 10-fold higher. Addition of 1 mM of ATP increased the reaction rate and resulted especially in increased sgRNA transcription and generation of RNA II. Reactions performed with the P15 fraction (not expected to contain endogenous NTPs) were strongly dependent on the supplied NTPs, as no ^{32}P-CTP incorporation was observed in the absence of NTPs (Fig. 3g). A final concentration of 10 μM CTP, UTP and GTP and 1 mM of ATP was optimal. Higher NTP concentrations did not substantially increase the activity (not shown).

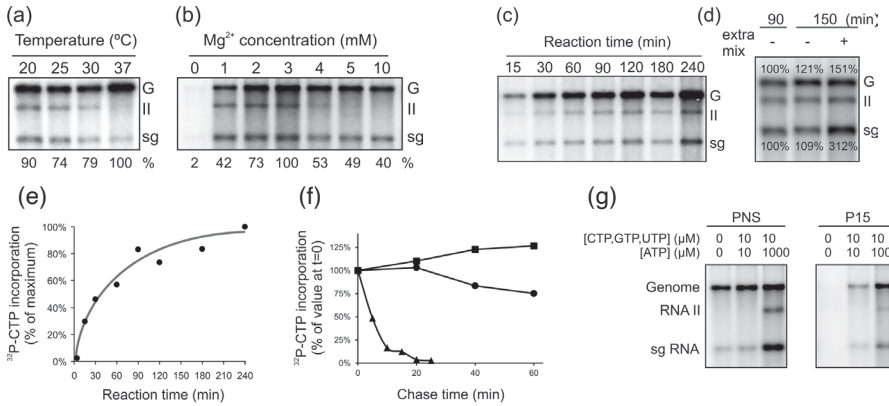

Figure 4. Optimization of reaction conditions for the *in vitro* RNA-synthesizing activity of CHIKV RTCs. The temperature (a), Mg^{2+} concentration (b) or incubation time (c) were varied and ^{32}P-CTP-labeled RNA products were analyzed (see Fig. 1). The percentages depicted under the lanes indicate ^{32}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}P-CTP into CHIKV genomic and sgRNA. (e) Stability of newly synthesized CHIKV RNA in PNS. The incorporation of ^{32}P-CTP into CHIKV RNA was allowed to proceed for 90 min, after which it was blocked by the addition of 0.1 mM 3'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'dUTP was included as a control (squares). The triangles show the decay of a *in vitro* transcribed control RNA that was incubated with PNS. (f) Assays performed with either PNS or the P15 fraction in the presence of various concentrations of (added) ATP and the other NTPs.

CHIKV RTCs incorporate ^{32}P-CTP into single-stranded +RNA

We next assessed whether *in vitro* synthesized radioactive CHIKV RNAs resulted from genuine RdRp-mediated incorporation of ^{32}P-label or whether they were merely the result of end-labeling of existing RNA molecules by a viral or host cell activity. This was done by hybridizing a small DNA probe at a specific position in the viral RNA, ~2.2 kb from the 3' end of both genome and sgRNA, and subsequently cleaving the DNA:RNA duplex using RNase H (Fig. 5a). The fact that, after this targeted cleavage, the ~9.6-kb 5'-terminal genome fragment was radioactively labeled confirmed that ^{32}P-CTP had been incorporated at other positions than merely the 3' end of the CHIKV RNAs (Fig. 5b). A radioactive fragment corresponding to the predicted ~2.1 kb 5' fragment of the sgRNA was also observed, although migrating very close to the ~2.2 kb 3'-terminal fragment. Treatment of ^{32}P-labeled reaction products with single strand-specific RNAse A/T1 resulted in the degradation of more than 90% of the *in vitro* synthesized RNA, along with the 18S RNA that was used as an internal control (Fig. 5c). Treatment with double-stranded RNA-specific RNase III led to a 10-20% decrease of radioactive CHIKV RNA, suggesting that radioactivity had mainly accumulated in single-stranded RNA.

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

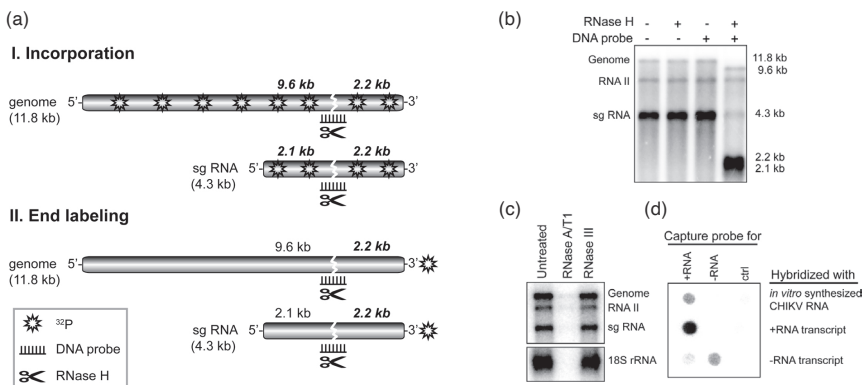

Figure 5. Characterization of *in vitro* synthesized CHIKV RNAs. (a) Schematic representation of the two possible mechanisms that could lead to generation of ^{32}P-labeled CHIKV RNAs and the targeted RNA cleavage assay with DNA probe and RNase H that was performed to distinguish between the two. Genuine RdRp activity would incorporate ^{32}P-CTP throughout newly synthesized RNA and would result in 4 radioactive fragments (sizes in bold italic) after cleavage. If RNAs would only be labeled at their 3' end, due to terminal transferase activity, only the 2.2 kb 3'-terminal fragment of genome and sgRNA would be radioactively labeled. (b) Result of the targeted cleavage assay using reaction products from an *in vitro* RTC assay. RNA II was not cleaved since it was not recognized by the DNA probe. (c) Treatment of *in vitro* synthesized CHIKV RNA with single strand-specific RNase A/T1 or the double-stranded RNA-specific RNase III, followed by denaturing agarose gel electrophoresis and direct detection of ^{32}P-labeled products by phosphor imaging. Ribosomal 18S RNA, was included as a control. (d) Binding of radioactive *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.

Distribution of CHIKV RNA and proteins between the P15 and S15 fraction

To obtain more insight into the composition of the P15 fraction that contains most of the RTC activity (Fig. 2b), the distribution of CHIKV RNA, viral proteins and several cellular marker proteins between P15 and S15 was studied.

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 6 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 E2 envelope protein was enriched in the membrane fraction (P15), which also contained about 15% of the capsid protein (Fig 6b). The absence of the nuclear marker fibrillarin indicated that the P15 and S15 fraction (and PNS) were not notably contaminated with nuclear material. The P15 fraction did not contain detectable amounts of the cytosolic marker 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 panel). The early endosome marker, Rab5, was predominantly found in the S15 fraction, whereas Rab7, a late endosome marker, was present both in P15 and S15. These results indicated that P15 was a rather crude fraction that contained membranes of various cellular origins.

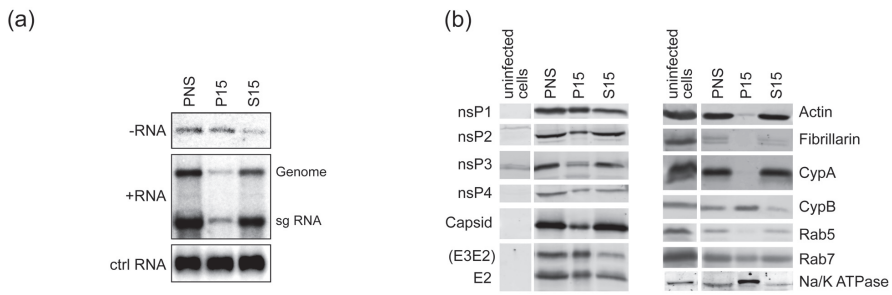

Figure 6. Distribution of CHIKV RNA and viral and cellular marker proteins between the P15 and S15 fractions. (a). Total RNA was isolated from PNS, P15 and S15 fractions (volumes reflecting equal cell numbers) obtained from CHIKV-infected Vero E6 cells. After denaturing agarose gel electrophoresis, -RNA and +RNA were specifically detected by hybridization with ^{32}P-labeled DNA 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.

The in vitro RTC assay as a tool for mode of action studies on inhibitors of CHIKV replication

To determine whether our *in vitro* assay for CHIKV RNA synthesis is a suitable tool for mode of action studies on inhibitors of CHIKV replication, we have studied the effect of a variety of compounds. We first tested a set of compounds that inhibit CHIKV replication in cell culture [24], but were not expected to directly inhibit RNA synthesis. These compounds either affect another step in the CHIKV replication cycle (chloroquine, 3-deaza-adenosine), act as antimetabolite (mycophenolic acid, ribavirin, 6-aza-uridine) and/or need to be first

converted by cellular enzymes to their active form (ribavirin, 6-aza-uridine). Indeed, none of these compounds had a measurable inhibitory effect in the *in vitro* assay for CHIKV RNA synthesis (Fig. 7a).

A set of nucleoside analogs that act as obligate chain terminators strongly inhibited the synthesis of CHIKV RNA in the *in vitro* assay in a dose-dependent manner (Fig. 7b). The weaker inhibitory effect of 3'dATP compared to that of the other 3'dNTPs was likely due to an excess of ATP in the reaction (1 mM supplied and also produced by the ATP-regenerating system). In line with this notion, the inhibitory effect of 3'dUTP, which was already very strong at a concentration of 10 μM (Fig 7b, lane 7) could be reversed by adding an excess of UTP. For example a 20-fold molar excess of UTP over 50 μM 3'dUTP restored the RNA-synthesizing activity to a level comparable to that observed in the presence of 1 μM of the inhibitor (Fig. 7b, compare lanes 6 and 11). Finally, we analyzed whether 500 μM of ribavirin triphosphate or 100 μM 6-aza-UTP had an effect on the kinetics of CHIKV RNA synthesis *in vitro*. Figure 7c shows that the kinetics of RNA synthesis in the presence of these compounds was indistinguishable from that of the control reaction, suggesting these compounds do not (directly) affect the reaction rate.

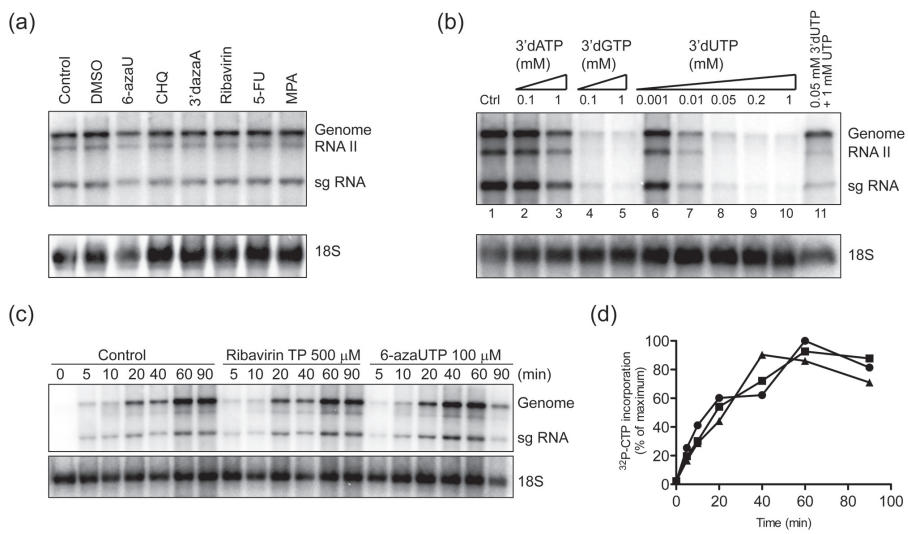

Figure 7. Effect of various compounds on the *in vitro* RNA synthesizing activity of CHIKV RTCs. (a) Standard assays were performed in the presence of 0.5% DMSO, 50 μM 6-azauridine, 100 μM chloroquine, 60 μM 3'-deaza-adenosine, 150 μM ribavirin, 500 μM fluorouracil, or 6 μM mycophenolic acid. (b) Standard 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) analyzed over a time course of 5-90 min. (d) Quantified *in vitro* ^{32}P-CTP incorporation into CHIKV RNA in the presence of ribavirin triphosphate (squares) or 6-aza-UTP (triangles) represented as % of the maximum incorporation in the control reaction (circles).

Discussion

We have successfully developed an *in vitro* system to study CHIKV RNA synthesis, in which ^{32}P-CTP is incorporated into CHIKV genome and sgRNA. Mainly single-stranded +RNA accumulated, which was not unexpected considering lysates were prepared at 6 h p.i., when -RNA synthesis has ceased and RTCs are exclusively involved in +RNA synthesis [24].

A crude membrane fraction (P15) had an approximately 10-fold higher RNA synthesizing activity than the cytosolic S15 fraction. It remains to be determined whether the low activity in S15 represents a pool of structurally and functionally distinct RTCs or whether the activity is merely due to RTCs that were released (dissociated) from the pool of P15-associated complexes. We are currently developing more elaborate subcellular fractionation procedures to study these possibilities in detail. Our fractionation data showed that the pelleted CHIKV RTCs were not dependent on cytosolic host factors for their activity, in contrast to what was found for nidoviruses [20, 25]. This is in line with previous studies on SINV and SFV [21].

The P15 fraction contained most of the -RNA, the template for +RNA synthesis. The bulk of the +RNA was found in the S15 fraction, suggesting it was released from the membrane-associated RTCs. The *in vitro* synthesized CHIKV RNA was relatively stable in the lysate and appeared to be somehow protected from cellular nucleases. This might be due to its structure, membrane association, presence within polysomes, or encapsidation soon after its synthesis (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 shut-off of host transcription and translation and interacting with a variety of host proteins. Compared to nsP2 and nsP3, the RdRp nsP4 and especially nsP1 were enriched in the P15 fraction. 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 genome and sgRNA. RNA II was synthesized both *in vitro* and *in vivo*, and we demonstrated that it represents the first 7.5 kb of the genome.

Earlier metabolic labeling studies already suggested the presence of similar molecules in SINV- and SFV-infected cells [26, 27] and Wielgosz *et al.* [22] have identified and mapped RNA II in SINV-infected cells [22]. RNA II was then believed to be unique for SINV, but we have now also identified it in CHIKV-infected cells. RNA II probably results from premature termination (near the sgRNA promoter) of RNAs initiated on the 3' end of a -RNA template that is also involved in transcription. This is supported by the observation that RNA II was only found under conditions that favored sgRNA synthesis. 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 the capping of CHIKV RNA [24]. In line with this, we found that 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. Otherwise the inhibitory effect of (novel) compounds might be underestimated or missed completely. Using the newly developed assay, we found that ribavirin triphosphate and 6-aza-UTP had no measurable effect on the kinetics of CHIKV RNA synthesis. This might indicate that either these compounds were not incorporated by the RTC or they were 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 IMPDH inhibitor mycophenolic acid had no effect *in vitro*, while it inhibited CHIKV in cell culture. Since ribavirin and 6-azauridine increase the viral mutation frequency [28, 29], 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, to screen for inhibitors, and to study the mode of action of antiviral compounds originating from other (cell-based) screens.

Methods

Cells, virus, antisera and compounds

Vero E6 cells were infected with CHIKV strain LS3 at an MOI of 5 as described [24]. Antisera against CHIKV nsP1, nsP2, nsP3, nsP4, and capsid protein were a generous gift from prof. Andres Merits (Tartu, Estonia). The E2 antiserum [30] was obtained from dr. Gorben Pijlman (Wageningen, The Netherlands). Antisera against cellular markers were purchased from Sigma (actin), Santa Cruz (fibrillarin, cyclophilins A&B), and Cell Signalling (Rab5, Rab7 and Na/K ATPase). 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 described [24].

RNA isolation, denaturing agarose gel electrophoresis, in gel hybridization and detection of ^{32}P- and ^{3}H-labeled RNA

RNA isolation [25] and denaturing formaldehyde agarose gel electrophoresis were performed as described [24]. For detection of ^{32}P-labeled RNA Phosphor Imager screens were directly exposed to dried gels, followed by scanning with a Typhoon 9410 imager (GE Healthcare). CHIKV -RNA and +RNA were specifically detected by in-gel hybridization with ^{32}P-labeled probe Hyb2 and Hyb4 [24]. Probes IA1 (5'-CAATATCGCAGTCTA-TGGAGATGTGCTCAT-3'), IA2 (5'-GTTATGACG-GGTCCTCTGAGCTTCTCGA-3') and IA3 (5'- CCTCCTATT-GTAAAAAGTTT-GGGTTGGG-ATG-3') are complementary to nt 4572-4601, 7452-7479, and 7575-7605 of the genome, resp. The 18S ribosomal RNA, detected with probe 5'-ATGCCCCCGGCCGTCCCTCT-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 labeling of CHIKV-infected cells

Metabolic labeling of CHIKV-infected cells with 40 µCi ^{3}H-uridine was done basically as described [24] except that we used Vero E6 cells seeded in 12-well clusters at a density of $2x10^5$ cells/well and infected at an MOI of 5.

Isolation of RTCs from CHIKV-infected cells

Approximately 1 x 10^8 CHIKV-infected VeroE6 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/µl RiboLock (Thermo Scientific), and 2 µg/ml 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 also added to a final concentration of 35 mM and 2.5 mM, respectively. The lysate was cleared by centrifugation at 1,000 x g for 10 min to remove unlysed cells, nuclei and cellular debris, yielding a post-nuclear supernatant (PNS). Part of the PNS was further separated by centrifugation at 15,000 x g for 15 min into a heavy membrane 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/ml ActD, and 0.2 U/µl RiboLock) using 1/5 of the volume of the PNS from which the pellet originated (now S15). This 5x concentrated P15 fraction and all other fractions were aliquoted and stored at -80°C.

In vitro RNA synthesis assay

These assays were performed inside the BSL-3 facility, since infectious CHIKV remained present in the lysates and fractions used. Standard 30-µl reactions contained either 25 µl PNS, or 25 µl S15, or 5 µl P15 (5x 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/ml ActD, 0.2 U/µl of RiboLock, 20 mM creatine phosphate, 10 U/ml creatine phosphokinase, 10 µCi (0.12 µM) of [α-^{32}P]-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 by the addition of 60 µl of 5% lithium dodecyl sulfate, 0.1 M Tris-HCl pH 8.0, 0.5 M LiCl, 10 mM EDTA, 5 mM DTT, and 0.1 mg/ml proteinase K. After a 15-min incubation at 42 °C, unincorporated label was removed using RNase-free Micro Bio-spin 30 columns (Bio-Rad), and total RNA was isolated and analyzed as described above.

Detection of ^{32}P-labeled reaction products with capture probes

In vitro transcribed RNAs (1 µg) representing the first 1348 nucleotides of the CHIKV negative strand, the 1100 3'-terminal nt of the CHIKV genome, or nt 1-2042 of the unrelated equine arteritis virus (negative control), were immobilized to Hybond N+ membrane (GE Healthcare) as described [20]. The membrane with the immobilized probes was incubated with half of the ^{32}P-labeled RNA isolated from a 30-µl *in vitro* reaction and washing and detection of bound material were done as described [20]. ^{32}P-labeled *in vitro* transcript that contained 5' and 3' UTR of CHIKV or a transcript with the complementary sequence were used as positive controls and to assess the specificity of the immobilized probes.

RNase treatments

RNA isolated from *in vitro* RTC reactions was treated with a RNase A/T1 mixture under high salt conditions to degrade single-stranded RNA, or with RNase III to degrade double-stranded RNA as described [25].

Targeted RNA cleavage assay

For targeted cleavage, RNA isolated 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, 1mM EDTA, and 80% deionized formamide with DNA probe AS25 (5'-GATAACTGCGGCCAATACTTAT-3') that is complementary to nt 9548 – 9569 of the CHIKV genome. RNase H (7.5 U; Fermentas) in a volume of 150 µl 10 mM PIPES pH 6.5, 0.1 M NaCl, 0.25 mM EDTA, and 10 mM DTT was added to the hybridized RNA. After an incubation for 60 min at 37°C, 225 µl of RNase inactivation solution (Ambion RPA kit) was added, followed by incubation for 30 min at -20°C. This treatment results in the targeted cleavage of a 2,242 nucleotide fragment of the 3' end of genomic and sgRNA, The digested RNA was precipitated in the presence of GlycoBlue (Ambion) at 4°C by centrifugation for 30 min at 15,000 x g. After resuspension in 5 µl of 1 mM sodium citrate, RNA was analyzed 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 done as described [24].

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