SUPPLEMENTARY INFORMATION FOR:

Reinitiated viral RNA-dependent RNA polymerase resumes replication at a reduced rate

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SUPPLEMENTARY TEXT

Proof of stalling during replication on short oligos

φ6 RdRP elongation complex (φ6EC) can be stalled *in vitro* using a limited selection of NTPs and a template molecule in which the 3’-terminal region is devoid of one or more of the coding nucleotides (in our experiments we deplete only adenosine on the template). Elongation can be reinitiated by the addition of the missing NTP(s) - in our case, UTP - yielding an entirely double-stranded product. To demonstrate this, we designed a 204 nucleotide long ssRNA (0.2kb RNA) in which the first occurrence of adenine was 50 nucleotides (nt) from the 3’-end. Heat-denatured 0.2kb RNA was incubated with φ6 RdRP in the φ6 RdRP reaction buffer in the presence of CTP, GTP, and ATP (Materials and Methods). The resulting decrease in electrophoretic mobility compared to free 0.2kb RNA suggested successful initiation of replication followed by stalling (Lanes 1 and 4 in Figure S3). Not all 0.2kb RNAs included a stalled φ6EC, as indicated by the presence of a band in Lane 4 with the same electrophoretic mobility as the free 0.2kb RNA.

To specifically test subsequent reinitiation of the stalled φ6EC, we first sought to prevent random initiation of replication on the remaining free 0.2kb RNA. For this reason, we hybridized a 50 nt DNA oligo (oligoDNA; Table S1) to the 3’-end of the free 0.2kb RNA to yield a blunt-end initiation site, which is an inefficient template for φ6 RdRP (1). The oligoDNA was successfully hybridized to the free 0.2kb RNA, as shown by the disappearance of the free 0.2kb RNA band and the appearance of a band corresponding to 0.2kb RNA hybridized to oligoDNA in the absence of φ6 RdRP (Lane 2 in Figure S3). Conversely, the oligoDNA did not form complexes with the stalled φ6EC, as no appreciable decrease in the electrophoretic mobility of the band corresponding to stalled φ6EC was observed (Lane 5 in
Figure S3). After blocking the remaining free 0.2kb RNA in this manner, the stalled φ6EC was successfully reinitiated by the addition of UTP, as indicated by the appearance of the final product band (Lane 6 in Figure S3). This demonstrates that φ6EC can be stalled using nucleotide starvation, and that it can be reinitiated by the addition of the missing nucleotide.

**Calibration curve relating the electrophoretic mobility of φ6EC and the number of replicated nucleotides**

In the case of replication, the φ6EC is a complex consisting of a template strand, a replicating φ6 RdRP, and a newly synthesized RNA strand hybridized to already replicated section of the template strand. In front of the replicating φ6 RdRP, there is thus a region of single-stranded RNA whereas behind it, there is a region of double-stranded RNA. Under our experimental conditions (i.e. replication on a 4kb ssRNA template strand), the φ6 RdRP itself contributes a negligible amount to the overall electrophoretic mobility of φ6EC (Figure S4). At any time during the replication, the electrophoretic mobility of φ6EC could thus be approximated by the electrophoretic mobility of an RNA hybrid with the corresponding lengths of double- and single-stranded regions (Figure S2A).

We used this fact to characterize the electrophoretic mobilities of intermediates of known lengths, which allowed us to construct a calibration curve relating the electrophoretic mobility of a φ6EC intermediate on agarose gel to a number of replicated nucleotides. To this effect, a 4193 nt long ssRNA (4kb main RNA) was hybridized to a complementary 500, 1000, 1500, 2500, 3500, or 4000 nt long ss RNA (0.5kb Hyb, 1kb Hyb, 1.5kb Hyb, 2.5kb Hyb, 3.5kb Hyb, or 4kb Hyb, respectively) in order to obtain a series of RNA hybrids with successively longer double-stranded regions (i.e. successively higher values of parameter α which represents
the fraction of the 4kb main RNA that has been hybridized) (Figure S2A). The RNA hybrids were exposed to similar treatment as the RNA templates during the φ6 RdRP replication reaction: i) heat-denaturation by incubation at 65 °C for 15 min, and fast-cooling to 4 °C, ii) equilibration for 5 min at 22 °C, iii) incubation under φ6 RdRP stalling conditions at 22 °C for 15 min (Materials and Methods), iv) addition of EDTA, and GTP, and v) incubation for 60 min at 22 °C. After the addition of 6× Loading buffer (Promega, USA), the RNA hybrids were loaded on a 1.5 % agarose gel together with a 2-log DNA ladder (New England BioLabs, USA) and run as described for replication intermediates in the Materials and Methods. The 2-log DNA ladder was diluted and mixed with 6× Loading buffer (Promega, USA) prior to loading it on the agarose gel. After electrophoresis, we used ethidium bromide staining of the replication intermediates rather than more involved radioactive labeling, since an accurate position of the replication intermediate band rather than its absolute intensity sufficed for this calibration (Figure S2).

An example of an agarose gel loaded with RNA hybrids (Lanes 1 to 6; corresponding values of α are indicated) and 2-log DNA ladder (Lane marked M) is shown in Figure S2B. The electrophoretic mobilities of the RNA hybrids were analyzed with the LabImage 1D 2006 gel analysis program (Kapelan Bioimaging, Germany). Briefly, the intensity profile of each lane was taken, and the bands of the individual RNA hybrids were identified and their electrophoretic mobility determined by the maximum of the corresponding band intensity profile. The electrophoretic mobility of each RNA hybrid was then evaluated relative to the electrophoretic mobilities of dsDNA fragments contained in the 2-log DNA ladder. The electrophoretic mobility of RNA hybrid, expressed in units of the length of dsRNA with an
equivalent electrophoretic mobility, was plotted as a function of parameter $\alpha$ (Figure S2c). The set of experimental points obtained was best fitted with a sigmoidal curve (solid red curve).

The obtained sigmoidal fit was used as a calibration curve to convert the electrophoretic mobilities of the reinitiated $\phi 6ECs$ to the corresponding number of replicated nucleotides. Briefly, after reinitiating $\phi 6ECs$, aliquots of the reaction mix were collected, and subsequently run on an agarose gel as described in Material and Methods. Examples of these gels are shown in Figures 2B and S5. Agarose gels were then stained with ethidium bromide, and imaged with a gel imager. The intensity profile of each lane was subsequently taken using LabImage 1D 2006 gel analysis program (Kapelan Bioimaging, Germany). The band of the reinitiated $\phi 6ECs$ in each lane was identified as a peak in the intensity profile. An average electrophoretic mobility of the reinitiated $\phi 6ECs$ ($\mu$(RNA)) was determined by the position of the maximum of the peak and expressed in the units of length of dsDNA exhibiting an equivalent electrophoretic mobility using the 2-log DNA ladder in Lane M of each gel. The relative electrophoretic mobility of the reinitiated $\phi 6ECs$ obtained in this manner was finally converted to the number of replicated nucleotides at a corresponding elongation time using the calibration curve in Figure S2c.

The measured elongation rate ($k_{elong}$) corresponds to the elongation rate during the first round of replication

We have measured $k_{elong}$ of reinitiated $\phi 6EC$ and the polymerization rate ($k_{poly}$) of randomly initiated $\phi 6EC$, as explained in the main text. While $k_{poly}$ is determined by all the replication steps (i.e. binding of $\phi 6 RdRP$ to the RNA template, initiation of replication, elongation, and replication termination), $k_{elong}$ can be defined more specifically as an elongation rate of
reinitiated ϕ6ECs that successfully complete the first round of replication of the RNA template strand (Figure S1). Should reinitiated ϕ6ECs prematurely dissociate from the template, they become unsynchronized with the remainder of reinitiated ϕ6ECs and thus do not contribute to the distinct band of the replication intermediates. A reactivation of the dissociated ϕ6ECs by ϕ6 RdRP rebinding is similarly unsynchronized and would not form a single distinct band; the same applies to subsequent rounds of replication that are also unsynchronized.

SUPPLEMENTARY FIGURES (CAPTIONS)

Figure S1 Replication reaction performed by ϕ6 RdRP can be devided into three stages: initiation, elongation, and termination. The initiation of ϕ6 RdRP replication occurs at the free 3’-end of the template strand (Step 1). An internal initiation (Step 2) does not occur as a free 3’-end is required for the initiation of ϕ6 RdRP replication. After initiation, an elongation complex (ϕ6EC) is formed to polymerize the complementary strand (Step 3). The replication is terminated when ϕ6EC reaches the 5’-end of the template strand, where it dissociates into dsRNA product and free ϕ6 RdRP (Step 4). The free ϕ6 RdRP can subsequently initiate a replication at the 3’-end of a new template strand (Step 5). A dissociation of ϕ6EC prior to the termination point (Step 6) may occur resulting in an incomplete elongation intermediates. The re-association of the ϕ6EC by ϕ6 RdRP rebinding (Step 7) may reinitiate the replication.

Figure S2 Calibration of the electrophoretic mobilities of RNA hybrids against a 2-log DNA ladder. (A) RNA hybrids were assembled consisting of a 4193 nt long ssRNA (4kb main RNA) hybridized to a complementary 500, 1000, 1500, 2500, 3500 or 4000 nt long ss RNA (0.5kb
Hyb, 1kb Hyb, 1.5kb Hyb, 2.5kb Hyb, 3.5kb Hyb, or 4kb Hyb, respectively). The details of the hybridization reactions are described in the Materials and Methods. (B) An example of an agarose gel loaded with RNA hybrids (Lanes 1 to 6) and 2-log DNA ladder (Lane marked M) with corresponding $\alpha$ values for the RNA hybrids indicated. (C) The RNA hybrid electrophoretic mobility ($\mu$(RNA)), expressed in units of the length of dsDNA that exhibits the equivalent electrophoretic mobility, as a function of parameter $\alpha$. The set of experimental points obtained was best fitted with a sigmoidal curve (solid red curve): $A = 4234 \pm 46$ bp, $B = -4657 \pm 286$ bp, $C = -0.54 \pm 0.07$, $D = 0.92 \pm 0.05$.

**Figure S3** Demonstration of *in vitro* stalling and reinitiation of $\phi$6EC. The reactions were carried out as explained in the Material and Methods as well as Supplementary Text. $\phi$6EC was stalled on a 0.2kb RNA (Lane 4), and the 3’-end of the free 0.2kb RNA was subsequently blocked by a 50 nt long oligo DNA (Lane 5). The stalled $\phi$6EC was reinitiated by the addition of UTP (Lane 6). The electrophoretic mobilities of free 0.2kb RNA, 0.2kb RNA:oligo DNA hybrid, and replication product (0.2kb dsRNA) are shown in Lanes 1, 2, 3, respectively.

**Figure S4** $\phi$6 RdRP binding to template strand (4kb ssRNA). $\phi$6 RdRP replication initiation as well as replication of the first 50 nt prior to stalling do not affect the electrophoretic mobility of 4kb ssRNA. Lane M: 2-log DNA ladder; Lane 1: 4kb ssRNA template in THE RNA storage solution; Lane 2: Replication of 4kb ssRNA template by $\phi$6 RdRP to 4k dsRNA; Lane 3: 4kb ssRNA incubated under the stalling conditions (2.6 $\mu$M $\phi$6 RdRP, 50 mM HEPES pH 7.9, 20 mM ammonium acetate, 5 mM MgCl$_2$, 2 mM MnCl$_2$, 0.1 mM EDTA pH 8.0, 0.1 % Triton X-
100, 5% (v/v) Superase•In, 2.5 mM in ATP, CTP, and GTP); Lane 4: 4kb ssRNA incubated under the stalling conditions in the absence of NTPs.

**Figure S5** Temperature dependence of $k_{\text{elong}}$ after reinitiation of the stalled φ6EC. The φ6EC were stalled at three different temperatures $T_1$ (16 °C, 22 °C, or 30 °C), and reinitiated at three different temperatures $T_2$ (16 °C, 22 °C, or 30 °C). For each combination of $T_1$ and $T_2$, a sample agarose gel is displayed. The 2-log DNA ladder is indicated by ‘M’, and the elongation times are indicated above each lane. The electrophoretic mobility of reinitiated φ6EC at each elongation time was converted to the number of replicated nucleotides as explained in the Supplementary Text. We converted only the earlier elongation time points (< 1200 s), because the decreasing differences in the electrophoretic mobilities in the later reaction stages precluded an accurate determination of the number of replicated nucleotides. The results are plotted in the graphs below the gel images and are grouped by common $T_1$. Within each plot, the green triangles, red circles, and blue squares and their corresponding linear fits represent data obtained at $T_2 = 16$ °C, $T_2 = 22$ °C, and $T_2 = 30$ °C, respectively. Occasionally, two distinct bands of replication intermediates were detected (e.g. for $(T_1, T_2)$ pairs (16 °C, 16 °C), (22 °C, 16 °C), or (30 °C, 16 °C)). Given that such double-banded patterns did not consistently form, we conservatively assigned the elongation rate to the faster of the two populations. The value of $k_{\text{elong}}$ for each $(T_1, T_2)$ pair was determined from the slope of the corresponding linear fit. The average $k_{\text{elong}}$ for each $(T_1, T_2)$ pair was deduced from three measurements, and was equal to $k_{\text{elong}} = 2 \pm 1 \text{ nt·s}^{-1}$ for all temperature regimes investigated.
Figure S6 Temperature dependence of $k_{poly}$ of the randomly-initiated $\phi 6EC$. The replication templates were incubated with $\phi 6 RdRP$ at three different $T_1$ (16 °C, 22 °C, or 30 °C), and the replication was randomly-initiated by the addition of all four NTPs at three different $T_2$ (16 °C, 22 °C, or 30 °C) (Materials and Methods). For each combination of $T_1$ and $T_2$, a sample agarose gel is displayed. The 2-log DNA ladder is labelled by the letter ‘M’ above the corresponding lane, and the polymerization times are indicated above the lanes. The intensities of the replication product bands were measured with the LabImage 1D 2006 Professional software (Kapelan, Germany) and normalized by dividing by the intensity of the 4kb ssRNA template present prior to the addition of all four nucleotides. The normalized intensities of the replication products were plotted as a function of polymerization time and fitted to a straight line. The intercept of each linear fit with the $x$-axis yielded a time $\tau$, which is the minimum time necessary for the conversion of a single template strand to its double-stranded replication product. Within each plot, the green, red, and blue experimental points and their corresponding linear fits represent data obtained at $T_2 = 16$ °C, $T_2 = 22$ °C, and $T_2 = 30$ °C, respectively. The $\tau$ values extracted and their corresponding uncertainties are displayed in the plots. $k_{poly}$ were calculated by dividing the total length of 4kb ssRNA (4193 nt) by the value of $\tau$. The mean values for $k_{poly}$ for all combinations of $T_1$ and $T_2$ were deduced from three experiments and are displayed below the corresponding plots. A quantitative analysis of the agarose gel obtained for the randomly-initiated replication at $T_1 = 16$ °C, and $T_2 = 30$ °C could not be performed due to a persistent background signal in the gel.

Figure S7 The effect of NTP concentration during the stalling stage on the reinitiated $\phi 6EC$. The stalling was carried out at different NTP concentrations. The reinitiated $\phi 6EC$ performed
the reaction at a constant NTP concentration of 2.5 mM per NTP. Aliquots at 4 min and 8 min were taken and analysed on an agarose gel. The replication reaction at each NTP concentration were studied in the absence (A) and presence (B) of heparin. Heparin was added after the stalling step, and prior to reinitiation.

**Figure S8** The synchronized population of reinitiated φ6EC is first observed after 2 min stalling time. The stalling, reinitiation, and sample analysis were carried out as described in the Material and Methods. The tested stalling times were 1 min, 2 min, and 15 min. The stalling times are indicated above the corresponding images of the agarose gels. A synchronized population of φ6ECs is characterized by a well-defined band on an agarose gel, while a population of randomly initiated φ6ECs should form a diffuse band typically undetectable under our experimental conditions. No detectable bands indicative of a synchronized population of reinitiated φ6ECs were detected at the shortest stalling times investigated (i.e. 1 min). However, a well-defined band of the synchronized reinitiated φ6ECs could be detected on the gel after a stalling time of 2 min. These reinitiated φ6ECs immediately exhibit slow elongation kinetics, indicating a correlation between stalling and slow elongation kinetics after reinitiation.

**Table S1** Pairs of forward and reverse primers used in PCR reaction to amplify DNA fragments for *in vitro* run-off transcriptions (Materials and Methods). The part of each primer carrying a sequence complementary to DNA template is indicated in **bold**. The T7 RNA polymerase promoter sequence is shown in *italic* and is present only in one of the primers per pair. The underlined sequences in the reverse primers of DNA fragments for 0.2kb RNA, 3kb
RNA, and 4kb RNA were introduced to extend the original DNA template sequence at the point of primer hybridization by fifty nucleotides. These extensions yielded an adenine-free 3’-ends of the corresponding in vitro transcripts.

SUPPLEMENTARY REFERENCES

1. Makeyev, E. V. & Bamford, D. H. (2000) Replicase activity of purified recombinant protein P2 of double-stranded RNA bacteriophage φ6 EMBO J. 19: 124-133.
SUPPLEMENTARY FIGURES

Figure S1
Figure S2

(A) 
\[ \alpha = \frac{I}{L} \]

(B) 
Lane 1: \( \alpha = 0.12 \)  
Lane 2: \( \alpha = 0.24 \)  
Lane 3: \( \alpha = 0.36 \)  
Lane 4: \( \alpha = 0.60 \)  
Lane 5: \( \alpha = 0.84 \)  
Lane 6: \( \alpha = 0.96 \)  

(C) 
\[ \mu_{RNA}(\alpha) = A + Be^{-(\alpha \beta)^2} \]  

\( \mu_{RNA} \) [dsDNA bp] vs. \( \alpha \)
Figure S3
| Lane     | M   | 1 | 2 | 3 | 4 |
|----------|-----|---|---|---|---|
| 46 RdRP  | -   | + | + | + |   |
| 46 RdRP buffer | - | + | + | + |   |
| ATP      | -   | + | + |   | - |
| CTP      | -   | + | + |   | - |
| GTP      | -   | + | + |   | - |
| UTP      | -   | + |   |   | - |

Figure S4
| $T_1$ | $T_2$ |
|-------|-------|
| 16 °C | 16 °C |
| 22 °C | 22 °C |
| 30 °C | 30 °C |

**Figure S5**
| (dNTP) | [hSRNA] | Polymerization time [s] |
|--------|--------|------------------------|
| T<sub>1</sub> = 16 °C | T<sub>4</sub> = 22 °C | T<sub>1</sub> = 30 °C |
| T<sub>2</sub> = 16 °C | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| T<sub>2</sub> = 22 °C | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| T<sub>2</sub> = 30 °C | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |

K<sub>poly</sub> ([dNTP] = 16 °C; T<sub>poly</sub> = 16 °C) = 9 ± 4 nM s<sup>-1</sup>
K<sub>poly</sub> ([dNTP] = 16 °C; T<sub>poly</sub> = 30 °C) = N.A
K<sub>poly</sub> ([dNTP] = 22 °C; T<sub>poly</sub> = 16 °C) = 9 ± 4 nM s<sup>-1</sup>
K<sub>poly</sub> ([dNTP] = 22 °C; T<sub>poly</sub> = 30 °C) = 43 ± 14 nM s<sup>-1</sup>
K<sub>poly</sub> ([dNTP] = 30 °C; T<sub>poly</sub> = 16 °C) = 9 ± 3 nM s<sup>-1</sup>
K<sub>poly</sub> ([dNTP] = 30 °C; T<sub>poly</sub> = 30 °C) = 66 ± 28 nM s<sup>-1</sup>

Figure S6
Figure S7
**Figure S8**

Stalling time = 1 min

Stalling time = 2 min

Stalling time = 15 min
## SUPPLEMENTARY TABLE

| PCR fragment | Forward & reverse primers |
|--------------|---------------------------|
| 0.2kb RNA    | **Forward primer:** TAATACGACTCACTATAAGGGTGATCCGAGAGGCGGCGAGG | **Reverse primer:** GGAACGTATGGTAATGACAGG |
| 0.5kb Hyb    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 1kb Hyb      | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 1.3kb RNA    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 1.5kb Hyb    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 2.5kb Hyb    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 3kb RNA      | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 3.5kb Hyb    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 4kb Hyb      | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 4kb RNA      | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 4kb main RNA | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| 4.2kb RNA    | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |
| oligoDNA     | **Forward primer:** TAATACGACTCACTATAGGGATCTACGAGAGGCCTCGAGG | **Reverse primer:** GGATCCGATCTGAGAGG |

Table S1