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Genome Replication, Synthesis, and Assembly of the Bacteriophage T7 in a Single Cell-Free Reaction

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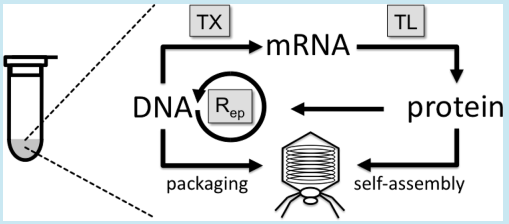
Supporting Information

ABSTRACT: The synthesis of living entities in the laboratory is a standing challenge that calls for innovative approaches. Using a cell-free transcription-translation system as a molecular programming platform, we show that the bacteriophage T7, encoded by a 40 kbp DNA program composed of about 60 genes, can be entirely synthesized from its genomic DNA in a test tube reaction. More than a billion infectious bacteriophages T7 per milliliter of reaction are produced after a few hours of incubation. The replication of the genomic DNA occurs concurrently with phage gene expression, protein synthesis, and viral assembly. The demonstration that genome-sized viral DNA can be expressed in a test tube, recapitulating the entire chain of information processing including the replication of the DNA instructions, opens new possibilities to program and to study complex biochemical systems *in vitro*.



KEYWORDS: *bacteriophage, cell-free protein synthesis, cell-free synthetic biology, DNA replication, self-assembly*

Cell-free protein synthesis, with its immediate impact on the elucidation of the genetic code,[1] has been an essential technique in providing an understanding of the process of protein synthesis in living organisms. DNA-dependent cell-free expression systems became widely used as a research tool in the late 1960s and the 1970s to analyze gene products and to unravel the regulation of gene expression systems such as the *E. coli* lactose[2] and tryptophan[3] operons. The development of highly efficient hybrid cell-free expression systems in the early 1990s marked a turning point for this technology.[4] The modern transcription-translation (TX-TL) cell-free systems, optimized for large-scale protein synthesis as an alternative to the recombinant protein technology,[5,6] are used in an increasing number of applications in biotechnology, industry, and proteomics.[7−9]

With the advent of the synthetic biology era, TX-TL cell-free systems could also become powerful platforms to build complex biochemical systems using a bottom-up molecular programming approach, offering the opportunity for investigation of emergent properties of complex biological systems.[10] Importantly, the construction of biological systems in a test tube using DNA programs provides a means to study biochemical behaviors in isolation, with a greater level of control and a greater freedom of design compared to *in vivo*. In addition to increasing our knowledge of the molecular repertoire found in biology, constructing information-based biochemical systems *in vitro* offers the possibility of expanding the capabilities of existing biological systems.[10] Elementary gene circuits,[11,12] pattern formation,[13] and prototypes of artificial cells[14,15] have been engineered with TX-TL cell-free systems. However, the development and the quantitative investigation of such complex systems in a cell-free context are limited by the current available technology, which has not been optimized to construct information-based molecular systems. The expression of genome-sized DNA programs that recapitulates the entire chain of biological information, with the circuitry and the self-organization of complex active biological entities has not been demonstrated in a cell-free context.

In this work, we use a cell-free TX-TL system specifically developed to construct complex biochemical systems *in vitro* to show that large DNA programs can be expressed outside living cells. First, we searched for natural DNA programs that, within range of the cell-free expression system's capacity, could be entirely expressed into a functioning entity. The bacteriophage T7 emerges as an almost ideal DNA program and biological system. We demonstrate that genome replication, synthesis and self-assembly of the coliphage occur concurrently in a single batch mode cell-free reaction. The approach is not limited to T7, as we show that the phage ΦX174 can be also synthesized with the same cell-free expression system.

Our experiments are based on a conceptual approach, which consists of expressing synthetic or natural DNA programs in a test tube. The DNA program acts as molecular software processed by the TX-TL machineries, the hardware provided by a cell-free platform recently developed for molecular programming.[12] This approach has proven to work for elementary synthetic gene circuits engineered in the laboratory. To estimate how large a DNA program could be processed *in vitro*, we performed the following calculation. A maximum concentration of 25−30 μM of active enhanced green fluorescent protein (eGFP), a medium size protein, is

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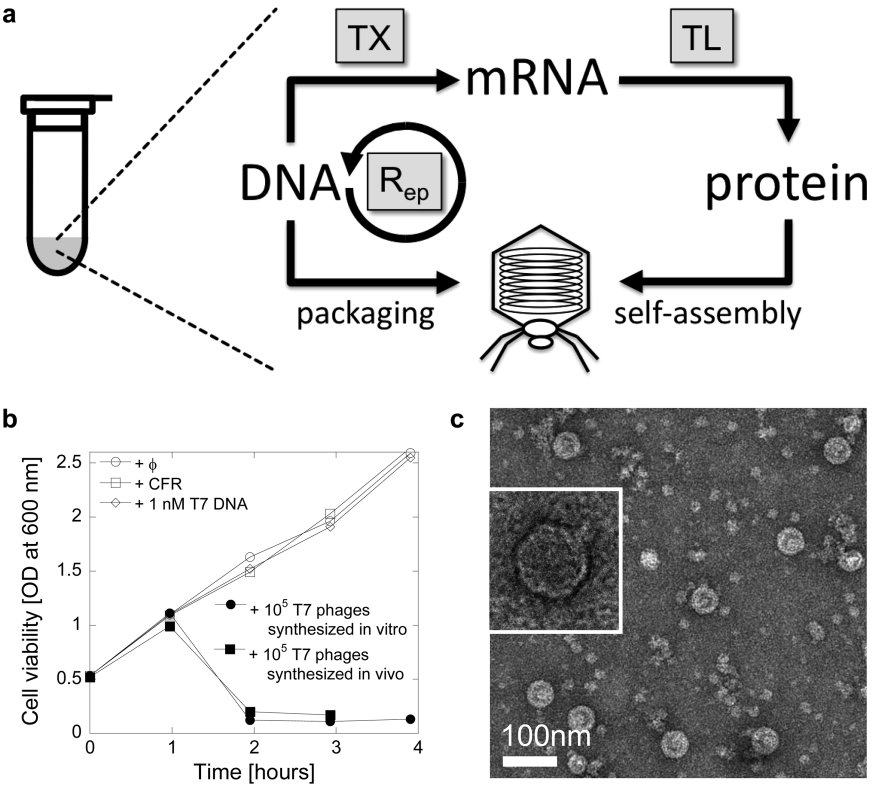

Figure 1. Cell-free synthesis of the bacteriophage T7. (a) Schematic of the transcription (TX) and translation (TL) reaction carried out in a test tube. DNA replication (R_{ep}) occurs concurrently with the phage synthesis. (b) *Escherichia coli* cell viability measured by absorbance at 600 nm. *E. coli* strain B cells were grown at 37 °C in LB medium. At an optical density of 0.5 ($\approx 0.5 \times 10^9$ cells mL^{-1}), five identical 5 mL cultures were inoculated with nothing (⌀), 1 μL of a cell-free TX-TL reaction (CFR) incubated at 29 °C for 12 h supplemented with a plasmid encoding for the reporter protein eGFP, 1 μL of a solution of T7 DNA genome resuspended in a cell-free reaction at a concentration of 1 nM (no incubation), 1 μL of a cell-free TX-TL reaction supplemented with 1 nM of T7 DNA incubated at 29 °C for 12 h containing at the end of the incubation 10^8 mL^{-1} T7 phages, and 1 μL of a solution containing 10^8 mL^{-1} T7 phages synthesized *in vivo* (*E. coli* strain B infection). (c) Transmission electron microscopy (TEM) image of the T7 phages synthesized in a cell-free reaction (magnification: 59000X). Inset: blow-up of a single T7 phage; the capsid (55 nm diameter) and the tail (20 nm) are visible.

synthesized in a batch mode reaction after 5 h of incubation. In *E. coli*, the average concentration of cytoplasmic protein is on the order of 500 nM.[16] This led us to conclude that DNA programs composed of up to 60 genes, the genomic size range of bacteriophages, could be entirely expressed as a functioning whole in a cell-free reaction. We identified bacteriophages encoded by DNA programs in this range, based on *E. coli* transcriptional regulatory parts, and with a dependence on the host limited to the TX-TL machineries. The dependence of most of the coliphages on the host goes far beyond the TX-TL machineries. For example, the phages Lambda and M13 rely, to a large extent, on the host *E. coli* for both gene circuits and self-organization.[17,18] The phage T7 and its relative T3, however, have a limited interaction with *E. coli* once in the cytoplasm. Besides the TX-TL hardware and the membrane receptors, it has been reported so far that the growth of the phage T7, encoded by a double-stranded DNA program of approximately 57 genes, depends only on the host protein thioredoxin for its DNA replication, which is independent from the phage synthesis, and on the CMP kinase.[19] In addition, the phage T7, isolated 60 years ago, is still actively studied,[20,21] and its DNA program presents two uncommon features: it has its own RNA polymerase, expressed early in the infection cycle, and its own DNA polymerase. In a context based on a hardware-software analogy we found that the phage T7 and its DNA instructions were an ideal biological system to be processed through our cell-free platform[12] (Figure 1a), which is prepared from an *E. coli* strain having both the genes encoding for the thioredoxin and the CMP kinase but none of the T7 DNA instruction parts.

The 40 kbp linear T7 DNA genome was first incubated at 29 °C for 12 h in a cell-free reaction containing the nutrients necessary for TX-TL. Phage production from the reaction was measured by counting plaque forming units (PFU) (Supporting Figure S1a). At a genome concentration of 1 nM, which in our system corresponds to one genome into one *E. coli* cell, 0.1 to 1 billion phage per milliliter of reaction was synthesized (Supporting Figure S1b). Negative controls included samples where only the T7 DNA genome or a cell-free reaction containing a plasmid encoding for eGFP were used. Phage particles produced in the cell-free system have the same biological capacity as *in vivo* assembled particles. When an equivalent amount of infectious T7 phages, either synthesized in a test tube or *in vivo*, was added to a 5-mL *E. coli* culture in log phase (OD_{600} = 0.5, 0.5×10^9 cells mL^{-1}), the kinetics of cell viability were identical (Figure 1b). The absorbance at 600 nm dropped to background level between 1 and 2 h after induction, as expected for a number of phage produced per cell of about 100, a multiplicity of infection of 2×10^{-4}, and a phage cycle of 25 min. Electron microscopy of material from the cell-free reaction revealed the appearance of fully

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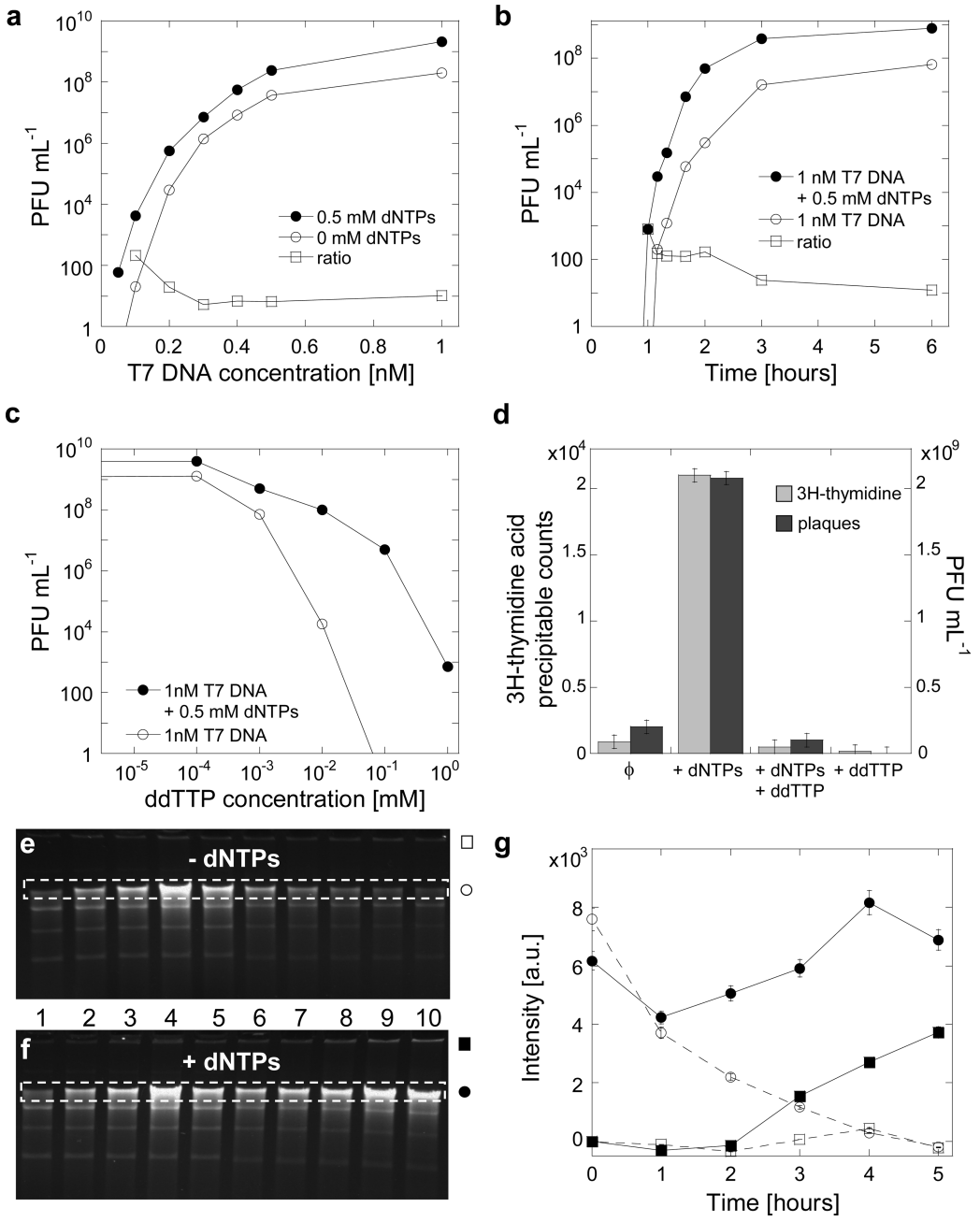

Figure 2. Characterization of the T7 phage cell-free synthesis and of the DNA replication. (a) Number of synthesized T7 phages (PFU mL^{-1}: plaque forming units per milliliter) as a function of the T7 DNA genome concentration in the cell-free reaction, with and without the addition of 0.5 mM of each of the DNA nucleotide triphosphates (dNTPs), measured after 12 h of incubation at 29 °C. (b) Number of T7 phages synthesized in cell-free reactions (1 nM T7 DNA genome, incubation at 29 °C) as a function of time, with and without the addition of 0.5 mM of each of the dNTPs. (c) Number of synthesized T7 phages as a function of the concentration of the DNA replication inhibitor ddTTP in the reaction (1 nM T7 DNA genome), with and without the addition of 0.5 mM of each of the dNTPs, measured after 12 h of incubation at 29 °C. (d) ^{3}H-Thymidine (2.5 μM) incorporation and number of plaques measured after 12 h of incubation at 29 °C (1 nM T7 DNA genome) for four different samples. ⌀: nothing added; dNTPs: addition of 0.5 mM of each of the four DNA bases; dNTPs + ddTTP: addition of 0.5 mM of each of the four DNA bases and 25 μM of chain terminator; ddTTP: addition of 0.5 mM of chain terminator. (e) Agarose gel electrophoresis (stained with ethidium bromide) of the T7 DNA present in a cell-free reaction as a function of time with no addition of dNTPs. Lanes 1−4: 0, 0.5, 1, and 2 nM of T7 DNA added to a cell-free reaction (no incubation) used for quantification. Lanes 5−10: 1 nM of T7 DNA incubated in a cell-free reaction measured every hour (lane 5: 0 h; lane 10: 5 h). The white dotted frame indicates the T7 DNA band. Symbols: open square (concatemers in wells) and circle (T7 genome unit-length DNA) used in (g). (f) Agarose gel electrophoresis of the T7 DNA present in a cell-free reaction as a function of time with 0.5 mM of each of the dNTPs added to the reaction. Lanes 1−4: 0, 0.5, 1, and 2 nM of T7 DNA added to a cell-free reaction (no incubation) used for quantification. Lanes 5−10: 1 nM of T7 DNA incubated in a cell-free reaction measured every hour (lane 5: 0 h; lane 10: 5 h). The white dotted frame indicates the T7 DNA band. Symbols: closed square (concatemers in wells) and circle (T7 genome unit-length DNA) used in (g). (g) Kinetic of T7 DNA genome present in a cell-free reaction, with and without 0.5 mM of dNTPs, constructed from the gels shown in (e) and (f). The intensity of the T7 DNA band (unit-length genomes) and the intensity of the DNA present into the wells (unprocessed concatemers) were measured.

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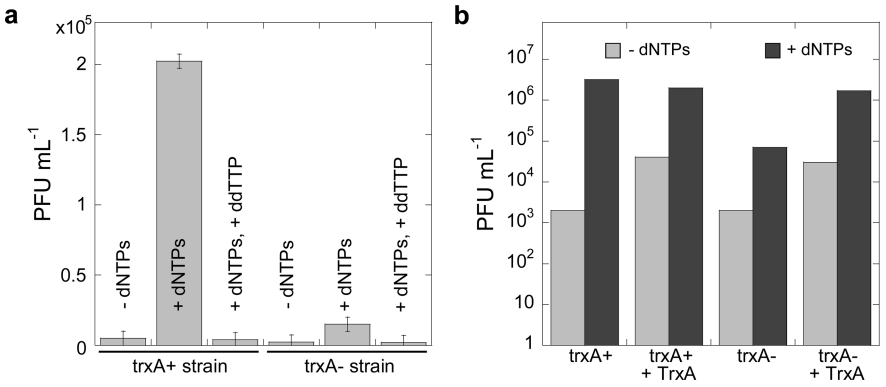

Figure 3. Effect of thioredoxin on phage synthesis. (a) Synthesis of the bacteriophage T7 using cell-free systems prepared from the *E. coli* BL21 *trxA* + strain and from an *E. coli* BL21 *trxA*− strain, with and without dNTPs (0.5 mM of each) or ddTTP (25 μM) added to the reaction. (b) Synthesis of the bacteriophage T7 using cell-free systems prepared from the *E. coli* BL21 *trxA*+ strain and from the *E. coli* BL21 *trxA*− strain, with and without dNTPs (0.5 mM of each) or thioredoxin (TrxA, 3 μM final concentration, synthesized in a separate cell-free reaction) added to the reaction.

synthesized T7 phages whose morphology is undistinguishable from *in vivo* synthesized phages (Figure 1c).

To further characterize the cell-free synthesis of T7, the yield of phage particles was determined as a function of the genome concentration, with and without the addition of the four deoxyribonucleotide-triphosphate bases (dNTPs) to the reaction (Figure 2a). Phage synthesis, observed above a genome concentration of 0.1 nM, reached a peak at a T7 DNA concentration of 2 nM, with a maximum yield of 5 billion phages per milliliter of reaction corresponding to ∼0.01 nM phage with the four dNTPs added to the reaction. In the absence of dNTPs, a maximum of 1 billion of phages per milliliter of reaction was produced (∼0.002 nM phage). Phage production increased up to 200-fold when the reaction was supplemented with dNTPs over the entire range of genome concentrations tested. The first phages were synthesized after 1 h of incubation, while their accumulation in the reaction stopped after 5 h (Figure 2b), the typical reaction time for a batch mode reaction. At 2 h of incubation, dNTPs supplemented reactions yielded ∼200 times more phages than reactions without dNTPs.

To confirm concurrent replication of the DNA instructions with phage synthesis, phage production was assessed in the presence of the DNA replication inhibitor ddTTP, a chain-terminator whose impact on cell-free gene expression is negligible (Supporting Figure S2). Interestingly, phage synthesis was entirely inhibited at high ddTTP concentration in the absence of exogenous dNTPs (Figure 2c). These results suggested that, in the absence of dNTP supplementation, T7 DNA genomes could be either newly replicated or repaired from an endogenous dNTP pool provided by degraded T7 genomes. When dNTPs were added to the reaction, phage synthesis could be quantitatively reduced by more than one million-fold at high chain-terminator concentration. Replication of new DNA was assessed by measuring the incorporation of radioactive 3H-thymidine (Figure 2d). The relative amount of DNA synthesized paralleled the number of viable phage produced.

The amount of phage DNA synthesized as a function of time was determined by gel electrophoresis. With no dNTPs added to the reaction (Figure 2e and g), the genomic DNA was degraded to background level after 4 h of incubation, despite the addition of the protein GamS to the reaction. GamS, used in all of the reactions, reduces the degradation of linear DNA by inhibiting the 3′ exonuclease activity of the RecBCD complex[22] present in the cell-free system. In addition, T7 synthesizes its own endonuclease, known to cleave the T7 genome, which might also be involved in genome degradation in our cell-free system. With dNTPs added to the reaction (Figure 2f and g), a reduction in the amount of genome-length T7 DNA was observed during the first hour followed by an increase of up to twice the initial concentration (Supporting Figure S3a and b). In addition, longer DNAs that could not enter agarose gels could be seen to appear in the wells. We attributed these longer DNAs to concatemers formed during replication.[23] Such observations could not be made in the absence of DNA (Figure S3c and d).

The dependence of phage synthesis on the host protein thioredoxin was assessed by measuring the production of phage T7 in TX-TL cell-free systems prepared from either a *trxA*− or a *trxA*+ *E. coli* strains. Although less efficient than our original system, cell-free gene expression with these two systems was sufficient to support phage synthesis. Addition of dNTPs resulted in a greater increase in phage production in the *trxA*+ system compared to the *trxA*− system (Figure 3a). We noted, however, a significant level of phage production with the *trxA*− system even though the lack of thioredoxin would completely impair DNA replication *in vivo*. No explanation can be provided for this observation. When exogenous thioredoxin, synthesized in a parallel cell-free reaction, was added to the *trxA*− system, the yield of phage synthesis returned to the level produced by the *trxA*+-based system (Figure 3b). Phage synthesis in the absence of supplemental dNTPs was 100- to 1000-fold lower than with dNTPs.

Despite extensive efforts over a period of 60 years, only 35 of the ∼60 proteins encoded by the T7 DNA instructions have a known function.[21] Developed from a strict software-hardware analogy, the TX-TL system used herein is capable of running a natural DNA program whose complexity exceeds our current knowledge, as well as our current capacity to understand such large networks as a whole. It is even more surprising when we consider that the cell-free system used in this study is a cytoplasm diluted 20−30 times relative to *in vivo*. The demonstration that a functioning entity as complex as the phage T7 can be synthesized in a test tube from its genome DNA opens broad possibilities to program and to interrogate synthetic biochemical systems *in vitro*. This bottom-up reductionist approach, which escapes the constraints of living

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organisms, may also find some applications in biotechnology and bioengineering. Our method, fundamentally different than the commercial T7 *in vitro* packaging mix, offers the possibility of doing purely *in vitro* evolution of phage genomes, perhaps even to select DNA and RNA polymerases that can grow in the presence of novel inhibitors (such as those that might kill the cell). Testing redesigned T7 DNA instructions would be a relevant extension to the present work,[21] in addition to the construction of entirely man-made synthetic circuits.[12] We point out that T7 DNA is certainly not the only natural DNA program that can be processed as described in this work. The phage ΦX174 is constructed from a 5.4-kbp genome encoding 13 proteins. ΦX174 has been synthesized *in vitro* using purified and preformed components.[24] With regard to this pioneering achievement, we thought that it would be interesting to pass the ΦX174 DNA software through the TX-TL hardware, even if the interaction of ΦX174 with *E. coli* is not completely known.[25] Using the same cell-free system, 1 million infectious ΦX174 phages were synthesized per milliliter of reaction (Figure 4, Supporting Figure S4). We note that a similar work

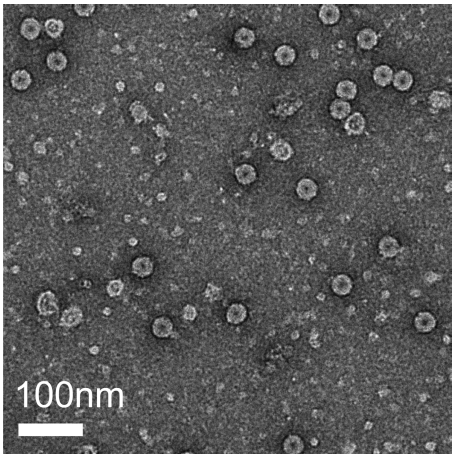

Figure 4. Cell-free synthesis of the phage ΦX174. The same cell-free TX-TL system used for the phage T7 was used to synthesize the phage ΦX174. Transmission electron microscopy (TEM) image of a cell-free reaction supplemented with ΦX174 double stranded genomes (30 nM). Magnification: 59000X.

was performed recently with a eukaryotic cell-free expression system. The encephalomyocarditis virus was reconstructed from cDNA in an *in vitro* transcription-translation system.[26]

METHODS

Cell-Free System and Reaction. The TX-TL cell-free system used in this study was prepared from the *E. coli* strain BL21 Rosetta2 (Novagen) according to a procedure described previously.[12] The cell-free reactions (CFRs) were composed of 33% (volume) crude extract and the other 66% (volume) of water, genomes and buffer with the following components: 50 mM HEPES pH 8, 1.5 mM ATP, 1.5 mM GTP, 0.9 mM CTP, 0.9 mM UTP, 0.2 mg/mL tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-phosphoglyceric acid, 2 mM DTT, 1.5 mM amino acids, 6.5 mM Mg-glutamate, 100 mM K-glutamate, and 2% PEG 8000. The CFRs were also supplemented with 3.3 μM concentration of the protein GamS to prevent degradation of linear DNA by the RecBCD complex. Cell-free expression systems prepared from the *E. coli* strains BL21 (*trxA*+) and BL21 *trxA*− were used for the thioredoxin part of the study. CFRs (10 μL) were incubated in 1.7 mL tubes at 29 °C for 12−18 h. The T7 genome (Boca Scientific Laboratory Products) and the ΦX174 genome (New England Biolabs) were directly added to the CFRs. The reporter gene eGFP, cloned under the *E. coli* sigma 70 regulatory part OR2-OR1-Pr,[12] was used for some control experiments. The gene encoding for thioredoxin was cloned under the same regulatory part.

Bacteriophage Titration and Gel Electrophoresis. The bacteriophages were counted by the standard plaque assay using the *E. coli* strain B for T7 and the *E. coli* strain C for ΦX174 (Carolina Biological Supply). The cells were grown in Luria−Bertani (LB) broth at 37 °C. The plates were prepared as follows: each sample was added to a solution composed of 3 mL of 0.55% liquid LB-agar (45 °C) and 0.2 mL of cell culture, poured on a 1.1% solid LB-agar plate. Plates were incubated at 37 °C and counted after 16 h.

Gel Electrophoresis, Radioactive Incorporation, and Transmission Electron Microscopy (TEM). DNA electrophoresis was carried out according to standard procedures. CFRs (10 μL) were run on 0.8% agarose gel (80 V, 2 h) stained with ethidium bromide. Prior to gel electrophoresis, the reactions were treated with 0.6 μM concentration of ribonuclease A (Sigma) at room temperature for 30 min, and with 0.1 mg/mL of proteinase K (Roche) at 62 °C for 30 min. Gels were analyzed with the software ImageJ. For radioactive labeling, the CFRs, supplemented with 2.5 μM concentration of 3H-thymidine (PerkinElmer), were incubated at 29 °C for 18 h. After incubation, the reactions were diluted 20-fold with water, mixed, and diluted 5-fold with 10% trichloroactetic acid (TCA). The samples were chilled on ice for 30 min and then filtered through GFC filters. Filters were washed twice with 1 vol of cold 10% TCA and twice with 1 vol of 95% ethanol and air-dried. Radioactivity was measured with a scintillation analyzer (PerkinElmer). TEM was performed with a FEI Tecna F30 (300 kV, negative staining, carbon-coated TEM grid).

ASSOCIATED CONTENT

Supporting Information

Supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.S. and P.J. designed and performed the experiments, analyzed the data, and edited the manuscript. V.N. guided the project, designed the experiments, discussed the data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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