Supporting Information

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SI Text

Plasmid Constructs. Plasmid pETORPHI68L was constructed designing oligonucleotides that, after hybridization, reconstitute the last 68 bp from the left end of Φ29 DNA plus an AATT extension to ligate to EcoRI sites; pETORPHI was digested with DraI and EcoRI, to eliminate the 194-bp right end of Φ29 DNA from the plasmid, and the 68-bp left end origin (made by hybridizing the oligonucleotides 68L and Comp68LEco, both phosphorylated) was cloned to replace the right end fragment. Plasmid pETORPHIBae was made by cutting pETORPHI with DraI and inserting a phosphorylated double-stranded oligonucleotide (oligonucleotides BaeIFwd and BaeIRev, see *Oligonucleotides List* below) that bears a recognition site for BaeI and places the cutting sites for that enzyme immediately adjacent to the Φ29 origins with respect to the 5′ strand while leaving a 5-nt extension at the 3′ strand (see Fig. 6*A*). Plasmid pEYFPBsm was made by creating a BsmBI recognition site (oligonucleotides BsmFwd and Rev; see *Oligonucleotides List*) at position 4702 of pEYFP-N1 (Clontech). Plasmid pEYFPORBae was made by PCR by amplifying a 982-bp DNA fragment from pETORPHI-Bae (with the oligonucleotides T7Univ and pETORPHIBsm; see *Oligonucleotides List*) comprising the Φ29 origins joint by the BaeI site and then cutting with the enzymes NdeI and BsmBI and cloning the corresponding restriction fragment between the BsmBI and NdeI sites of pEYFPBsm. Plasmid pETORPHI3-2 was prepared by cloning between the sites EcoRI and NcoI of pETORPHI, a DNA fragment amplified by PCR from the phage Φ29 genome corresponding to positions 1,112 to 3,687 (oligonucleotides p3Nco and p2Eco; see *Oligonucleotides List*) with the sites EcoRI and NcoI added. The Φ29 origins including the BaeI site were amplified by PCR with the oligonucleotides T7univ and T7term from plasmid pETORPHIBae and cloned between the sites NotI and SacI of pETORPHI3-2 to obtain pETORPHI3-2Bae. In a similar way, pETORPHI6-2 was prepared by cloning between the sites EcoRI and NcoI of pETORPHI, a DNA fragment containing from position 1,112 to 4,921 from the phage Φ29 genome (oligonucleotides p6Nco and p2Eco; see *Oligonucleotides List*). The Φ29 origins plus the BaeI site were PCR amplified from pETORPHIBae and cloned between the NotI and SacI sites of pETORPHI6-2 to generate pETORPHI6-2Bae.

Plasmid Multimerization. The plasmids pETORPHI3-2Bae and pETORPHI6-2Bae were multimerized by digestion with SacI in New Englad Biolabs (NEB) Buffer 1, inactivation of SacI by incubation for 20 min at 65 °C, ligation in the same buffer with ATP to 1 mM, inactivation again, digestion with BaeI in NEB Buffer 4 plus S-adenosylmethionine at 40 μM, and a final inactivation as before. The ligation after the SacI treatment generates three types of fragments with two origins at the ends, depending on the orientation of the plasmid ligation: "head to tail," "tail to tail," or "head to head"; these fragments are (see Fig. S5*A*), (*i*) the original plasmid cut with BaeI (pETORPHI3-2Bae, 8.4 kb, pETORPHI6-2Bae, 9.5 kb), (*ii*) a short fragment of 466 bp formed by the dimerizarion of the right origin between SacI and BaeI, and (*iii*) a dimer of the corresponding plasmid minus the 466 bp of the short fragment, which has 16.2 kb for pETORPHI3-2Bae and 18.6 kb for pETORPHI6-2Bae. The amplification reactions contained 25 ng of Φ29 terminal protein (TP)-DNA or pETORPHI, 50 ng of pETORPHI3-2Bae, or pETORPHI6-2Bae, both cut with BaeI, or a total of 50 ng of DNA from the restriction-ligation reactions with pETORPHI3-2Bae or pETORPHI6-2Bae.

Protein Purification. The TP was purified from BL21(DE3) *Escherichia coli* cells transformed with a TP-overproduction plasmid (1) derived from pT7-3 (2). The overproducing cells were incubated at 30 °C up to an OD_{600} of 0.9, then expression of the protein was induced by addition of IPTG to 0.5 mM and, after 45 min of induction, the culture was centrifuged, lysed by grinding with alumina, and resuspended in Buffer A [50 mM Tris·HCl (pH 7.5), 7 mM β-mercaptoethanol, 1 mM EDTA, 5% glycerol] supplemented with 0.8 M NaCl, utilizing 6 mL of buffer per gram of cells. After centrifugation, polyethyleneimine was added to the supernatant up to 0.3% (vol/vol) and the precipitated DNA was removed by centrifugation. The TP was precipitated by addition of ammonium sulfate between 35% and 70% saturation and the pellet was resuspended in Buffer A containing 0.2 M NaCl and was loaded onto a phosphocellulose Whatman P11 column; after washing with Buffer A with 0.4 M NaCl, the TP was eluted with Buffer A with 0.45 M NaCl. The sample was then diluted until it was 0.25 M NaCl and loaded on a DNA-cellulose column prepared as described (3); after washing with Buffer A with 0.3 M NaCl, the TP was eluted with Buffer A with 1 M NaCl. Then, ammonium sulfate was added to the sample to 70% saturation, followed by centrifugation, and the protein pellet was resuspended in Buffer A with 0.3 M NaCl and 50% glycerol. Gene 6 encoding protein p6 was PCR-amplified using primers p6N and p6R (*Oligonucleotides List*). The DNA fragment was cleaved with NdeI and PstI and cloned between the corresponding sites of the expression plasmid pT7-7 (2) to obtain pT7-7-p6. *E. coli* BL21 (DE3) cells harboring plasmid pT7-7-p6 were incubated at 37 °C and induced at an OD_{600} of 1.6 by adding IPTG at 0.5 mM final concentration, followed by a 30 min incubation, addition of rifampicin to 120 μg/mL, and another incubation of 90 min. The cell pellet was lysed by grinding with alumina and resuspended in Buffer A supplemented with 0.8 M NaCl, utilizing 6 mL of buffer per gram of cells. After centrifugation, polyethyleneimine was added to the supernatant up to 0.3% (vol/vol) and the precipitated DNA was removed by centrifugation. The supernatant was diluted with an equal volume of Buffer A containing 0.4 M NaCl plus 0.04% polyethyleneimine and, after centrifugation, the supernatant was discarded and the p6-containing pellet was resuspended with 0.4 mL/g of cells of Buffer A plus 1 M NaCl by stirring vigorously. The protein was precipitated with ammonium sulfate at 70% saturation and, after centrifugation, the pellet was resuspended in Buffer A supplemented to 25% glycerol and loaded onto a phosphocellulose Whatman P11 column. After washing with Buffer A plus 25 mM NaCl, protein p6 was eluted with Buffer A plus 100 mM NaCl, the sample was then diluted with the same volume of Buffer A, and it was loaded on a Q Sepharose column. This column was washed with Buffer A with 50 mM NaCl and p6 was eluted with Buffer A with 175 mM NaCl. The sample was again diluted with Buffer A to 50 mM NaCl, loaded on a new Q Sepharose column, eluted with a small volume of Buffer A with 1 M NaCl, and precipitated with ammonium sulfate at 70% saturation. After centrifugation, the protein pellet was resuspended in Buffer A with 50% glycerol.

Sequences of Multiple Cloning Sites (MCSs). The sequence of MCS1 is CTCGAGTGCGGCCGC AAGCTTGCATGCAGGCCTCTGCAGTCGAC with the unique restriction sites for XhoI, NotI, PstI, and SalI underlined. The sequence of MCS2 is GGTACCGAGCTC GAATTCGGATCCCGACCCATTTGCTGTCCACCAGTCATGCTAGCCATATG with the unique restriction sites for KpnI, SacI, EcoRI, NheI, and NdeI underlined.

Oligonucleotides List. -68L
AAAGTAAGCCCCCACCCTCACATGATACCATTCTCCT-AATATCGACATAATCCGTCGATCCTCGGCAT
-Comp68LEco
AATTATGCCGAGGATCGACGGATTATGTCGATATTAG-GAGAATGGTATCATGTGAGGGTGGGGGCTTACTTT
-T7Univ
TAATACGACTCACTATAGGG
-T7term
TAATACGACTCACTATAGGG
-BsmFwd
GTTATCCCCTGCGTCTCTGGATAACCGTAT
-BsmRev
ATACGGTTATCCAGAGACGCAGGGGATAAC
-pETORPHIBsm
TAGCAGCCGCGTCTCAGGATTGGTGGTGGT
-Comp68LBsm
ATCCATGCCGAGGATCGACGGATTATGTCGATATTA-GGAGAATGGTATCATGTGAGGGTGGGGGCTTACTTT
-Comp68LEco109
[Phos]GCCATGCCGAGGATCGACGGATTATGTCGATA-TTAGGAGAATGGTATCATGTGAGGGTGGGGGCTTACT-TT
-BaeIFwd
[Phos]TCTCCTAATTATTTGACTGTCGTATCTGTCATCA-ACCA
-BaeIRev
[Phos]TGGTTGATGACAGATACGACAGTCAAATAATT-AGGAGA
-p6Nco
GGGACGAAGCCATGGCAAAAATGATG
-p3Nco
GGAGATAACGCACCATGGCGAGAAGTCC
-p2Eco
CTTCTCTTGATCGAAGAATTCACCCATTAC
-p6N
GTGGGACGAACATATGGCAAAAATGATGCAGAGAG
-p6R
CCCTCCTGCAGTCATTCAGCAACCTGTTCTTC
[Phos] indicates 5′ phosphorylation.

1. Longás E, Villar L, Lázaro JM, de Vega M, Salas M (2008) Phage Φ29 and Nf terminal protein-priming domain specifies the internal template nucleotide to initiate DNA replication. *Proc Natl Acad Sci USA* 105:18290–18295.
2. Tabor S, Richardson CC (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* 82:1074–1078.
3. Litman RM (1968) A deoxyribonucleic acid polymerase from *Micrococcus luteus (Micrococcus lysodeikticus)* isolated on deoxyribonucleic acid-cellulose. *J Biol Chem* 243:6222–6233.

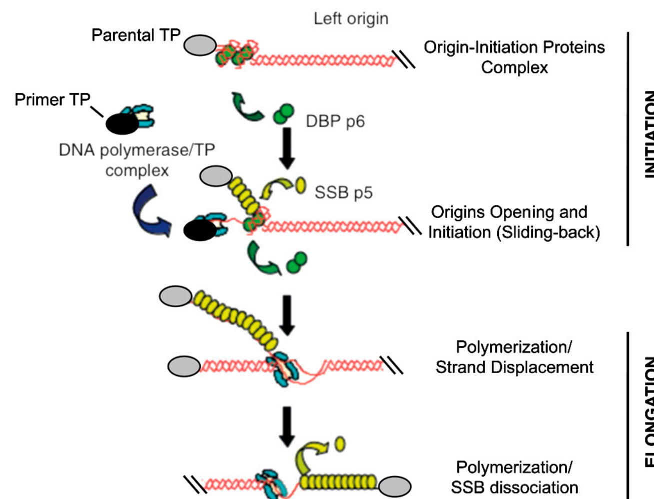

Fig. S1. Schematic representation of the mechanism of protein-primed Φ29 DNA replication (figure modified from ref. 1). Primer and parental TP are shown in black and gray, respectively; DNA polymerase is shown in blue. Protein p5 (single-stranded DNA binding protein, SSB) and p6 (double-stranded DNA binding protein, DBP) are shown in yellow and green, respectively. The different stages of replication are indicated at the right part of the figure.

1 Blanco L, Lázaro JM, de Vega M, Bonnin A, Salas M (1994) Terminal protein-primed DNA amplification. *Proc Natl Acad Sci USA* 91:12198–12202.

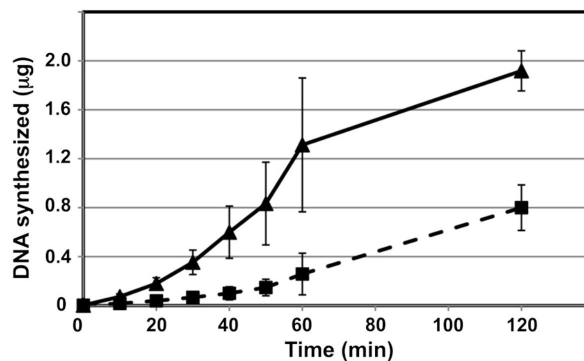

Fig. S2. Amplification kinetics of TP-DNA and pETORPHI. The amounts of synthesized DNA obtained using as template TP-DNA (▲) or pETORPHI (■) are represented in the y axis as a function of time. The synthesized DNA was calculated as described in *Materials and Methods*. The error bars correspond to the standard deviations from three independent experiments.

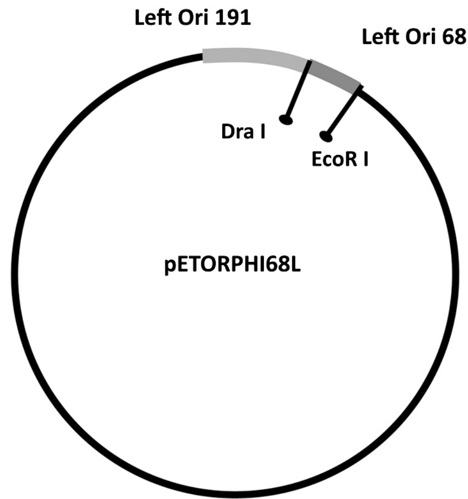

Fig. S3. Plasmid pETORPHI68L was constructed cutting pETORPHI with DraI and EcoRI to excise the 194-bp fragment corresponding to the right end of the Φ29 genome and substituting it by a DNA fragment comprising the 68 bp from the left end of the Φ29 genome.

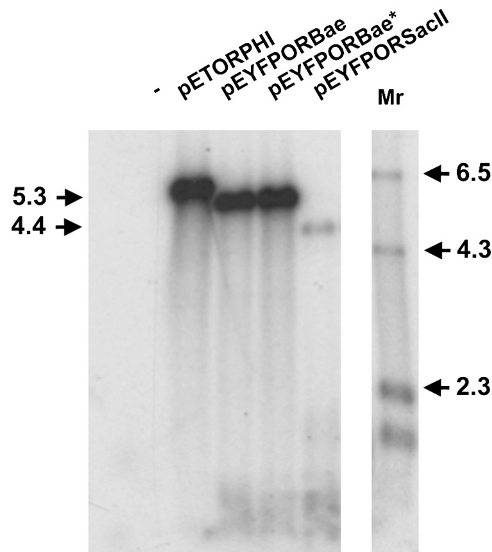

Fig. S4. Amplification of pEYFPORBae linearized with BaeI requires two Φ29 DNA ends in the same molecule. Plasmid pEYFPORBae was cut with BaeI (pEYF-PORBae), and then set in a reaction without (pEYFPORBae*) or with SacII (pEYFPORBaeSacII). Digestion with SacII of pEYFPORBae cut with BaeI was performed in New England Biolabs Buffer 4 for 6 h at 37 °C. After restriction enzyme heat inactivation, the DNAs were used as templates in amplification reactions. The size of the band corresponding to the plasmid cut with BaeI is 5.3 kb and the cut with BaeI plus SacII generates 4.4- and 0.9-kb bands.

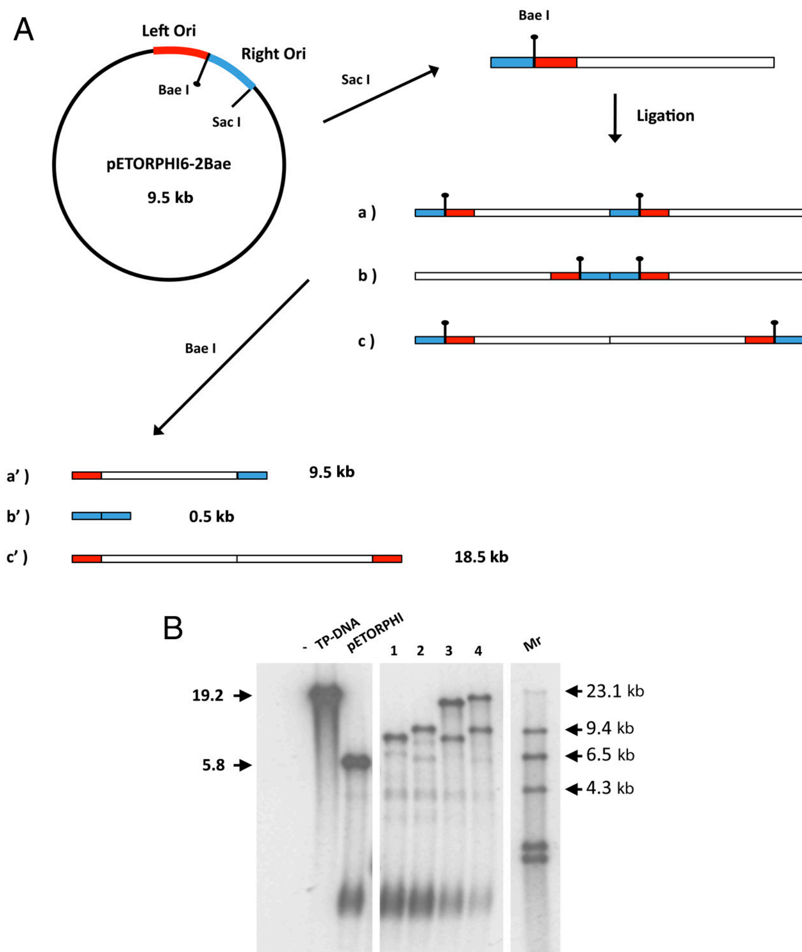

Fig. S5. The Φ29 DNA replication system can amplify DNA of 18 kb. (*A*) Ligation reactions were performed as described in *SI Text*. Plasmid pETORPHI6-2Bae is depicted as example. The left (red rectangles) and right (blue rectangles) Φ29 DNA replication origins and the BaeI and SacI restriction site positions are shown. The linear DNA fragments after digestion with SacI and ligation (limited to two plasmid copies, as examples [a, b, c]) are represented. The DNA fragments containing two Φ29 origins (a′, b′, c′), derived from each of the ligation products after cutting with BaeI are also depicted. (*B*) The amplification reactions contained no DNA (–), 25 ng of Φ29 TP-DNA (TP-DNA) or pETORPHI, 50 ng of pETORPHI3-2Bae (lane 1) or pETORPHI6-2Bae (lane 2), both cut with BaeI, or a total of 50 ng of DNA from the restriction-ligation reactions with pETORPHI3-2Bae (lane 3), or pETORPHI6-2Bae (lane 4). The reactions were performed under the standard conditions and stopped after 2 h at 22°. The lambda phage DNA cut with HindIII is shown as size marker. The different DNA sizes expected are explained in *SI Text*.

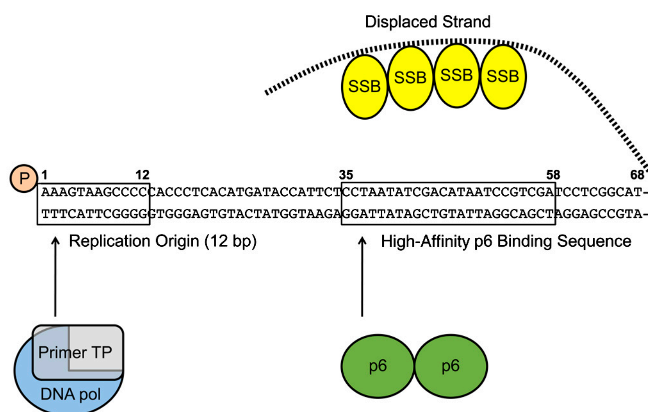

Fig. S6. Φ29 DNA origin requirements for in vitro amplification (see main text). The Φ29 DNA 68-bp left end origin is shown. The 12 bp of the very end (rectangle) are required for the initiation and elongation reactions performed by the Φ29 DNA polymerase that forms part of the heterodimer primer TP-DNA polymerase (1) (primer TP in gray, DNA polymerase in blue). The phosphate group at the 5′ end (P, orange circle) has been shown to be essential for in vitro amplification. The protein p6 (green) high-affinity binding sequences (rectangle at positions between 35 and 58) are necessary for stimulation of initiation by this protein (2). The binding of single-stranded DNA binding protein (SSB, yellow) to the displaced DNA strand (discontinuous line) is required for amplification (3).

1 Gutiérrez J, Garmendia C, Salas M (1988) Characterization of the origins of replication of bacteriophage Φ29 DNA. *Nucleic Acids Res* 16:5895–5914.
2 Serrano M, Gutiérrez J, Prieto I, Hermoso JM, Salas M (1989) Signals at the bacteriophage Φ29 DNA replication origins required for protein p6 binding and activity. *EMBO J* 8:1879–1885.
3 Blanco L, Lázaro JM, de Vega M, Bonnin A, Salas M (1994) Terminal protein-primed DNA amplification. *Proc Natl Acad Sci USA* 91:12198–12202.