1-1-2018

Design of DNA rolling-circle templates with controlled fork topology to study mechanisms of DNA replication

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Abstract

Rolling-circle DNA amplification is a powerful tool employed in biotechnology to produce large from small amounts of DNA. This mode of DNA replication proceeds via a DNA topology that resembles a replication fork, thus also providing experimental access to the molecular mechanisms of DNA replication. However, conventional templates do not allow controlled access to multiple fork topologies, which is an important factor in mechanistic studies. Here we present the design and production of a rolling-circle substrate with a tunable length of both the gap and the overhang, and we show its application to the bacterial DNA-replication reaction.

Disciplines

Medicine and Health Sciences

Publication Details

Monachino, E., Ghodke, H., Spinks, R. R., Hoatson, B. S., Jergic, S., Xu, Z., Dixon, N. E. & van Oijen, A. M. (2018). Design of DNA rolling-circle templates with controlled fork topology to study mechanisms of DNA replication. Analytical Biochemistry, 557 42-45.

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Conflict of interest: none

Abstract

Rolling-circle DNA amplification is a powerful tool employed in biotechnology to produce large from small amounts of DNA. This mode of DNA replication proceeds *via* a DNA topology that resembles a replication fork, thus also providing experimental access to the molecular mechanisms of DNA replication. However, conventional templates do not allow controlled access to multiple fork topologies, which is an important factor in mechanistic studies. Here we present the design and production of a rolling-circle substrate with a tunable length of both the gap and the overhang, and we show its application to the bacterial DNA-replication reaction.

Keywords: Rolling-circle amplification, DNA replication, Replisome, Nucleic-acid biochemistry

Introduction

Rolling-circle amplification (RCA) refers to the synthesis of DNA using a circular, covalently-closed template strand (Fig. 1A). First identified as a natural mechanism for replication of the DNA of bacteriophages [1], RCA has proven to be extremely useful in many fields from those addressing important mechanistic questions concerning DNA replication [2–5] to applications in materials sciences, embracing biomedical and diagnostic technologies, DNA sequencing, and nanotechnology [6–10]. The success of RCA is largely due to its simplicity and robustness. Unlike the polymerase chain reaction (PCR), RCA is isothermal. Nicked plasmid [11] or circular single-stranded (ss) DNA molecules annealed to a complementary oligonucleotide [2,4] are commonly employed as rolling-circle substrates because they are easy to develop and enable processive replication.

Loading of a bacterial (5'–3') replicative helicase requires the use of a so-called tailed-form II DNA substrate (TFII-DNA; Fig. 1A); form II is a historical nomenclature for nicked, or relaxed, covalently-closed circular double-stranded plasmid or bacteriophage DNA, and helicase loading is facilitated by a 5'-unpaired single-stranded overhang. These substrates with a single-stranded overhang resemble the replication fork in a living cell, and make ideal templates for *in vitro* studies of DNA replication. Most often, TFII-DNA substrates have been created by primer extension by a

DNA polymerase of a tailed complementary oligonucleotide primer annealed to a closed-circular single-stranded DNA template such as a phage M13 derivative [3,12]. A disadvantage of this approach is that it does not allow control over the size of the ssDNA gap at the fork on the leading-strand template arm.

Alternatively, TFII-DNA substrates have been created using strand displacement DNA synthesis at sites of nicks on plasmid DNA templates, resulting in substrates lacking a gap at the fork, but with 5'-tails of variable lengths [13].

The inability to control fork topology and ssDNA gap sizes in either approach limits its utility and translatability in studying DNA replication mechanisms. For example, studies on forked linear DNA molecules have revealed that the length of both the gap and the 5' overhang greatly influences the loading of the *Escherichia coli* DnaB helicase in PriA- and PriC-mediated replication restart pathways [14,15]. Synthetic TFII mini-rolling circles have been created to overcome some of the limitations of the traditional approaches used for making RCA substrates. This approach combines the advantages of RCA with a fork topology that is fully defined by the user, even at the sequence level [16–18]. However, the small size of these mini-rolling circles (70–100 bp) results in a very poor eukaryotic helicase loading efficiency [19], thus limiting their utility. This might be due to the strong rigidity of short double-stranded (ds) DNA segments and the consequently high topological strain in mini-rolling circles [6].

Here we report a quick, efficient and generalizable method to create substrates for the study of DNA replication on rolling-circle templates with control of gap size as well as length of overhang, with single-nucleotide accuracy (Fig. 1B). We used the plasmid pSCW01 (2030 bp) [20] to develop a rolling-circle template for use in *in vitro* studies of DNA replication. Briefly, the Nt.*Bst*NBI nickase recognizes and introduces nicks at four sites on the same strand in the pSCW01 plasmid in a 37-nt-long region. The three nicked oligonucleotides are displaced by heating at 85°C to obtain a 37-nt-long single-stranded region. A partially complementary fork oligonucleotide is then annealed to generate a gap and an overhang, whose lengths are both controllable. In the final step, the fork oligonucleotide is ligated to the gapped plasmid, yielding a TFII-DNA substrate with the desired fork topology.

Material and Methods

Materials

We used the following reagents:

Chemicals: acetic acid, glacial (Ajax Finechem), agarose (Bioline), ATP (Sigma-Aldrich), dNTPs (dATP, dCTP, dGTP, dTTP) (Bioline), dithiothreitol (Astral Scientific), EDTA (Ajax Finechem), ethanol (Chem-Supply), ethidium bromide (Amresco), HCl (Ajax Finechem), potassium glutamate (Sigma-Aldrich), $MgCl_2$ (Ajax Finechem), $Mg(OAc)_2$ (Sigma-Aldrich), Na_2EDTA (Ajax Finechem), PEG-8000 (Sigma-Aldrich), SDS (Sigma-Aldrich), Tris (Astral Scientific), Tween-20 (Sigma-Aldrich);

DNA Purification kits: QIAGEN Spin Miniprep kit;

Gel Electrophoresis: 6x DNA Gel Loading Dye (ThermoFisher Scientific), GeneRuler DNA Ladder mix (ThermoFisher Scientific), 10,000x SybrGold (Life Technologies);

DNA replication proteins from *E. coli* (purified according to previously published procedures): $\chi\psi\tau_3\delta\delta$' clamp loader complex [21], β_2 clamp [22], and co-purified $DnaB_6/C_6$ helicase/helicase loader complex and the DNA Pol III $\alpha\varepsilon\theta$ polymerase core [4], with the α subunit purified according to [23];

Restriction enzymes and ligase (New England Biolabs): *Bam*HI-HF (R3136S), *Nco*I (R0193S), Nt.*Bst*NBI (R0607L), *Pst*I-HF (R3140S), T4 DNA ligase (M0202L);

Buffers: NEB buffer 3.1 (50 mM Tris.HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mg/mL BSA), NEB CutSmart buffer (20 mM Tris-acetate pH 7.9, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 0.1 mg/mL BSA), replication buffer (30 mM Tris.HCl pH 7.6, 12 mM $Mg(OAc)_2$, 50 mM potassium glutamate, 0.5 mM EDTA, 0.025% (*v*/*v*) Tween-20, 10 mM dithiothreitol), LES buffer (2x DNA Gel Loading Dye, 200 mM EDTA, 2% (*w*/*v*) SDS), TE buffer (10 mM Tris.HCl pH 7.6, 1 mM EDTA), Tris acetate EDTA buffer (TAE; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3).

Oligonucleotide sequences

Oligo 1: 5'-ATT TGA CTC C
Oligo 2: 5'-CAT GGA CTC GCT GCA G
Oligo 3: 5'-GAA TGA CTC GG
Oligo 4: 5'-AAA AAA AAA AAA AAA AGA GTA CTG TAC GAT CTA GCA TCA ATC ACA GGG TCA GGT TCG TTT GGG AGT CAA AT
Oligo 5: 5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GGG AGT CAA AT

Oligos 1, 2, 3, and 5 were purchased from Integrated DNA Technologies, USA. Oligo 4 was purchased from GeneWorks, Australia.

Leading-strand synthesis bulk assay

Rolling-circle DNA template (3.8 nM) was incubated with 1 mM ATP, 125 μM dNTPs, 30 nM $\chi\psi\tau_3\delta\delta$', 90 nM $\alpha\varepsilon\theta$, 200 nM β_2, 60 nM $DnaB_6/C_6$ at 37°C in replication buffer. Replication was terminated by mixing equal volumes of replication mixture with LES buffer.

Gel electrophoresis

Ethidium bromide-stained gels:

Agarose gels (1% *w*/*v*) were cast with 0.8 μg/mL ethidium bromide. Electrophoresis in 1x TAE buffer was at 82 V for 85 min in a Wide Mini-Sub Cell GT System (Bio-Rad). DNA was visualized using a Bio-Rad Gel Doc XR (302 nm trans-UV light).

Cy5-labeled DNA gels:

Cy5-labeled DNA products were loaded in 1% (*w*/*v*) agarose gels and separated in 2x TAE buffer at 82 V for 85 min in a Mini-Sub Cell GT System (Bio-Rad). The Cy5 signal was detected with a GE Healthcare Life Science "Amersham Imager 600RGB" (630 nm light). The DNA molecules were stained with 1x SybrGold in 2x TAE buffer for 2 h and then detected with a Bio-Rad Gel Doc XR (302 nm trans-UV light).

SybrGold-stained gels:

Agarose gels (1% *w*/*v*) were run in 2x TAE buffer at 60 V for 150 min in a Wide Mini-Sub Cell GT System (Bio-Rad). The gel was stained after electrophoresis with 1x SybrGold in 2x TAE buffer for 2 h. The SybrGold-stained DNA molecules were detected with a Bio-Rad Gel Doc XR (302 nm trans-UV light).

Protocol

We adapted previously published protocols that use the pSCW01 plasmid [20,24]. Plasmid pSCW01 was maintained in *E. coli* DH5α cells. A freezer stock was streaked on LB-agar plates containing 100 μg/mL of ampicillin. A single colony of DH5α/pSCW01 was amplified in a 3 mL culture in LB broth and grown for 8 h at 37°C. LB (100 mL) supplemented with 100 μg/mL ampicillin was inoculated with 0.1 mL of overnight culture and grown for 12 h. Cells were pelleted by centrifugation at 3,000 x *g* for 20 min at 6°C. Pellets (~1.6 g from 100 mL culture) were flash frozen and stored at –80°C until further use. Plasmid DNA was isolated from the cell pellets using QIAGEN Spin Miniprep columns. Typically 60 μg of DNA were obtained for each gram of cells; 100–200 μg of pSCW01 were treated with 1.5 units/μg of Nt.*Bst*NBI and 100x molar excess of displacer oligonucleotides complementary to the fragments to be removed to create the gap (Oligos 1, 2, 3) in 1x NEB buffer 3.1 at 55°C for 4 h. The nickase was inactivated according to manufacturer's instruction by heating at 85°C for 10 min. Following this, displacer oligos were annealed in a thermal cycler at a cooling rate of 1°C/min until the reaction reached 12°C. Excess displacer oligonucleotides were purified away from the gapped plasmid by PEG purification [20]. Specifically, an equal volume of a freshly made 2x solution containing 26% (*w*/*v*) PEG-8000 and 20 mM $MgCl_2$ in Milli-Q water was added to the cooled reaction mixture containing the DNA and centrifuged at 6°C for 1 h at 21,000 x *g*. The supernatant was discarded and the pellet was gently resuspended and washed with 1.5 mL of 70% (*v*/*v*) ethanol followed by centrifugation at 6°C for 15 min at 21,000 x *g*. Finally, the gapped plasmid (≥60% yield efficiency) was resuspended in previously warmed (65°C) Milli-Q water to a concentration of 500 μg/mL.

In the next step, the fork oligonucleotide (Oligo 4) was annealed to the gapped substrate. Annealing was performed in the presence of a three-fold molar excess of fork oligo over DNA substrate in 1x CutSmart buffer at 50°C for 10 min, followed by slow cooling to 16°C. The fork oligonucleotide is a 71-mer ssDNA molecule with a 12-nt 3'-sequence complementary to pSCW01. Hybridization to the gapped pSCW01 plasmid results in a 25-nt gap. Next, ligation was performed by addition of 62.5 units of T4 DNA ligase per μg of DNA substrate in the reaction mixture supplemented with 8 mM ATP and 10 mM dithiothreitol, followed by incubation at 16°C for 18 h. The ligase was then inactivated according to manufacturer's instruction by heating at 65°C for 10 min. Finally, the rolling-circle substrate was purified by precipitation with PEG (as before), resuspended in Milli-Q water and stored at –20°C. For long-term storage, the DNA substrates are resuspended in TE buffer.

Validation

Prior to use in a rolling-circle DNA replication assay, the DNA substrate was assayed to verify efficiency of gap creation and ligation of the fork oligonucleotide. First, the efficiency of gap creation was assayed by restriction digestion using *Bam*HI-HF, *Pst*I-HF, and *Nco*I (see Fig. 2A). These restriction endonucleases digest the pSCW01 plasmid at single sites (Fig. 2A; lanes 2–4) in the region destined to yield the gap, and all three sites are lost when the plasmid has been successfully

nicked by Nt.*Bst*NBI. Annealing and ligation of the fork Oligo 4 does not restore any of the restriction sites. As expected, none of the three restriction enzymes digest the gapped pSCW01 (not shown) or the TFII DNA substrate (Fig. 2A; lanes 5–8). Efficiency of gap creation was calculated by measuring the intensity of the bands corresponding to the linearized and untreated DNA substrate in ethidium bromide-stained agarose gels. Efficient gapping resulted in an undetectable band corresponding to the linearized DNA template (Fig. 2A).

We then performed a parallel ligation reaction in every batch using a 5'-Cy5 modified fork oligo (5Cy5 Oligo 5) to create a DNA substrate termed 'FluoRC'. To measure the efficiency of ligation, we ran four different samples on an agarose gel and imaged the gel using the Amersham RGB imager to detect DNA containing the Cy5 label (Fig. 2B, magenta), followed by staining the gel with SYBR-gold to detect non-fluorescently modified DNA (Fig. 2B, green). pSCW01 substrate (lane 1) shows the migration of the super coiled and nicked plasmids. Sample containing gapped pSCW01 annealed to the 5Cy5 Oligo 5 (lane 2) exhibited a fluorescent band that migrates at the same position as 5Cy5 Oligo 5 control (lane 4) and a higher band that is consistent with the migration of the gapped pSCW01 substrate. On the other hand, the ligation reaction exhibits a shift in the migration of the Cy5 containing oligo, consistent with the formation of the ligated fork template (lane 3). Greater than 90% Cy5 signal overlaps with the relaxed DNA signal (lane 3).

Finally, to assess the efficiency of the DNA substrate as a rolling-circle template, we examined its utilization in a DNA replication assay. In this experiment, we used the subset of proteins from the *E. coli* replisome that are necessary and sufficient for performing leading-strand synthesis. Under these conditions, we observed products that are several tens of thousands of nucleotides long [2,5,6,9], with 75% of the original template being consumed after 60 min (Fig. 2C).

In summary, we present a straightforward, customizable and efficient strategy to create RCA templates with defined fork topology. This strategy can be exploited to optimize experimental conditions and can prove very valuable especially in single-molecule experiments, where a high throughput allows a better characterization of subpopulations, transient states, and rare events [23,25].

Figure

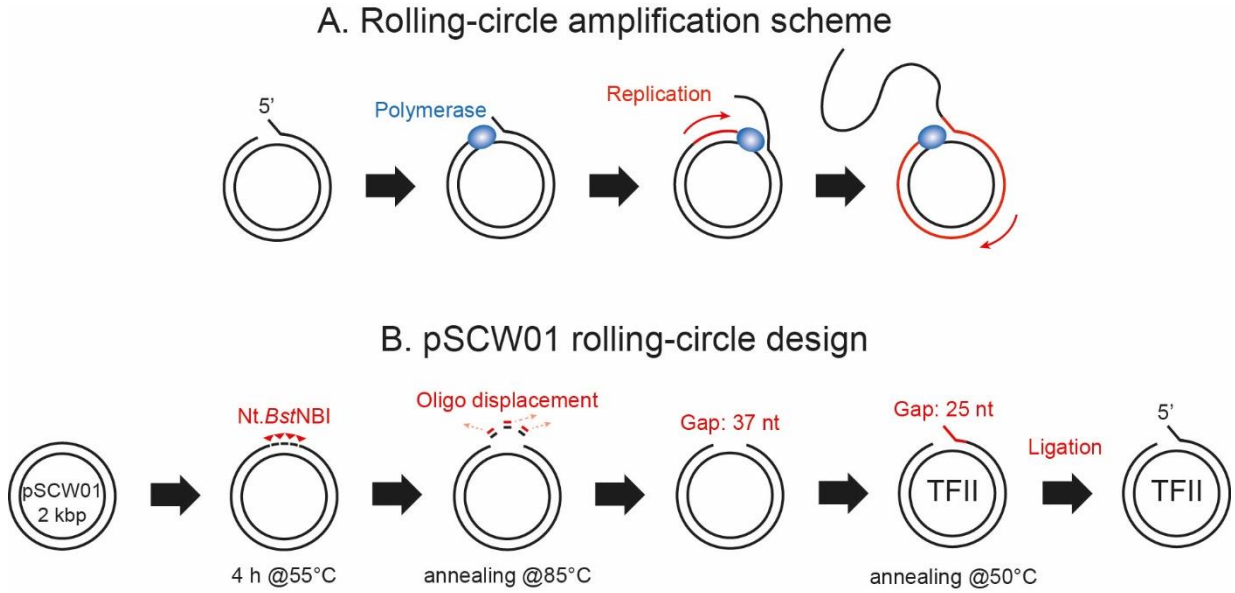

Fig. 1: pSCW01 plasmid conversion into a rolling-circle TFII-DNA template

(A) Rolling-circle amplification scheme. The internal strand serves as template for the leading strand. In this way, the template can be replicated perpetually; (B) pSCW01 rolling-circle design. The TFII-

DNA substrate is obtained through nicking of the pSCW01 plasmid, creation of a ssDNA gap, annealing and ligation of a partially complementary fork oligonucleotide.

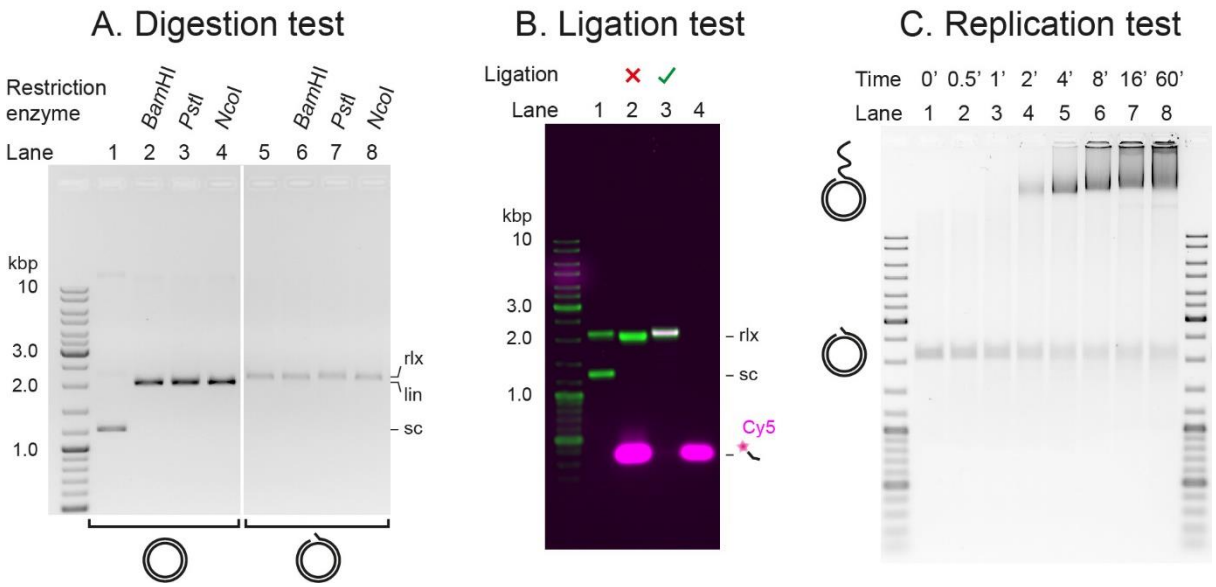

Fig. 2: Validation

(A) Digestion test. Plasmid and form TFII pSCW01 were treated with restriction endonucleases and separated in a 1% agarose gel. Plasmid pSCW01 (2.03 kb) migrates faster (lane 1) because it is supercoiled (sc; form I). After linearization with *Bam*HI, *Pst*I, or *Nco*I (linear; marked "lin"), it migrates as expected at 2 kb (lanes 2–4). Form TFII pSCW01 migrates slower than linear pSCW01 (lane 5) because it is no longer supercoiled (i.e., it is relaxed; marked "rlx"), but it is still circular. *Bam*HI, *Pst*I, and *Nco*I recognition sequences are completely or partially overlapping with the 25-nt gap of pSCW01. Therefore, these restriction enzymes no longer cleave the TFII pSCW01 template or affect the way the DNA migrates (lanes 6–8); (B) Ligation test. A sample of not-ligated (lane 2) and of ligated (lane 3) 5'-Cy5 labeled TFII pSCW01 were run in a 1% agarose gel. Only after ligation, we obtained that ≥90% Cy5 signal overlapped with the relaxed DNA signal. As controls for the migration of the DNA molecules, we ran a mixed sample of supercoiled and relaxed pSCW01 plasmid in lane 1 and a sample of the Cy5-labeled fork oligo in lane 4; (C) Replication test. A leading-strand synthesis experiment was carried out using TFII pSCW01 and *E. coli* proteins. The reaction was terminated after 0, 0.5, 1, 2, 4, 8, 16, 60 min of incubation and the reaction products were separated in a 1% agarose gel (lanes 1–8, as shown).

Acknowledgements

E.M., H.G., and A.M.v.O. thank Drs Peggy Hsieh (NIH) and Bennett Van Houten (University of Pittsburgh) for providing the pSCW01 plasmid. This work was supported by the Australian Research Council: DP150100956 and DP180100858 to A.M.v.O and N.E.D., an Australian Laureate Fellowship to A.M.v.O. (FL140100027), and Netherlands Organisation for Scientific Research (NWO) for E.M. (12CMCE03).

Authors contribution

E.M., H.G., and A.M.v.O designed the DNA substrate and wrote the manuscript draft; E.M., R.R.S., and B.S.H. produced and validated the DNA substrate; S.J. and Z-Q.X. provided methods and reagents; E.M., H.G., N.E.D., and A.M.v.O finalized the manuscript.

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