Analytical Biochemistry 557 (2018) 42–45

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

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

Enrico Monachino[a,b], Harshad Ghodke[a], Richard R. Spinks[a], Ben S. Hoatson[a], Slobodan Jergic[a], Zhi-Qiang Xu[a], Nicholas E. Dixon[a], Antoine M. van Oijen[a,*]

[a] Molecular Horizons and School of Chemistry and Molecular Bioscience, University of Wollongong, and Illawarra Health and Medical Research Institute, Wollongong, New South Wales, 2522, Australia
[b] Zernike Institute for Advanced Materials, University of Groningen, 9747 AG, Groningen, The Netherlands

ARTICLE INFO

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

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.

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-

* Corresponding author.
E-mail address: vanoijen@uow.edu.au (A.M. van Oijen).

https://doi.org/10.1016/j.ab.2018.07.008
Received 6 April 2018; Received in revised form 12 July 2018; Accepted 13 July 2018

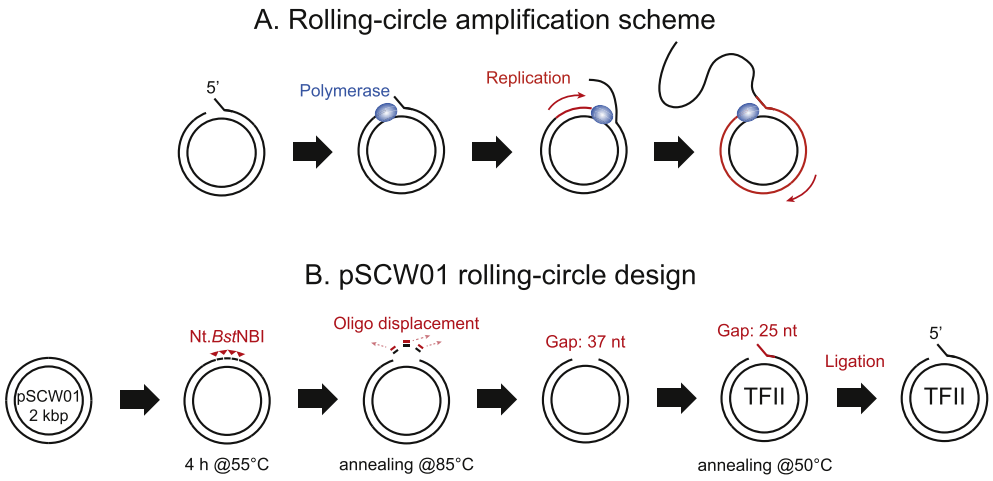

Fig. 1. pSCW01 plasmid conversion into a rolling-circle TFII-DNA template.
(A) Rolling-circle amplification scheme. The internal strand serves as template for the leading strand. In this way, the template can be replicated perpetually; (B) pSCW01 rolling-circle design. The TFII-DNA substrate is obtained through nicking of the pSCW01 plasmid, creation of a ssDNA gap, annealing and ligation of a partially complementary fork oligonucleotide.

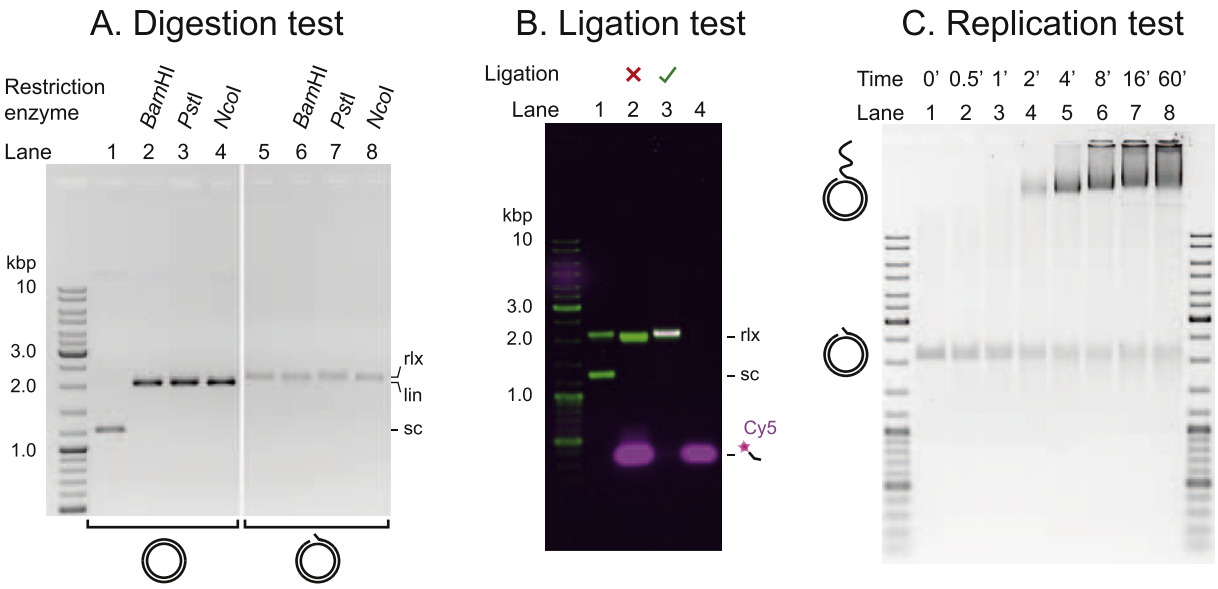

Fig. 2. Validation.
(A) Digestion test. Plasmid and form TFII pSCW01 were treated with restriction endonucleases and separated in a 1% agarose gel. Plasmid pSCW01 (2.03 kb) migrates faster (lane 1) because it is supercoiled (sc; form I). After linearization with *Bam*HI, *Pst*I, or *Nco*I (linear; marked "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).

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: 6× DNA Gel Loading Dye (ThermoFisher Scientific), GeneRuler DNA Ladder mix (ThermoFisher Scientific),