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Brief report

Construction of a circular single-stranded DNA template containing a defined lesion

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ABSTRACT

We report a concise and efficient method to make a circular single-stranded DNA containing a defined DNA lesion. In this protocol, phagemid DNA containing Uracil is used as a template to synthesize a complementary DNA strand using T7 DNA polymerase and an oligonucleotide primer including a site-specific DNA lesion. The ligated lesion-containing strand can be recovered after the phage-derived template DNA is degraded by treatment with *E. coli* Uracil DNA glycosylase and Exonucleases I and III. The resulting product is a circular single-stranded DNA containing a defined DNA lesion suitable for *in vitro* translesion replication assays.

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1. Introduction

Replicative DNA polymerases are unable to bypass bulky lesions in DNA. As a result, the replication fork may collapse leading to cell cycle arrest and possibly cell death. To avoid such deleterious consequences, organisms possess specialized DNA polymerases that can circumvent DNA lesions in a process referred to as "Translesion DNA synthesis" (TLS). *Escherichia coli* possesses three TLS polymerases and humans possibly as many as ten TLS polymerases [1]. The biological importance of TLS is typified by *E. coli* polV, which is responsible for the majority of mutagenic TLS *in vivo* [2] and DNA polymerase η (polη), which protects humans from UV-induced cancers by accurately replicating past *cis–syn* cyclobutane pyrimidine dimers (CPDs). Indeed, defects in human *POLH,* encoding polη, lead to the sunlight-sensitive and cancer-prone *xeroderma pigmentosum* variant (XP-V) syndrome [3,4].

The TLS polymerases gain access to a nascent primer terminus via an interaction with the cell's replicative, ring-shaped, clamp (β-clamp in *E. coli* and PCNA in eukaryotes). The process is initiated by a clamp loader (γ-complex in *E. coli* and replication factor C in eukaryotes), which recognizes the DNA primer terminus and opens and assembles the clamp around the nascent DNA [5]. Each clamp has two (prokaryotes), or three (eukaryotes) potential DNA polymerase binding sites and may, therefore, engage multiple polymerases simultaneously. Indeed, such interactions are believed to be critical for switching between replicative and TLS polymerases [6].

In vitro studies investigating the effects of the replicative clamps on TLS have been hampered because the clamps readily slide off of linear DNA substrates. One option is to cap the DNA ends using large biomolecules such as Streptavidin beads linked to biotinylated oligonucleotides. However, this imposes large steric constraints and may affect the ability of the DNA polymerase to access the primer terminus. Circular, single-stranded templates are, therefore, more likely to provide more informative data on the effects of the replicative clamps on TLS and polymerase switching *in vitro*.

Protocols to generate such substrates have previously been reported by the Lawrence [7] and Fuchs laboratories [8]. Lawrence *et al.* used a long oligomer scaffold to anneal and subsequently ligate a short lesion-containing oligonucleotide into M13 DNA. Using a similar "scaffold" approach, Napolitano and Fuchs hybridized two related plasmids of slightly different sizes, so as to generate a gapped double-stranded substrate, to which the lesion-containing oligonucleotide was annealed and ligated. The non-adducted DNA strand was subsequently degraded, so as to generate a single-stranded lesion-containing substrate [8]. While both protocols achieve their desired goal, both are somewhat time consuming and include labor-intensive steps.

Abbreviations: pol, DNA polymerase; TLS, translesion DNA synthesis; XP-V, xeroderma pigmentosum variant.

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We were therefore interested in developing a protocol for the rapid and efficient purification of circular, single-stranded DNA containing a defined lesion. To achieve our goal, we took advantage of the methodology previously described by Kunkel *et al.* to generate site-directed mutations in DNA [9,10]. In the Kunkel protocol, a primer containing the desired mutation is annealed to a ssDNA template containing Uracil. After primer extension and ligation, the dsDNA is used to transfect *ung*$^+$ *E. coli* wherein the Uracil-containing DNA is degraded. The Uracil-free ssDNA is converted back into duplex DNA by host polymerases and in the process, the nucleotide change in the original oligonucleotide primer is fixed as a mutation *in vivo*. We have used a conceptually similar approach in our protocol, but instead of using a primer with a mutation, we used a primer containing a site-specific DNA lesion and instead of degrading the Uracil-containing template *in vivo*, it is degraded *in vitro* using the combined actions of *E. coli* Uracil DNA glycosylase and Exonucleases I and III. The final product is a circular, single-stranded DNA molecule containing a defined lesion that can be used for *in vitro* replication and repair assays.

2. Materials and methods

2.1. Reagents

E. coli DNA polymerase I (Klenow fragment) [pol I (Kf)], *E. coli* Exonuclease III, *E. coli* Exonuclease I, *E. coli* Uracil DNA glycosylase, *E. coli* RecA, T7 DNA polymerase, T4 Polynucleotide kinase, and M13KO7 helper phage were all purchased from New England Biolabs (Ipswich, MA). ATP was from Roche Applied Science (Indianapolis, IN). T4 DNA ligase and deoxyribose nucleoside triphosphates (dNTPs) were purchased from Invitrogen (Carlsbad, CA). Polyethylene glycol 6000 and 8000 was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. DNAs

The two 60mer oligonucleotides U60T and U60B were synthesized by Lofstrand Laboratories (Gaithersburg, MD). The *cis–syn* cyclobutane pyrimidine dimer (CPD)-containing oligonucleotide TTC48P was synthesized by Phoenix BioTechnologies (Hunstville, AL). pBluescript II KS(+) was purchased from Stratagene (San Diego, CA). The QIAquick PCR purification kit was purchased from QIAGEN (Valencia, CA).

3. Results

3.1. Construction of a single-stranded circular DNA containing a CPD lesion

The first step of the protocol is to clone a short insert into a double-stranded plasmid vector capable of producing single-stranded phagemid DNA, so that one can subsequently anneal a lesion-containing oligonucleotide to the phagemid-derived ssDNA. In our case, we synthesized two 60mer oligonucleotides, U60T (5′-AAT TCG ATT CGA TAC TGG TAC TAA TGA TTA ACG AAT TAA GCA CGT CCG TAC CAT CGA TCA-3′) and U60B (5′-AGC TTG ATC GAT GGT ACG GAC GTG CTT AAT TCG TTA ATC ATT AGT ACC AGT ATC GAA TCG-3′), which were annealed to each other and cloned into EcoRI–HindIII digested pBluescript II KS(+) to generate a 3010 bp plasmid, pAVR88.

pAVR88 was introduced into *E. coli* strain CJ236 (*dut*, *ung1*), which allows for high levels of dUMP incorporation into DNA. A fresh single colony of CJ236 harboring pAVR88 was inoculated into 200 ml of LB broth with 0.75 μg/ml of Uridine and incubated at 37 °C with aeration for several hours. When the culture became slightly turbid, 1×10^{10} pfu of M13KO7 helper phage was added and cultured for an additional 1 h prior to the addition of kanamycin (50 μg/ml) and subsequent overnight growth at 37 °C.

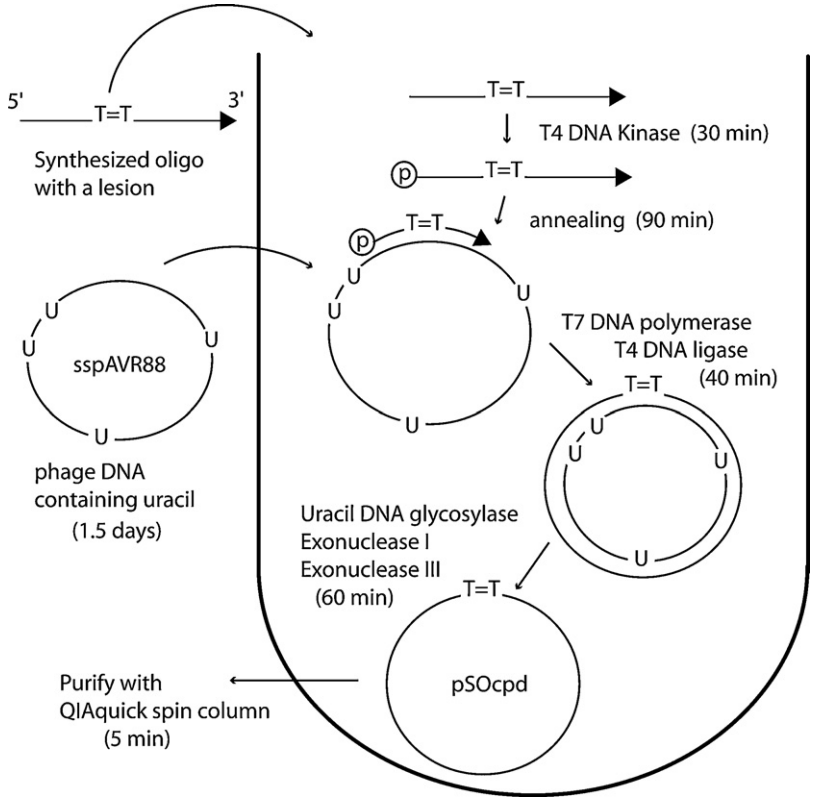

Fig. 1. Flow chart for the construction of pSOcpd. All reactions were performed in one tube, except for the preparation of phage DNA and the purification of the final product. The approximate time to complete each step is shown in parentheses.