ATP-independent Loading of the Proliferating Cell Nuclear Antigen Requires DNA Ends*

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The proliferating cell nuclear antigen (PCNA) is a processivity subunit for eukaryotic DNA polymerase δ. We present biochemical evidence that yeast PCNA likely adopts a toroidal structure containing an inside cavity through which double-stranded DNA slides. A comparative study of DNA replication reactions on circular versus linear model substrates shows that PCNA can only interact productively with DNA polymerase δ if the substrate is linear. This combined with the observation that a large molar excess of PCNA is required for maximal stimulatory activity is consistent with a model in which PCNA slips onto the end of the DNA in an ATP-independent manner.

Three DNA polymerases are essential for cell growth in the yeast Saccharomyces cerevisiae (1–4). Two of these, DNA polymerase δ (pol δ) and DNA polymerase ε, interact with an accessory factor, the proliferating cell nuclear antigen (PCNA), in order to effect processive DNA synthesis under physiological conditions (5–7). PCNA is functionally homologous to the β subunit of Escherichia coli DNA polymerase III holoenzyme. The structure of the β subunit has been determined by x-ray diffraction analysis (8). As a homodimer it resembles a torus with the dimensions of the circular middle cavity suitable for binding double-stranded B form DNA. The structure of the β subunit is consistent with its biochemical properties (9). Despite this functional homology, there is virtually no conservation at the sequence level between the β subunit and the eukaryotic PCNAs.

Efficient loading of PCNA at the primer terminus requires another replication factor, replication factor C (RF-C) and energy provided by the hydrolysis of ATP (7, 10–12). This complex can then interact with polymerase δ or ε and assemble into the respective holoenzymes. However, in the absence of RF-C or ATP, a productive interaction between DNA polymerase δ and PCNA can still be observed under special conditions. In particular, PCNA stimulates processive synthesis by pol δ on the homopolymeric template poly(dA) sparsely primed with oligo(dT). In fact, PCNA was isolated first from calf thymus, and later from yeast, using this assay system (5, 13). PCNA also effectively promotes replication by pol δ across ultraviolet light-induced thymine dimers present on model oligonucleotide templates, indicating a productive interaction in this system as well (14). In contrast, we and others have been unable to observe stimulation of pol δ by PCNA on primed circular single-stranded phage DNA templates, e.g. M13mp18, in the absence of RF-C or ATP (5, 15).

This paradox can be explained if PCNA, like the β subunit of E. coli DNA polymerase III holoenzyme, has a torus-like structure. Then, loading of PCNA at the primer terminus in such a way that the DNA is encircled by PCNA could proceed by two pathways. First, the PCNA homotrimer (5, 8) could (partially) dissociate and reassociate around the double-stranded DNA at or near the primer terminus (route A; see Fig. 1). Presumably, RF-C loads PCNA in this fashion by partial opening of the homotrimer in an ATP-dependent step. Alternatively, in the case of linear DNA templates such as poly(dA)–oligo(dT) or oligonucleotides, PCNA could diffuse onto the DNA at one end followed by sliding over the double-stranded DNA to a primer terminus site (route B). Such a mechanism would be inoperable with circular DNA templates such as M13mp18.

Here we present evidence that, in the absence of RF-C and ATP, PCNA loads onto the DNA via a double-stranded end (route B) and, based on this mode of loading, PCNA probably has a toroidal structure.

EXPERIMENTAL PROCEDURES

Materials—All enzymes and proteins were as described previously (6).

DNA Substrates—A 44-mer oligonucleotide was hybridized to circular single-stranded mp18 DNA preparations typically contained <20% linear molecules) at nucleotide positions 6278–6235. The primed DNA was then cut with HincII at position 6267, XbaI at 6259, BamHI at 6253, or KpnI at 6248, whereas control DNA templates were treated similarly without restriction enzyme. Cutting was confirmed by agarose gel electrophoresis and was >80% complete for all digests except KpnI, which proceeded for about 40%. After addition of EDTA to 10 mM, the substrates were purified by phenol extraction followed by several ether backwashes. Residual ether was removed in a SpinVac. Since hybridization and restriction cutting were performed at a 0.5–2 mg/ml phase DNA concentration and the final DNA concentration in the assays was about 3 μg/ml, carryover of buffer components and EDTA into the assay was negligible.

Assays—Replication assays (30 μl) contained 40 mM Tris-HCl, pH 7.8, 8 mM magnesium acetate, 0.2 mg/ml bovine serum albumin, 4% (v/v) polyethylene glycol 8000, 1 mM dithiothreitol, 80 μM each of dATP, dCTP, and dGTP, and 100 μM of [α-32P]dTP. 100 ng of primed single-stranded (SS) circular or HincII-cut mp18 DNA (0.04 pmol of template molecules), 1.3 μg of yeast RF-A, 380 ng (4.5 pmol) of PCNA (as indicated), and 15 ng (about 0.06 pmol) of pol δ. Variations are indicated in the legends to the figures. Reactions containing RF-C (20 ng) also contained 0.5 mM ATP, and the polyethylene glycol was omitted. Reactions were incubated at 37 °C for 5 min or as indicated, then stopped with EDTA to 10 mM and sodium dodecyl sulfate to 0.2%. One half of the reaction was acid-precipitated to quantify DNA synthesis and the other half was analyzed by alkaline agarose gel electrophoresis (16).

RESULTS AND DISCUSSION

Single-stranded circular mp18 DNA was primed with a 44-mer oligonucleotide at the polylinker region and coated with single-stranded binding protein. This substrate was incubated with pol δ in the presence of [α-32P]dNTPs and with increasing amounts of PCNA. The products were separated on an
alkaline agarose gel and visualized by autoradiography. The data in Fig. 2 show that no increase in processive DNA synthesis was observed. In fact, at very high levels of PCNA, a marked inhibition occurred (Fig. 2, lanes 6 and 7). This was probably due to a non-productive protein-protein interaction between pol δ and PCNA, which either inhibited binding of the polymerase to the primer terminus or caused dissociation of the enzyme during DNA synthesis.

In sharp contrast to these results are replication reactions with primed DNA that had been linearized with an appropriate restriction enzyme, e.g. HincII (Fig. 2, top panel). In the absence of PCNA, identical size products were made by pol δ on the linear and the circular DNA (Fig. 2, lanes 1 versus 8). However, addition of PCNA gave full-length replication products in addition to the smaller size products made in its absence. Two observations are important here. First, a large molar excess of PCNA is required to effect this type of synthesis on linear DNA, about 10-100 trimers/input primer terminus, or 100–1000 trimers/phage DNA molecule replicated. Second, this reaction is quite inefficient, with only about 10% of the input DNA replicated during the 5-min reaction period. In contrast, DNA synthesis, in which PCNA is enzymatically loaded onto the DNA by RF-C and ATP, is both efficient and requires stoichiometric amounts of PCNA (Fig. 2, bottom panel) (6); moreover, results with circular and linear DNA are identical (Fig. 2, bottom panel).

The dramatic difference in properties between circular and linear DNA was observed under a variety of different reaction conditions, at each pH value between 6.0 and 8.0 (Fig. 3), in the presence or absence of hydrophilic polymers such as polyethylene glycol, and regardless of whether the SS mp18 DNA was coated with E. coli single-stranded binding protein (SSB, Fig. 2) or yeast single-stranded binding protein (RF-A, Figs. 3–5). Interestingly, PCNA-mediated inhibition of DNA synthesis by pol δ on primed circular SS DNA was most apparent at higher pH values (Fig. 3). Finally, the efficiency of processive synthesis was independent of the restriction enzyme used to linearize the DNA, as long as a suitable primer for DNA synthesis was provided (data not shown).

The different modes of DNA synthesis by pol δ, i.e. non-processive in the absence of PCNA and on circular templates and processive in the presence of PCNA on linear templates, was most clearly revealed when lengths of products synthesized as a function of reaction time were measured. On primed circular DNA in the absence or presence of PCNA, the mean size of products increased as a function of time, indicating a distributive mode of DNA synthesis (Fig. 4, panels A and B). Results identical to those in panel A were observed with linear DNA in the absence of PCNA (data not shown). In the presence of PCNA, however, the number of completely replicated molecules increased with time, indicating an extremely processive, as well as fast, mode of DNA synthesis (panel C). These observations were confirmed by carrying out replication reactions with a 5'-end-labeled primer and with non-radioactive dNTPs. Again, on circular DNA templates, all labeled primer termini were extended and the mean size of products increased with time. No full-length products were synthesized during the 15-min incubation (panel D). On linear DNA, all primer termini were extended, but most of those via a distributive process.
**Fig. 3.** PCNA loads on linear DNA at various pH levels. Assays were as described under “Experimental Procedures,” except that MES-NaOH was used for pH values of 6.0 and 6.5 and HEPES-NaOH was used for pH values of 7.0, 7.5, and 8.0. Reaction times were 15 min.

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**Fig. 4.** Replication products synthesized as a function of time. A–C, Replication reactions (60 µl) were as described under “Experimental Procedures.” Aliquots (15 µl) were taken as indicated and analyzed on a 1.5% alkaline agarose gel. D and E, primed circular (D) or HincII-cut linear (E) DNA was passed through a Bio-Gel A-5m column in 10 mM Tris-HCl, 1 mM EDTA to dissociate and separate the primer from the template. The void volume fractions containing unprimed phage DNA were neutralized with acetic acid, and 5'32P-labeled 44-mer oligonucleotide was hybridized to the phage DNA. Unbound primer was then removed by passage through a Bio-Gel A-5m column in 10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl. Replication reactions (60 µl) with these DNA substrates were as described under “Experimental Procedures,” except that 80 µM non-radioactive dTTP replaced labeled TTP and the 15-µl aliquots were analyzed on a 2% alkaline agarose gel. Autoradiograms were scanned with a densitometer for quantitation.

**Fig. 5.** PCNA does not load onto single-stranded DNA ends. Unprimed SS mp18 DNA linearized at the HincII site (nucleotide 6267) and purified as described for Fig. 4 was hybridized to a 24-mer complementary to nucleotides 5920–5897. The DNA was incubated in restriction enzyme buffer (lanes 1 and 3) or restriction enzyme buffer plus Avall (lanes 2 and 4), repurified as described under “Experimental Procedures,” and used in standard replication reactions. PCNA was added as indicated.

These studies reveal the following properties of PCNA: (i) it can traverse double-stranded, but not single-stranded, DNA when the latter is coated with a SSB; (ii) it can mediate rapid processive synthesis by pol δ in the absence of RF-C, with rates similar to observed for the complete holoenzyme (12); (iii) it can load onto DNA ends with the primer terminus far removed from that end. The latter property indicates that PCNA can load onto the DNA independently of pol δ. However, presumably because of the inefficiency and transient nature of this process, we have not been able to show directly, e.g. by trapping or staged experiments, that PCNA associates with linear DNA in the absence of pol δ. The properties of PCNA described under (i) and (ii) are remarkably similar to those of the β subunit of E. coli DNA polymerase III holoenzyme (9, 17). In addition, on the template primer poly(dA)-oligo(dT) β subunit stimulates the DNA polymerase III core in a manner similar to PCNA stimulation of pol δ (18). Finally, β subunit enzymatically loaded onto circular DNA fails to remain associated with the DNA when it is linearized with a restriction enzyme (9). These parallel properties indicate a remarkable conservation of function between prokaryotes and eukaryotes and predict a similar conservation in structure for this component of the replication apparatus (8).

**REFERENCES**


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5 An 0.7-kb EcoRI-BglII double-stranded DNA fragment from mp18 was heat-denatured and hybridized to SS mp18 DNA. The partial duplex was linearized with ClaI, providing an 0.55-kb duplex region at the template 3'-end. The linear but not the circular DNA provided a good substrate for PCNA-mediated processive DNA synthesis.

6 Little or no DNA synthesis by pol δ was observed when the SS DNA was not coated with a SSB.
ATP-independent Loading of PCNA

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