THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 2, Issue of January 14, pp. 1421–1432, 2000

Printed in U.S.A.

Dynamics of β and Proliferating Cell Nuclear Antigen Sliding Clamps in Traversing DNA Secondary Structure*

(Received for publication, October 1, 1999)

Nina Yao‡§, Jerard Hurwitz¶, and Mike O'Donnell§‖**

From the ‡Joan and Sanford I. Weill Graduate School of Medical Sciences of Cornell University, Microbiology Department, New York, New York 10021, the §Rockefeller University and the ‖Howard Hughes Medical Institute, Laboratory of DNA Replication, New York, New York 10021, and the ¶Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Chromosomal replicases of cellular organisms utilize a ring shaped protein that encircles DNA as a mobile tether for high processivity in DNA synthesis. These "sliding clamps" have sufficiently large linear diameters to encircle duplex DNA and are perhaps even large enough to slide over certain DNA secondary structural elements. This report examines the *Escherichia coli* β and human proliferating cell nuclear antigen clamps for their ability to slide over various DNA secondary structures. The results show that these clamps are capable of traversing a 13-nucleotide ssDNA loop, a 4-base pair stem-loop, a 4-nucleotide 5′ tail, and a 15-mer bubble within the duplex. However, upon increasing the size of these structures (20-nucleotide loop, 12-base pair stem-loop, 28-nucleotide 5′ tail, and 20-nucleotide bubble) the sliding motion of the β and proliferating cell nuclear antigen over these elements is halted. Studies of the *E. coli* replicase, DNA polymerase III holoenzyme, in chain elongation with the β clamp demonstrate that upon encounter with an oligonucleotide annealed in its path, it traverses the duplex and resumes synthesis on the 3′ terminus of the oligonucleotide. This sliding and resumption of synthesis occurs even when the oligonucleotide contains a secondary structure element, provided the β clamp can traverse the structure. However, upon encounter with a downstream oligonucleotide containing a large internal secondary structure, the holoenzyme clears the obstacle by strand displacing the oligonucleotide from the template. Implications of these protein dynamics to DNA transactions are discussed.

The *Escherichia coli* chromosomal replicase, DNA polymerase III (Pol III)[1] holoenzyme, is rapid and processive, in keeping with the need to replicate a 4 million-bp chromosome within 30 min (1). This enzyme is a multisubunit complex made up of three subcomplexes: the catalytic Pol III core, the β sliding clamp, and the γ complex clamp loader (2).

The catalytic core, comprised of α (polymerase), ε (3′-5′ exonuclease), and θ subunits (3–5), incorporates nucleotides into a growing nascent chain and proofreads the replicated sequence. Pol III core by itself is not processive; it extends a primed site by only 10–15 nucleotides each binding event (6). High processivity by the holoenzyme is achieved through the use of accessory proteins, the β sliding clamp, and the γ complex.

The β sliding clamp is composed of two identical protomers, each having three globular domains forming a six-domain closed ring that encircles DNA (7). This topological linkage of β to DNA, rather than direct chemical contact, underlies the ability of β to bind DNA tightly yet freely slide bidirectionally along a duplex (8). The β subunit also binds the catalytic Pol III core through specific protein-protein interactions (8–10). By simultaneously encircling DNA and binding to Pol III core, β tethers the polymerase to the template during elongation, thereby constraining it to act in a highly processive fashion (8).

The β clamp does not assemble onto DNA by itself. For this, it requires the γ complex, which acts as a clamp loader. This clamp loader is a multisubunit complex (five different subunits: γ, δ, δ′, χ, and ψ) that acts upon a primed template to assemble the β ring around the duplex in an ATP hydrolysis-driven reaction (11, 12).

At the replication fork, the leading strand is synthesized continuously, whereas the lagging strand is synthesized discontinuously in segments called Okazaki fragments. Semidiscontinuous DNA replication is rooted in the fact that the two strands of the duplex are antiparallel; yet DNA polymerases can only extend DNA in one direction. On the leading strand, a highly processive enzyme is beneficial for the continuous mode of DNA synthesis. However, on the discontinuous lagging strand, the polymerase must rapidly recycle from the end of one Okazaki fragment to a new upstream primed site synthesized by primase. Okazaki fragments are synthesized about once every second or two. How can a polymerase that is tightly held to DNA by a ring rapidly hop from one fragment to the next? This has been addressed in previous studies. Upon completing an Okazaki fragment, the polymerase rapidly departs from the β ring, leaving it behind on the completed lagging strand fragment (12, 13). The polymerase is then free to reassociate with a β clamp that has been assembled onto a new primed site by γ complex.

The strategy of coupling a DNA polymerase to DNA with a clamp and ATP-driven clamp loader generalizes to eukaryotes (14–16). For example, the human DNA polymerase δ replicase (Pol δ) relies on a ring shaped clamp and a clamp loader for processive synthesis (16, 17). The human clamp, referred to as the proliferating cell nuclear antigen (PCNA), is a ring-shaped protein with dimensions similar to *E. coli* β (18). The PCNA ring, however, is formed from three identical subunits each having only two globular domains which trimerize to form a six-domain ring. PCNA is assembled onto the primed template by the ATPase action of the human clamp loader, replication factor C (RF-C) (19, 20). Like *E. coli* γ complex, RF-C is a five subunit complex, and after it assembles the PCNA ring around

* This work was supported by National Institutes of Health Grant 38839. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Tel.: 212-327-7255; Fax: 212-327-7253; E-mail: odonnel@rockvax.rockefeller.edu.

[1] The abbreviations used are: Pol III, DNA polymerase III; bp, base pair(s); Pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; ssDNA, single-stranded DNA; BSA, bovine serum albumin; DTT, dithiothreitol; kb, kilobase(s).

a primed template, Pol δ binds the clamp for processivity in DNA synthesis (19–23). Pol δ is thought to act on the lagging strand in similar fashion to the *E. coli* system, hopping among PCNA clamps and leaving the used clamps behind on the DNA where they may interact with other proteins (24).

Recently, several proteins involved in replication, repair, and cell cycle control have been reported to interact with PCNA (reviewed in Ref. 25). For example, FEN-1, a nuclease responsible for removing ssDNA 5′ tails during Okazaki fragment maturation (26) and PCNA-dependent base excision repair (27), binds PCNA leading to a stimulation of the nuclease activity (28). PCNA is also thought to interact with human homologues of *E. coli* MutS and MutL (hMSH-2 and hMLH1, respectively; (29)). hMSH2 specifically binds single base pair mismatches as well as short loops of 4–16 nucleotides (30, 31). Other proteins that interact with PCNA include DNA ligase I (32), Xeroderma pigmentosum G (33), and p21 (Cip1) (34, 35).

This report examines the dynamics of β and PCNA upon encountering a variety of DNA structures. Earlier studies have shown that β and PCNA slide freely over duplex DNA, consistent with their large inner diameters; β measures approximately 35 Å (not including extended side chains) (7), and the inner diameter of the PCNA ring measures approximately 34 Å (36). These dimensions are sufficiently large to accommodate B-form duplex DNA (diameter, ~18 Å) and the A-form RNA-DNA heteroduplex (diameter, ~21 Å). Given the relatively large inner diameters of β and PCNA, they may be capable of sliding across some types of DNA secondary structures, although one may expect their sliding dynamics to be halted by secondary structures of a certain size.

This report examines DNA secondary structures to define those that β and PCNA can traverse, and those that block their sliding motion. The results obtained demonstrate that both β and PCNA slide over rather bulky DNA structures such as bubbles (long stretches of continuous mismatched bases), 5′ tailed flaps, loops, and stem-loops, but the sliding motion is finally blocked by each of these elements when they reach a sufficiently large size. The study also examines the consequences on DNA polymerization when the *E. coli* Pol III holoenzyme encounters these DNA structures. Previous studies have demonstrated that Pol III holoenzyme, upon encountering a short flush duplex in its path, simply slides over the duplex and restarts synthesis at the new 3′ end (37). We find here that upon encounter with a DNA structure that β is incapable of traversing, Pol III holoenzyme does not stop but instead strand displaces the oligonucleotide containing the obstacle.

MATERIALS AND METHODS

Reagents and Proteins

Reagents were purchased as follows: unlabeled ribonucleoside and deoxyribonucleoside triphosphates (Amersham Pharmacia Biotech), radioactive nucleoside triphosphates (NEN Life Science Products), Bio-Gel A15m agarose (Bio-Rad), restriction enzymes and T4 polynucleotide kinase (New England Biolabs), and RNasin Ribonuclease Inhibitor (Promega). DNA oligonucleotides used for polymerase chain reaction and template primers were synthesized by Oligos etc. and Life Technologies, Inc. Proteins were purified as described: α, ε, γ, τ (38), β (7), δ and δ′ (39), χ and ψ (40), and θ (41). γ complex and Pol III* (a subcomplex of Pol III holoenzyme that lacks only β) were reconstituted from individual subunits and purified from unassociated subunits as described in Refs. 42 and 43, respectively. β (7), β^{PK} with a C-terminal kinase site (13), human RF-C from HeLa cells (44), $PCNA^{PK}$ with an N-terminal kinase site (42), and $EBNA1^{PK}$ (45) were purified according to previous protocols. β^{PK}, $PCNA^{PK}$, and $EBNA1^{PK}$ were labeled with [γ-^{32}P]ATP using cAMP-dependent protein kinase to specific activities of approximately 100 cpm/fmol as described (46). SSB was purified from induced BL21(DE3) cells (400 g of wet weight) harboring pET SSB following disruption in the French press in buffer D (1500 ml). The clarified lysate (1200 ml) was treated with 0.24 g/ml ammonium sulfate, and the pellet was repeatedly resuspended and pelleted in 100 ml of buffer E containing 0.15 g/ml ammonium sulfate (6 times) and then using 100 ml of buffer E containing 0.13 g/ml ammonium sulfate (2 times). The pellet was resuspended in buffer F containing 0.2 M NaCl, dialyzed against the same buffer, and then applied to a 200 ml of heparin-agarose column (Bio-Rad) equilibrated in buffer F plus 150 mM NaCl. The SSB was eluted with a 1.5-liter linear gradient of buffer F from 150 to 500 mM NaCl, and 80 fractions were collected. Fractions 46–60 were pooled (197 mg in 38 ml), dialyzed against buffer E containing 0.2 M NaCl, diluted to a conductivity equal to 160 mM NaCl, and applied to a 20-ml Mono Q column. SSB was eluted with a 200-ml linear gradient of buffer E from 150 to 500 mM NaCl, and 80 fractions were collected. Fractions 45–64, containing homogeneous SSB, were pooled (140 mg in 40 ml), aliquoted, and stored at −70 °C.

Buffers

Buffer A is 10 mM Tris-HCl (pH 7.5), 300 mM NaCl and 30 mM sodium citrate. Buffer B is 20 mM Tris-HCl (pH 7.5), 4% (v/v) glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin (BSA), 5 mM DTT, 1 mM ATP, and 8 mM $MgCl_2$. Buffer C is 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 4% (v/v) glycerol, 0.5 mM EDTA, 100 μg/ml BSA, 2 mM DTT, and 50 mM NaCl. *Bam*HI storage buffer is 50 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, and 50% glycerol. Buffer D is 5 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 30 mM spermidine, and 10% sucrose. Buffer E is 20 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 10% glycerol. Buffer F is 20 mM Hepes (pH 6.5), 0.5 mM EDTA, 5 mM DTT, and 10% glycerol. *Hinc*II storage buffer is 200 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, and 50% glycerol. *Xba*I storage buffer is 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 200 μg/ml BSA, and 50% glycerol.

Construction of M13EBNA1 Phage

A single 18-bp EBNA1-binding site sequence was inserted into an M13mp18 vector (Roche Molecular Biochemicals) by polymerase chain reaction amplification. The upstream primer 5′-TAC<u>GAATTC</u>**GGGAA-G<u>CATATG</u>CTACCC**TCCTCTAGAGTCGACCTGCAGGCATGCAA-G-3′ has an *Eco*RI site (underlined) that is also present in the M13mp18 vector as well as a unique *Nde*I site (bold and underlined) located within the single EBNA1-binding site sequence (shown in bold). The downstream primer 5′-ATTTTTGAG<u>AGATCT</u>ACAAAGGCTATCAG-3′ is complementary to the M13mp18 template sequence and hybridizes over a *Bgl*II site (underlined). Polymerase chain reaction conditions were 72 °C for 1.5 min, 94 °C for 1 min, and 60 °C for 40 s for 40 cycles. The resulting 718-bp DNA fragment was purified using a polymerase chain reaction Wizard Prep column (Promega) and then digested with 200 units of *Eco*RI and *Bgl*II. The final 704-bp insert was isolated through agarose gel electrophoresis followed by butanol extraction and then ligated into an M13mp18 vector that had been previously cut with *Eco*RI and *Bgl*II and gel purified. The construct (M13EBNA1) was transformed into *E. coli* XL-1 Blue competent cells. Phage containing single-stranded circular M13EBNA1 DNA was purified using two cesium chloride gradients as described (47). The presence of the EBNA1-binding sequence in the recombinant construct was verified by DNA sequencing. The number of nucleotides in the M13EBNA1 genome is identical to that of M13mp18 because the same number of nucleotides were deleted as were inserted. Thus, the map position numbers of M13EBNA1 are the same as those of M13mp18.

Template Construction

Hybridization Conditions—All of the oligonucleotides used in these constructs were purified by polyacrylamide gel electrophoresis. Oligonucleotides (250 pmol) were mixed with circular M13EBNA1 ssDNA (12.5 pmol) in 20 μl of buffer A in a 1.5-ml Eppendorf tube. The tube was placed in a 13 × 100-mm test tube containing nearly boiling water then allowed to cool to room temperature (~22 °C). Templates containing two oligonucleotides were formed by the same procedure, except that both oligonucleotides (250 pmol each) were present in the reaction.

Formation of Templates to Which "Bubble" Oligonucleotides Are Annealed—Two DNA oligonucleotides were synthesized such that they form either 15- or 20-nucleotide bubbles (*i.e.* continuous mismatches; shown in boldface in Table I) flanked by duplex upon hybridization to M13EBNA1 ssDNA. The bubble is flanked by 19–20 base pairs at the 3′ end and 59–62 bp at the 5′ end. Near the 5′ end is a *Hinc*II site for linearization of the circular primed templates (Table I, underlined).

Formation of Templates Hybridized with "5′ Flap" Oligonucleotides—The 5′ flap templates contain two oligonucleotides. The first has a 5′ tail comprised of either 4, 10, or 28 T residues (Table I, shown in boldface)

TABLE I

Oligonucleotides used in the study of β and PCNA sliding over DNA containing secondary structure

The name of each oligonucleotide indicates the type and length of secondary structure formed. The secondary structure forming sequence is shown in bold, and the restriction enzyme site is underlined. The map position of the 5′ nucleotide on the M13EBNA1 ssDNA is noted.

Name	Map position (5′ end)	Restriction site	Sequence (5′ to 3′)
Bubble-15	6277	*Hinc*II	GCCTGCAGGTCGACTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCATA**A AAAAAAAAAAAAAA**AAATTGTTATCCGCTCACA
Bubble-20	6274	*Hinc*II	TGCAGGTCGACTCTAGAGGAGGGTAGCATATCCTTCCCGAATTCGTAATCATGGTCATA**CGAC AAAGGACACACTTTAA**GTTATCCGCTCACAATTCCA
5′ Flap-4 nucleotide	6191	*Scr*FI	**TTTT**TCCGCTCACAATTCCACACAACATACGATCCGGAAGCATAAAGTGTAAAGCCTGGGGTG CCTGATGAGTGA
5′ Flap-10 nucleotide	6191	*Scr*FI	**TTTTTTTTTT**TCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT GGGGTGCCTAATGAGTGA
5′ Flap-28 nucleotide	6191	*Scr*FI	**TTTTTTTTTTTTTTTTTTTTTTTTTTTT**TCCGCTCCCAATTCCACACAACATACGAGCCGGAA GCATAATGTGTAAAGCCTGGGGTGCCTAATGAGTGA
Adjacent to flap	6722	*Hinc*II	GCCTGCAGGTCGACTCTAGAGGA**GGGTAGCATATGCTTCCC**GAATTCGTAATCATGGTCATAG CTGTTTCCTGTGTGAAATTGTTA
Loop-13 nucleotide	6722	*Xba*I	GCCTGCAGGTCGACTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCATAG CTG**AAAAAAAAAAAAA**TTTCCTGTGTGAAATTGTTA
Loop-20 nucleotide	6722	*Xba*I	GCCTGCAGGTCGACTCTAGAGGAGGGTAGCAT?TGCTTCCCGAATTCGTAATCATGGTCATAG CTG**AAAAAAAAAAAAAAAAAAAA**TTTCCTGTGTGAAATTGTTA
Loop-25 nucleotide	6722	*Xba*I	GCCTGCAGGTCGACTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCATAG CTG**AAAAAAAAAAAAAAAAAAAAAAAAA**TTTCCTGTGTGAAATTGTTA
Stem-loop-4 bp	6722	*Xba*I	GCCTGCAGGTCGACTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCATAG CT**GGGGTTTTTCCCC**TTCCTGTGTGAAATTGTTA
Stem-loop-8 bp	6719	*Xba*I	TGCAGGTCGACTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCAT**AAAGG CCTTTTTTTAAGGCCTT**GCTGTTTCCTGTGTGAAATTGTTA
Stem-loop-12 bp	6719	*Xba*I	TGCAGGTCGACCTCTAGAGGAGGGTAGCATATGCTTCCCGAATTCGTAATCATGGTCATA**GGA AGGCCTTGGTTTTTCCAAGGCCTTCC**GCTGTTTCCTGTGTGAAATTGTTA

followed by a length of 72 nucleotides complementary to M13EBNA1 ssDNA. The second "adjacent-to-flap" oligonucleotide is a 30-mer of which, when annealed to the template, the 3′ end abuts the 5′ tail of the 5′ flap oligonucleotide. This second oligonucleotide also contains an EBNA1 site (Table I, shown in underlined boldface) and a *Hinc*II site (Table I, shown underlined). To verify that each template contained both oligonucleotides, the DNA was digested with either *Hinc*II (which cleaves only the adjacent-to-flap oligonucleotide) or *Scr*FI (which cleaves only the 5′ flap primers). Separation of products on an agarose gel revealed that all the DNA was linearized in both digests.

Formation of Template Hybridized with "Loop" Oligonucleotides—The loop oligonucleotides contained a 13, 20, or 25 dA residue insert in a sequence complementary to M13EBNA1 ssDNA (Table I, shown in boldface). Hybridization to M13EBNA1 ssDNA results in formation of a single-stranded loop flanked by 66 bp on the 5′ side and 20 bp on the 3′ side. Linearization of the circular primed template on the 5′ side of the loop can be achieved by cleavage with *Xba*I (Table I, shown underlined).

Formation of Template Hybridized with Stem-loop Oligonucleotides—The three stem-loop oligonucleotides contain two inverted sequences separated by 5 T residues that form a stem-loop with a stem of 4–12 bp and a loop of 5 nucleotides. Upon hybridization to M13EBNA1 ssDNA, the stem-loop is flanked by 60–69 bp on the 5′ side and 19–24 bp on the 3′ side. An *Xba*I site on the 5′ side of the stem-loop allows linearization of the circular primed template.

Isolation of Clamps Bound to DNA by Gel Filtration Chromatography

Reactions were performed in Buffer B in a volume of 100 μl. The exact amounts of clamp, clamp loader, and primed template used in each experiment are given in the figure legends. Reactions were incubated for 10 min at 37 °C before treatment with hexokinase (0.25 unit) and 300 nmol of glucose to remove ATP, which prevents further clamp loading events. DNA-bound protein was then separated from unbound protein on 5-ml Bio-Gel A15m gel filtration columns equilibrated in Buffer C at 4 °C. Fractions (180 μl) were collected, and aliquots (150 μl) were analyzed by liquid scintillation counting. Moles of protein bound to DNA were calculated from the area under the peak in the excluded fractions (five peak fractions, typically 12–16) using the known specific activity of the protein. Values were normalized for the total volume of each fraction.

Replication of Linear ssDNA Templates Primed with Multiple Oligonucleotides

Three types of oligonucleotides were synthesized for this experiment (refer to Table II for exact sequences). Oligonucleotide 1 is a 31-mer that anneals to the M13EBNA1 template (map positions 6160–6190). Oligonucleotide 2 is designed to form a specific secondary DNA structure such as a 5′ flap, stem-loop, loop, or bubble. Each secondary structure is flanked by 15 bp on the 5′ side (except for the flap structure, which consists of a 5′ floppy tail) and 16–30 base pairs on the 3′ side. Oligonucleotide 2 anneals approximately 2.5 kb downstream of oligonucleotide 1; oligonucleotide 3 is a short 15-mer that anneals to M13EBNA1 ssDNA approximately 1 kb downstream of oligonucleotide 2. This oligonucleotide contains a *Cla*I site that allows linearization of the multiply primed template.

The multiply primed template was prepared by annealing 500 pmol of oligonucleotide 1 along with 500 pmol of oligonucleotide 2 and 1500 pmol of oligonucleotide 3 to 15 pmol of M13EBNA1 ssDNA in 20 μl of Buffer A. The final primed template was then linearized: 3.8 pmol multiply primed template (as circles) was cleaved with 25 units of *Cla*I in 50 μl of buffer containing 50 mM potassium acetate, 20 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 1 mM DTT, and 100 μg/ml BSA at 37 °C for 1 h. Replication reactions were as follows: 152 fmol of linearized multiply primed template, 55 pmol of *E. coli* SSB, 25 fmol of β_2, 60 μM each dCTP and dGTP in 23 μl of buffer B. The DNA mixture was then preincubated with 25 fmol of *E. coli* Pol III* (25 fmol/μl) for 1.5 min at 30 °C. Replication was initiated upon adding 1 μl of 1.5 mM dATP and 0.5 mM [α-^{32}P]dTTP (3000 Ci/mmol). Individual reactions were allowed to proceed for either 6, 9, 12, or 30 s before being quenched with 25 μl of stop solution (40 mM EDTA, 1% SDS). Half of each reaction was subjected to electrophoresis through a 1% alkaline agarose gel, and the other half was subjected to electrophoresis through a 0.8% neutral agarose gel at 30 volts for approximately 18 h. Both gels were then dried and exposed to PhosphorImager screens to visualize the replicated DNA products.

RESULTS

The β and PCNA clamps bind tightly to circular DNA and slide freely along the duplex, but on linear DNA they readily fall off the end into solution (8, 24). The ability of these clamps to slide off DNA ends is used in this study as an assay to determine which DNA secondary structures the clamps can slide over. Thus, DNA templates were constructed that require β (or PCNA) to slide over a DNA structure to reach the DNA double strand break. If the clamp is capable of sliding over the DNA obstacle, it will have access to an end and fall off the DNA. On the other hand, if the clamp is unable to traverse the DNA structure, the clamp will be retained on the linear DNA. Linear DNA has two ends, and therefore access to one end is blocked in these experiments through the use of either EBNA1, a site-

TABLE II

Oligonucleotides used in replication of multiprimed linear templates by Pol III holoenzyme

The primer used to linearize M13EBNA1 ssDNA is called "Linearization 3," and the upstream primer is primer 1. The primer 2 oligonucleotides are those containing the secondary structure (underlined). The names of these number 2 oligonucleotides denote the length and type of secondary structure that they form. Map positions on the M13EBNA1 ssDNA are indicated for the 5′ nucleotide of each oligonucleotide.

Name	Map position (5′ end)	Sequence (5′ to 3′)
Primer 1	6190	CCGCTCACAATTCCACACAACATACGAGCCG
No 2 structure 2	3628	AAACAACATGTTCAGCTAATGCAGAACGCG
Flap 2–4 nucleotide	3628	TTTTAAACAACATGTTCAGCTAATGCAGAACGCG
Flap 2–30 nucleotide	3628	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAACAACATGTTCAGCTAATGCAGAACGCG
Hairpin 2–4 bp	3631	AATAAACAACATGTTGGGGTTTTTCCCCCAGCTAATCAGAACGCGC
Hairpin 2–12 bp	3631	AATAAACAACATGTTGGAAGGCCTTGGTTTTTCCAAGGCCTTCCCAGCTAATGCAGAACGCGC
Loop 2–5 nucleotide	3631	AATAAACAACATGTTTTTTTCAGCTAATGCAGAACGCGC
Loop 2–30 nucleotide	3631	AATAAACAACATGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACAGCTAATGCAGAACGCG
Bubble 2–6 nucleotide	3634	GACAATAAACAACATCAAGTCCTAATGCAGAACGCGC
Bubble 2–20 nucleotide	3649	TTCTGTCCAGACGACCTGTTATTTGTTGTACAAGTGCTAATGCAGAACGCGC
Linearization 3	2539	GAAACCATCGATAGC

specific DNA binding protein, or by the presence of ssDNA, to which SSB is attached. These proteins, bound to the ends of linear DNA, have been shown previously to block β and PCNA from sliding off DNA (8, 24).

The study utilizes ^{32}P-labeled proteins to follow their presence on DNA or in solution. To separate ^{32}P-labeled protein on DNA from ^{32}P-labeled protein in solution, reactions are analyzed by gel filtration over the large pore resin, Bio-Gel A15m. Large DNA molecules and ^{32}P-labeled protein bound to the DNA and elute in the excluded volume of this resin, but free ^{32}P-labeled protein is included and elutes later. The molar amount of ^{32}P-labeled protein bound to DNA can then be calculated from the total radioactivity in the excluded fractions and the known specific activity of the ^{32}P-labeled protein.

Clamps Are Blocked by a 12-bp Stem-loop—In the first experiment, we examined ability of β to slide over a stem-loop comprised of a 12-bp duplex with a 5-nucleotide loop. A 93-mer DNA oligonucleotide was designed containing two inverted repeats, each 12 nucleotides long, separated by five dT residues, and flanked 5′ and 3′ by 60 and 24 nucleotides that hybridize to one continuous 84-mer stretch on the M13EBNA1 ssDNA template. Hybridization of this oligonucleotide to the circular ssDNA template yields a stem-loop structure flanked by short duplex regions (refer to the *scheme* in Fig. 1). The duplex on the 5′ side of the stem-loop contains an *Xba*I site. Upon linearization with *Xba*I, a β clamp assembled onto the 3′ end of the stem-loop will not be able to slide over the long stretch of SSB-coated ssDNA. However, if β is capable of sliding over the stem-loop structure it will slide over the double strand break and dissociate from the DNA.

This experiment was performed by assembling ^{32}P-β onto the stem-loop template using γ complex and ATP, and then the reaction was quenched upon removing ATP by treatment with hexokinase and glucose. Following this, the reaction mixture was divided. One half was treated with *Xba*I (5 min, 37 °C); the other half was treated with *Xba*I storage buffer (5 min, 37 °C) and thus will remain circular providing a positive control for the amount of ^{32}P-β initially assembled onto the DNA. In all experiments in this report, verification that the DNA was completely linearized was determined by saving a sample of the reaction, quenching it with SDS/EDTA followed by analysis (after the experiment) in an agarose gel. To quantitate ^{32}P-β clamps that remained bound to the linearized DNA, reactions were analyzed by gel filtration through Bio-Gel A15m. The results, in *right panel* of Fig. 1*A*, demonstrate that approximately 83% of β was retained on the linear template relative to the circular DNA control (see excluded volume in fractions 10–15, also refer to Table III for quantitation). Therefore, β clamps do not slide over a 12-bp stem-loop during the time of this experiment (6 min at 37 °C and 15 min during gel filtration at 22 °C).

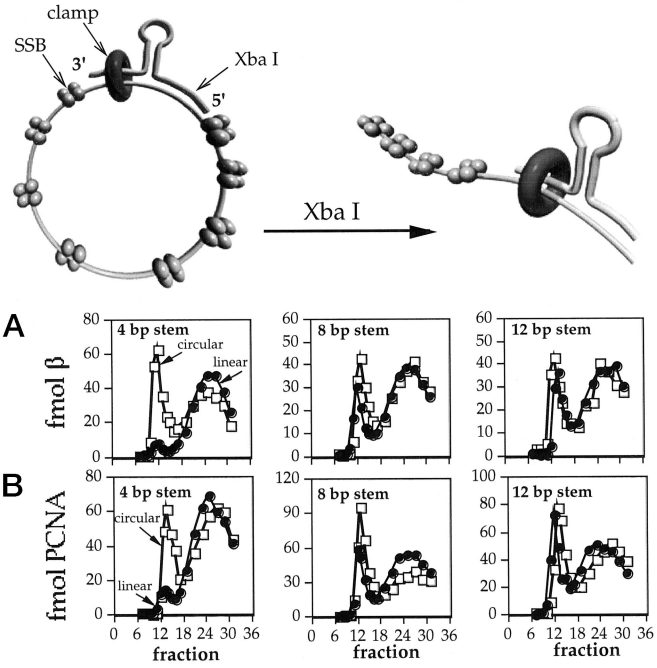


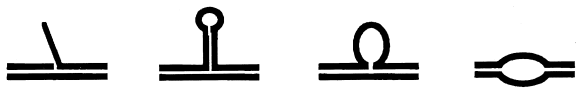
FIG. 1. **β and PCNA are blocked by a 12-bp stem-loop.** β and PCNA were assembled onto stem-loop templates in reactions containing 846 fmol of template, 212 pmol of SSB, 1872 fmol of ^{32}P-β or 1822 fmol of ^{32}P-PCNA, and 400 fmol of γ complex or 1600 fmol of RF-C in 100 μl of buffer B containing 50 mM NaCl. After 5 min at 37 °C, the reaction mixture was divided. In one half, the template was linearized with 100 units (10 μl) of *Hin*cII, and to the other 10 μl of *Hin*cII storage buffer was added. After a further 5 min at 37 °C, clamp loading activity was arrested by adding 300 nmol of glucose and 0.25 unit of hexokinase. Half of each reaction (55 μl) was analyzed by gel filtration on 5-ml columns of Bio-Gel A15m equilibrated with Buffer C containing either 100 mM NaCl (when using β and γ complex) or 250 mM NaCl (PCNA and RF-C). The remaining 55 μl of each reaction was analyzed on an agarose gel to confirm cleavage of the templates (data not shown). *A*, results using β and γ complex on stem-loop templates containing either a 4-bp stem (*left panel*), an 8-bp stem (*middle panel*), or a 12-bp stem (*right panel*). *Filled circles* represent reactions containing linearized template, and *open squares* represent reactions in which the DNA remained circular. *B*, results using PCNA and RF-C on stem-loop templates containing either a 4-bp stem (*left panel*), an 8-bp stem (*middle panel*), or a 12-bp stem (*right panel*).

Next, templates were constructed that had stem-loops having either an 8- or a 4-bp stem. The results of an experiment using the 8-bp stem-loop, in the *middle panel* of Fig. 1*A*, demonstrates that only 58% of β remained on the linear DNA relative to the circular DNA control. This partial loss of β may

TABLE III

Summary of the effectiveness of specific DNA secondary structures at blocking clamp sliding

Values given are percentages of β (or PCNA) that remains on linear DNA relative to a DNA control that quantitates total β (or PCNA) that initially assembles onto the DNA. nt, nucleotide.



Clamp	5′ flap			Stem-loop			Loop			Bubble	
	4 nt	10 nt	28 nt	4 bp	8 bp	12 bp	13 nt	20 nt	25 nt	15-mer	20-mer
E. coli β	17.3	50.3	99.9	11.4	58.0	83.2	10.4	81.2	78.1	21.9	74.4
human PCNA	19.3	65.8	77.3	26.4	63.2	75.0	15.9	64.1	99.9	19.3	88.7

indicate that β slowly slides off this DNA and that only half of the β traversed the 8-bp stem-loop during the time of this experiment. To test this, a separate experiment, not shown here, demonstrated that a further incubation of 15 min at 22 °C before applying the reaction to the gel filtration column was sufficient for an additional 30% of the β to slide off the DNA (relative to a similar further incubation of the circular template). Hence, β can slide over an 8-bp stem-loop, but slowly. The results using the 4-bp stem-loop, in the *left panel* of Fig. 4*A*, indicate that β is capable of sliding over this 4-bp stem-loop structure (11.4% remained relative to the circular DNA control).

In Fig. 1*B*, these experiments were repeated in the human system. In the first experiment, RF-C and ATP were used to assemble ^{32}P-PCNA onto the circular template containing a 12-bp stem-loop, and then the reaction was quenched with hexokinase, divided, and treated with either *Sba*I or *Sba*I storage buffer. Gel filtration analysis, in the *right panel* in Fig. 1*B*, indicates that 75% of ^{32}P-PCNA was retained on the DNA, similar to the result using ^{32}P-β. This experiment was repeated using templates having either an 8- or a 4-bp stem-loop. The results using the 8-bp stem-loop template, in the *middle panel* of Fig. 1*B*, show that 63% of the ^{32}P-PCNA remained on the linear DNA relative to the circular DNA control (Table III). Therefore, PCNA is probably blocked by this structure but given time can slide over it, like β. The result using a 4-bp stem-loop, in the *left panel* of Fig. 1*B*, indicates that PCNA readily slides over a 4-bp stem-loop (26% of the ^{32}P-PCNA was retained on the linear DNA relative to the circular DNA control; Table III). In conclusion, β and PCNA are blocked from sliding over a 12-bp stem-loop, slowly slide over an 8-bp stem-loop, and readily slide over a 4-bp stem-loop.

Clamps Are Blocked by a 25-Nucleotide Loop—The preceding experiment demonstrates that β and PCNA are capable of sliding over a stem-loop, provided it is not too large. Next we examined these clamps for their ability to slide over a ssDNA loop. In the first experiment, a primed template containing a 25 nucleotide loop was constructed using an oligonucleotide that possessed 25 adenosine residues flanked by 66 and 20 nucleotides of contiguous sequence complementary to the ssDNA template. This oligonucleotide contains an *Xba*I site on the 5′ side of the loop allowing formation of a linear DNA molecule with a duplex end behind the loop and a long stretch (>7 kb) of ssDNA 3′ to the loop (refer to the *scheme* in Fig. 2). Clamps cannot slide off the SSB-coated ssDNA end, and thus the only egress is by sliding over the loop and off the duplex end. Thus, study of whether β can slide off the linearized DNA provides an assay for the ability of β to slide over loops.

In the experiment shown in the *right panel* of Fig. 2*A*, ^{32}P-β was assembled onto the 25-nucleotide loop template using γ complex and ATP, and then hexokinase and glucose were added to remove ATP, thereby quenching the reaction. The reaction was divided, and one half was treated with *Xba*I,

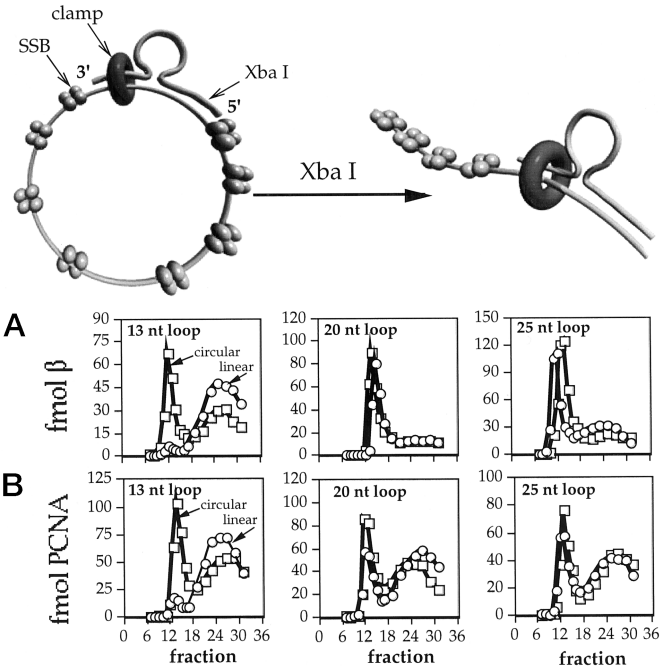


FIG. 2. **β and PCNA are blocked by a 20 nucleotide loop.** The experimental protocol for the loop-containing templates was as described in the legend to Fig. 1 for the stem-loop containing templates, except *Xba*I and *Xba*I storage buffer were used in place of *Hinc*II and *Hinc*II storage buffer. *A*, results using β and γ complex on templates containing a 13-nucleotide loop (*left panel*), a 20-nucleotide loop (*middle panel*), and a 25-nucleotide loop (*right panel*). *Open circles* are reactions in which the DNA was linearized. *Open squares* are reactions in which the DNA remained circular. *B*, *R*esults using PCNA and RF-C on templates containing either a 13-nucleotide loop (*left panel*), a 20-nucleotide loop (*middle panel*), or 25-nucleotide loop (*right panel*).

whereas *Xba*I storage buffer was added to the other half. After 5 min at 37 °C, the reactions were analyzed by gel filtration to quantitate the amount of ^{32}P-β that remained bound to the DNA. The results demonstrate that nearly 80% of the ^{32}P-β remained on the linear DNA compared with the circular DNA control (also refer to Table III). Next, templates were constructed with smaller loops, 20 and 13 nucleotides in size, and the experiment was repeated (*middle* and *left panels* of Fig. 2*A*, respectively). The results show that a 20-nucleotide loop is still an effective barrier; approximately 80% of the ^{32}P-β remained on the linear DNA compared with the circular DNA control. However, the β clamp was capable of sliding over the 13-nucleotide loop; nearly 90% of the β exited from the linearized template compared with the circular DNA control (Table III).

A comparison of these sliding clamp dynamics in the human system using the same DNA templates is shown in Fig. 2*B*. The results demonstrate that ^{32}P-PCNA is unable to slide over the 25-nucleotide loop; 64% of PCNA is retained on linear DNA

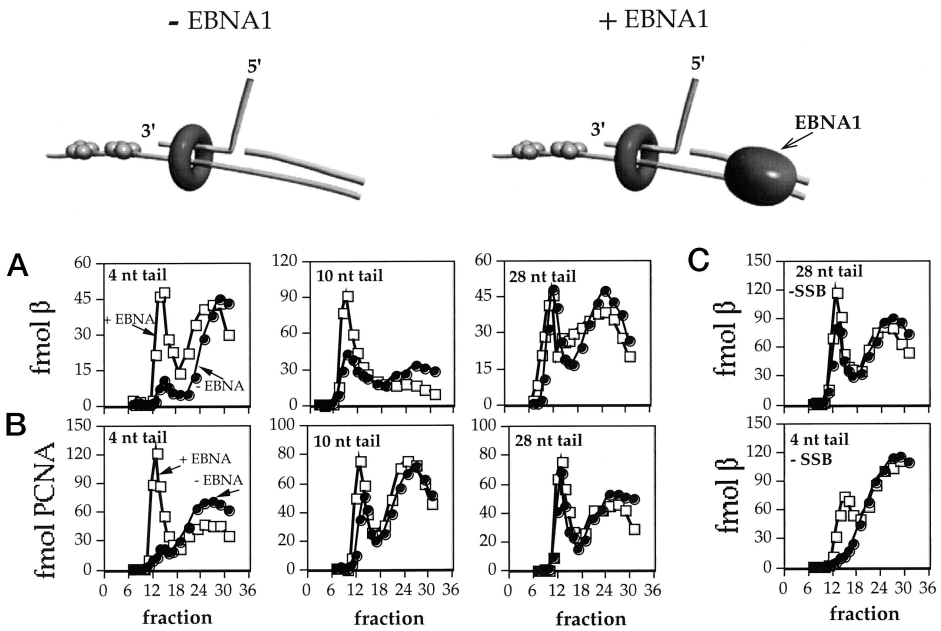


FIG. 3. ***β* and PCNA are blocked by a 28 nucleotide 5′ flap.** Circular 5′ flap templates were first linearized in a reaction containing 846 fmol of circular M13EBNA1 ssDNA primed with a 5′ flap oligonucleotide, 212 pmol of SSB, and 200 units (10 μl) of *Hin*cII in 100 μl of buffer B containing 50 mM NaCl for 5 min at 37 °C. Radiolabeled clamps were assembled onto 846 fmol of linear template upon adding 1352 fmol ^{32}P-β and 400 fmol γ complex (*A*) or 1822 fmol of ^{32}P-PCNA and 1600 fmol of RF-C (*B*). After 5 min at 37 °C, reactions were divided, 300 nmol of glucose and 0.25 unit of hexokinase were added to each half, and 1.8 pmol of EBNA1 (as dimer) was added to one half. Reactions were incubated an additional 1 min at 37 °C and then analyzed by gel filtration as described in the legend to Fig. 1. To verify cleavage of templates, half of each reaction was also analyzed on an agarose gel (data not shown). *A*, reactions containing ^{32}P-β and γ complex on templates having either a 4-nucleotide 5′ flap (*left panel*), a 10-nucleotide 5′ flap (*middle panel*), or a 28-nucleotide 5′ flap (*right panel*). *Open squares* represent reactions containing EBNA1, and *closed circles* represent reactions lacking EBNA1. *B*, reactions containing ^{32}P-PCNA and RF-C on templates having a 4-nucleotide 5′ flap (*left panel*), a 10-nucleotide 5′ flap (*middle panel*), or a 28-nucleotide 5′ flap (*right panel*). *C*, results of reaction lacking SSB using ^{32}P-β and γ complex on either the 28-nucleotide 5′ flap template (*top panel*) or the template containing only a 5-nucleotide 5′ flap (*bottom panel*). These reactions were performed as described above, except the β and γ complex were increased to 3200 fmol and 14 pmol, respectively, to compensate for their decreased activity in the absence of SSB.

containing the 20-nucleotide loop, and only 16% of the PCNA remained on linear DNA containing a 13-nucleotide loop. In summary, both β and PCNA can traverse a 13-nucleotide loop but not a 25-nucleotide loop.

Clamps Are Blocked by a 28-Nucleotide 5′ Flap—The third structure that we examined was 5′ unannealed ssDNA tailed structure. Henceforth, the 5′ ssDNA tail structure will be referred to as a 5′ flap. Flap structures have been proposed as intermediates in a variety of processes including DNA-end joining (48, 49), homologous recombination (50), DNA replication (1, 49, 51, 52), and PCNA-dependent base excision repair (49).

To examine the ability of *E. coli* β and human PCNA to slide over a 5′ flap, we designed a circular ssDNA template to which two oligonucleotides were hybridized: 1) a 99-mer oligonucleotide having 71 nucleotides complementary to the M13EBNA1 ssDNA substrate and 28 5′ nucleotides noncomplementary to the ssDNA and 2) an 86-mer oligonucleotide that fully anneals over the EBNA1 binding site (18 bp) in the M13EBNA1 ssDNA (the 86-mer also anneals over a downstream *Hin*cII site). The two oligonucleotides were annealed immediately adjacent to one another, as illustrated in the *scheme* in Fig. 3. The template was then cleaved with *Hin*cII to generate a linear molecular in which one end contains a 73-bp duplex positioned just behind the 5′ tail.

^{32}P-β was assembled onto the 5′ flap template using γ complex and ATP. ^{32}P-β can presumably be assembled at two possible sites on this DNA substrate: the 3′ end of the tailed 99-mer, and the 3′ end of the 86-mer (*i.e.* at the nick between the two oligonucleotides). However, ^{32}P-β that assembles at the nick should rapidly slide off the duplex end of the linear template (as in Ref. 8). In contrast, ^{32}P-β assembled at the ssDNA-double-stranded DNA junction located on the 3′ end of the 99mer with the 5′ tail will be prevented from sliding across the ssDNA ahead of the oligonucleotide by SSB that coats the large region of ssDNA. Therefore, only if ^{32}P-β can slide over the 5′ flap would it also slide over the duplex end and into solution. The use of templates with different flap lengths and the examination of whether ^{32}P-β remains bound or falls off the linear form of these templates provide a method to determine the size of 5′ flap that β can slide over.

First, we examined the ability of β to slide over a 28-nucleotide 5′ flap template (*right panel* in Fig. 3). In this experiment, ^{32}P-β was assembled onto the linear template using γ complex and ATP. After 10 min at 37 °C, the reaction mixture was divided, and saturating EBNA1 was added to one half. EBNA1 binds near the 3′ end of the 86-mer oligonucleotide and prevents clamp assembly at the nick (data not shown), whereas clamps assembled at the 3′ end of the 99-mer are prevented from sliding off the DNA (*i.e.* by SSB and EBNA1). Therefore, reactions to which EBNA1 is added serve as a positive control for the total number of β clamps assembled onto the 5′ flap oligonucleotide. Next, hexokinase and glucose were added to both reactions to remove ATP, thus preventing further clamp loading events catalyzed by γ complex. The reactions were then analyzed by gel filtration to quantitate clamps on DNA. The results demonstrate that nearly all of the β is blocked from sliding off DNA by the 28-nucleotide 5′ flap.

Next, templates having shorter 5′ flaps were constructed to determine which size of the 5′ flap that β can slide over. In the *middle panel* of Fig. 3*A*, the experiment was repeated using a 10-nucleotide 5′ flap. The results indicate that approximately 50% of β slides off the DNA during the time frame of this experiment. This lower level of β recovered on the DNA was not

due to an overall reduction in the amount of ^{32}P-β assembled onto DNA because in the presence of EBNA1 just as much, if not more, ^{32}P-β was loaded onto the template having the 10-nucleotide 5′ flap as on the template containing the 28-nucleotide 5′ flap. Presumably, β clamps slowly slide over the 10-nucleotide 5′ flap. In the *left panel* of Fig. 3*A*, β was examined for its ability to slide over a 4-nucleotide 5′ flap. The results demonstrate that β can slide over this short flap. Less than 20% of the total β assembled onto the DNA was retained relative to the control experiment in the presence of EBNA1.

E. coli SSB is estimated to have a binding site size of approximately 33 ± 3 nucleotides (53), and thus it seemed possible that SSB may bind the 5′ 28-nucleotide flap, thereby preventing β from sliding over the ssDNA 5′ tail. To address this possibility, these experiments were repeated using the 28- and 4-nucleotide 5′ flap templates in the absence of SSB.[2] The results, shown in Fig. 3*C*, indicate that β is still capable of sliding over a 4-nucleotide 5′ flap but cannot slide over a 28-nucleotide 5′ flap (~ 84% of β was retained on DNA in the absence of EBNA1 compared with the control in the presence of EBNA1). Therefore, β cannot slide over a 28-nucleotide 5′ flap in the presence or absence of SSB.

In Fig. 3*B*, these experiments were repeated in the human system. ^{32}P-PCNA was assembled onto the linear template containing a 28-nucleotide 5′ flap using RF-C and ATP and analyzed with and without EBNA1 as described above. The gel filtration analysis, in the *right panel*, demonstrates that ^{32}P-PCNA is retained on the DNA, similar to the result using ^{32}P-β. Comparable experiments using linear templates having 5′ flaps of 10 and 4 nucleotides show that PCNA can slide over these smaller sized flaps in nearly the same manner as β. In conclusion, β and PCNA cannot slide over a 28-nucleotide 5′ flap, slowly slide over a 10-nucleotide 5′ flap, and readily slide over a 4-nucleotide 5′ flap.

Clamps Are Blocked by a 20-mer Bubble—A bubble, as defined in this study, is a region of two unpaired DNA strands flanked on both sides by duplex DNA. Two unpaired strands of DNA would be expected to have a larger cross-sectional diameter than a duplex because it lacks the hydrogen bonding that would allow the two helices to pack. Are the central cavities of β and PCNA large enough to slide over bubbles in duplex DNA? If so, is there a size of bubble the clamps cannot traverse? To test this, we designed a 99-mer oligonucleotide with ends that are complementary to the circular ssDNA template but having a noncomplementary internal region thus forming a 20-nucleotide bubble flanked by 59 and 20 bp of duplex at the 5′ and 3′ ends, respectively. This template contains a *Hinc*II cleavage site 6 bp in from the 5′ end of the oligonucleotide. Therefore, linearization of this DNA template results in a double strand break behind the bubble as shown in the scheme in Fig. 4. β clamps that are assembled onto this linear template cannot slide over the long stretch of SSB-coated ssDNA that is present on the 3′ side of the oligonucleotide. The only route for dissociation of the clamp from the linear DNA is to slide over the bubble and off the duplex end.

In the *right panel* of Fig. 4*A*, β was tested for its ability to slide over a 20-mer bubble. ^{32}P-β was assembled onto the 20-mer bubble template using γ complex and ATP, and then the reaction was quenched with hexokinase/glucose and divided. *Hinc*II was added to one half, and *Hinc*II storage buffer was added to the other. After 5 min at 37 °C, linearization of the DNA was complete, and ^{32}P-β bound to the circular and linearized DNA was quantitated by gel filtration analysis. The

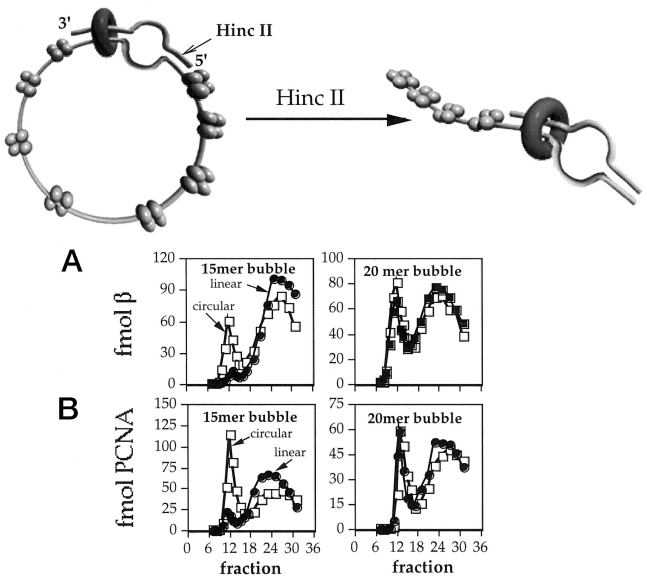


FIG. 4. ***β* and PCNA are blocked by a 20-mer bubble.** The experimental protocol for analysis of β and PCNA sliding on bubble-containing templates was as described in the legend to Fig. 1. *A*, results using ^{32}P-β and γ complex on templates having either a 15-mer bubble (*left panel*) a 20-mer bubble (*right panel*). *Closed circles* represent reactions containing linear templates, and *open squares* represent reactions containing circular templates. *B*, results using ^{32}P-PCNA and RF-C on templates having either a 15-mer bubble (*left panel*) or a 20-mer bubble (*right panel*).

results demonstrate that approximately 75% of β is retained on the linear template compared with the corresponding circular template. Therefore, β does not appreciably slide over a 20-mer bubble during the time of the experiment.

In the next experiment, the size of the bubble was reduced to 15 residues to determine whether this bubble is small enough for β to slide over. The results, in the *left panel* of Fig. 4*A*, demonstrate that β can diffuse over a 15-mer bubble; only 22% of the ^{32}P-β clamps on DNA were retained on the linear DNA compared with the circular DNA control. Therefore, β clamps can slide over a 15-mer bubble but not a 20-mer bubble.

To address the possibility that an SSB molecule bound to the 20-mer bubble might prevent β from sliding over it, the experiment using the 20-mer bubble template was repeated in the absence of SSB. The results show that β is not capable of sliding over the 20-mer bubble whether SSB is present or not (data not shown).[2]

In Fig. 4*B* we performed the same experiment using either the 15- or the 20-mer bubble template with human ^{32}P-PCNA and RF-C. As observed with *E. coli* β, the results show that PCNA cannot slide over a 20-mer bubble (~89% of ^{32}P-PCNA was retained on the linear DNA compared with the circular DNA; Table III) but is capable of sliding over a 15-mer bubble (~19% of ^{32}P-PCNA retained on linear DNA compared with the circular DNA; Table III).

E. coli Pol III Holoenzyme Diffuses over Small DNA Structures and Strand Displaces at Obstacles—In the next series of experiments, we examined the behavior of Pol III holoenzyme upon encountering a duplex containing a DNA structure. Pol III holoenzyme extends DNA rapidly, and thus the time scale of these replication based experiments (6–30 s) is much shorter than the duration of the clamp sliding assay. A previous study using templates to which multiple DNA oligonucleotides were annealed demonstrated that Pol III holoenzyme rapidly diffuses over duplexes annealed in its path (37). The previous study was performed with fully annealed oligonucleotides. Below, we examine the consequences on Pol III holoenzyme action

[2] The β clamp cannot slide over a long stretch (>500 nucleotides) of ssDNA even in the absence of SSB (8). Presumably the ssDNA forms secondary structures such as hairpins tha β cannot traverse.

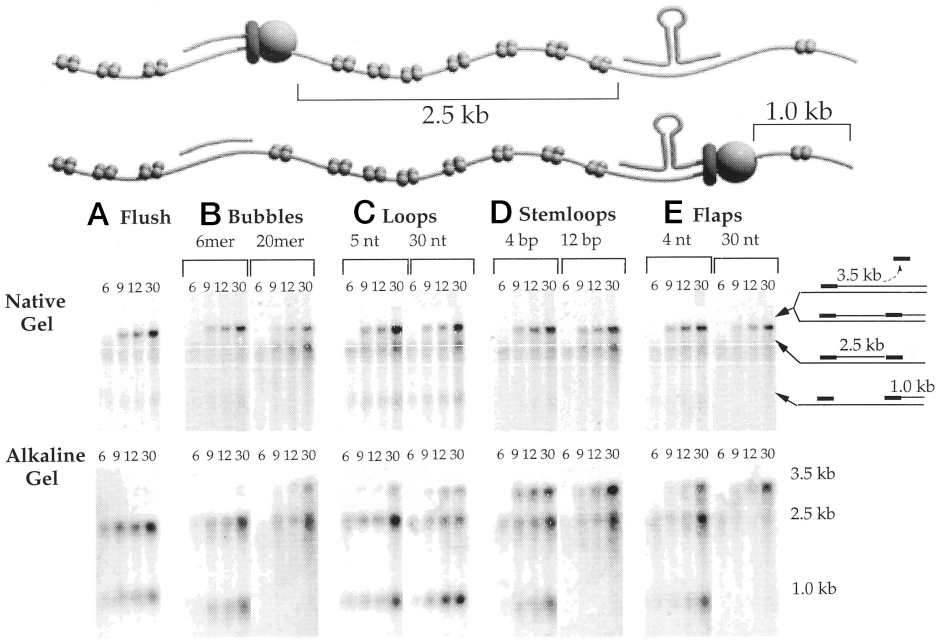


FIG. 5. **Pol III holoenzyme strand displaces oligonucleotides with large secondary structures.** Doubly primed linear templates were prepared as described under "Materials and Methods." 24.7 fmol of Pol III* was incubated with 152 fmol of doubly primed linear template, 55 pmol of SSB, and 24.6 fmol of β in 24 μl of buffer B containing 60 μM dCTP and 60 μM dGTP for 1.5 min at 30 °C. Replication was initiated with 1 μl of a mixture containing dATP and [α-^{32}P]dTTP (final concentrations, 60 and 20 μM, respectively) and then quenched after either 6, 9, 12, or 30 s by adding 25 μl of 1% SDS/40 mM EDTA (each time point is a separate reaction). The products were analyzed in both a 0.8% native agarose gel (*top*) and a 1% alkaline agarose gel (*bottom*). The *scheme* at the *top* illustrates that Pol III holoenzyme can initiate synthesis from either the upstream primer 1 (*top diagram*) or from the 3′ terminus of primer 2 containing the secondary structure (*bottom diagram*). Experiments were performed on doubly primed templates in which primer 2 was either a completely flush annealed oligonucleotide (*A*) or contained the following secondary structure elements: a 6- or 20-mer bubble (*B*), a 5- or 30-nucleotide loop (*C*), a stem-loop with stem sizes of either 4 or 12 bp (*D*), and a 5′ flap of either 4 or 30 nucleotides (*E*). Autoradiograms on the *top* are of native agarose gels, and those on the *bottom* of the native gels identify the DNA structure associated with each band. The slowest migrating (*top*) band is full-length and is the result of extension of primer 1 either all the way to the end of the DNA, by either displacing primer 2 (gives a 3.5-kb band in the alkaline gel) or sliding over primer 2 followed by extension of primer 2 to the end of the linear template (produces two fragments of 2.5 and 1 kb in the alkaline gel).

upon encountering DNA structures that β either can traverse or cannot slide over, as demonstrated in this report. For DNA structures that block β sliding, one might have expected the polymerase to simply halt. However, this was not the observed result.

In the experiments described in Fig. 5, we prepared linear ssDNA templates having an upstream primer (primer 1) and a downstream oligonucleotide containing different types of secondary structures (primer 2). Different primer 2 oligonucleotides containing either a bubble, 5′ tail, loop, or stem-loop were annealed to the template approximately 2.5 kb downstream of primer 1. Pol III holoenzyme was assembled onto the primed template using β and Pol III*. Pol III holoenzyme may assemble onto either primer as illustrated in the scheme of Fig. 5. In these experiments, primed template was present in molar excess over β and Pol III* to ensure that on average there will not be more than one holoenzyme/template. Two of the four dNTPs were included in the 5-min assembly reaction preventing chain extension while at the same time preventing the proofreading 3′ to 5′ exonuclease activity from degrading the primer. Replication was initiated by the addition of dATP and [^{32}P]dTTP and then quenched after only 6, 9, 12, or 30 s with SDS and EDTA. Reaction products were analyzed on native and alkaline agarose gels. Then two gel analyses were used to distinguish the structure of the products formed as described below. Autoradiograms from the two gels are shown in Fig. 5 (*A*—*E*).

Replication of the doubly primed templates by Pol III holoenzyme results in three different sized products (illustrated to the *right* of Fig. 5). The fastest migrating product on the native gel derives from Pol III holoenzyme that initially assembled on primer 2, extending it 1 kb to the end. This product will be ignored, because this report is concerned only with extension products from primer 1. Pol III holoenzyme that extends primer 1 2.5 kb will encounter primer 2. If the enzyme is halted at this point (*e.g.* cannot slide over the primer), a 2.5-kb extension product will be observed in the alkaline gel. In the native gel, this product migrates as a band between the full-length product and the smallest product, initiated from primer 2. If the enzyme traverses primer 2 and extends it 1.0 kb to the 5′ end of the template, then a full-length band is observed on the native gel (the slowest migrating band). This full-length product appears as two fragments, 2.5 and 1.0 kb, on the alkaline gel.

Product analysis was required in both native and alkaline gels for the following reasons. Firstly, a full-length product in the native gel could be due to polymerase, upon extending primer 1 to primer 2, either sliding over primer 2 (two products in the alkaline gel of 2.5 and 1 kb) or displacing primer 2 from the template to produce a continuous 3.5-kb extension product of primer 1 (3.5 kb in the alkaline gel). Secondly, if only alkaline gel analysis were performed, the appearance of 2.5- and 1-kb products could be due to polymerase extension of both primer 1 and 2 on the same template (full-length band in the native gel) or extension of these primers on separate templates (the two faster migrating products on the native gel).

The first experiment, shown in Fig. 5*A*, is a control reaction in which primer 2 contained no secondary structure at all. On this template, Pol III holoenzyme is expected to extend primer 1 until it encounters primer 2, and then the enzyme should simply slide over primer 2 and extend it into a full-length product that will appear as two fragments on the alkaline gel. The result, in Fig. 5*A*, is in keeping with this expectation. Full-length product is observed on the native gel (*top*), and the alkaline gel (*bottom*) shows two fragments of 2.5 and 1.0 kb.

Therefore, Pol III holoenzyme extended primer 1, diffused over primer 2, and extended it to the end of the template.

Next, we examined the ability of Pol III holoenenzyme to traverse duplex DNA containing secondary structure elements. In Fig. 5*B*, primer 2 contained either a 6-mer or 20-mer bubble. The replication products on the 6-mer bubble template showed a full-length species in the native gel, and both 2.5- and 1.0-kb fragments in the alkaline gel, indicating the enzyme diffused over the 6-mer bubble. This result is consistent with the earlier results of this report showing that β slides over a bubble as large as 15 nucleotides (Fig. 4*A*). Replication of the 20-mer bubble template showed both full-length and intermediate products of primer 1 extension on the native agarose gel. The intermediate sized product was produced first and represented an extension product of primer 1 that was paused or terminated at primer 2. This indicates that a 20-mer bubble, which was previously shown to block β sliding (Fig. 4*A*), poses an obstacle to DNA synthesis by the holoenzyme. However, at 12 s a full-length product appeared on the native gel, indicating that the polymerase only pauses at the 20-mer bubble and then diffuses over it. In this case, the alkaline gel analysis should show two products of 2.5 and 1.0 kb. Surprisingly, the alkaline gel showed no 1-kb fragment, only full-length 3.5-kb product and 2.5-kb fragment are observed. Therefore, the paused holoenzyme does not slide over the 20-mer bubble and sometimes displaces primer 2 producing a full-length 3.5-kb extension product of primer 1. This finding was not an artifact resulting from incomplete hybridization of primer 2 to the template; the primed templates used in all the replication assays described in Fig. 5 were analyzed by restriction enzyme digestion for the presence of both primer 1 and primer 2 (*i.e.* each primer contains a restriction enzyme site; data not shown).

It is interesting to note that products of the 20-mer bubble template show no fast migrating band on the native gel (*i.e.* 1-kb primer 2 extension product) or 1-kb product on the alkaline gel, suggesting that Pol III holoenzyme does not initially assemble onto the 20-mer bubble primer 2 (*e.g.* as in the *bottom diagram* in the *scheme* at the *top* of Fig. 5). The 1-kb primer 2 product is also missing from experiments using the 30-nucleotide loop template (Fig. 5*C*) and 12-bp stem-loop (Fig. 5*D*). We have looked into this in great detail and find that the underlying reason for this is that the holoenzyme cannot initiate synthesis on primed sites containing a large secondary structure located within 22 nucleotides from the 3′ terminus.[3] Consistent with this, the large bubble stem-loop and loop are located within 17–19 nucleotides upstream of the 3′ terminus. The 1-kb primer 2 extension product is observed using templates with small secondary structures and on the 5′ flap templates (over 30 bp to the 5′ flap).

Next, the holoenzyme was examined for ability to slide over a loop. In Fig. 5*C*, primer 2 contained a loop of either 5 or 30 nucleotides. Replication of the template with the 5-nucleotide loop resulted in mostly full-length product on the native gel. The corresponding alkaline gel shows that even before 12 s, the full-length product was composed of 2.5- and 1.0-kb fragments, indicating that Pol III holoenzyme is capable of rapidly diffusing over the 5-nucleotide loop. This result is consistent with results of the β clamp sliding assay, showing that β slides over a 13-nucleotide loop (Fig. 2*A*). Surprisingly, at 12 s and later, a strand displacement product (3.5 kb) is observed on the alkaline gel. Therefore, the polymerase often diffuses over the 5-nucleotide loop, but in a slower reaction it occasionally displaces the primer entirely.

Replication of the template containing a 30-nucleotide loop rapidly generates only full-length product in the native gel. The alkaline gel shows that the product is a continuous 3.5-kb fragment, indicating that Pol III holoenzyme, upon encountering a loop too large for β to diffuse over, efficiently displaces the oligonucleotide containing the 30-nucleotide loop.

In Fig. 5*D*, primer 2 contained either a 4- or 12-bp stem-loop. Replication of the template containing the 4-bp stem-loop generated primarily full-length product in the native gel. The corresponding alkaline gel shows that this was the combined result of diffusion over the primer (2.5 kb + 1.0 kb) and strand displacement (3.5 kb). Strand displacement took place very early in the replication assay because products are observed at 9 s, indicating that Pol III holoenzyme can rapidly displace stem-loop-containing primers. Nevertheless, the alkaline gel analysis indicates that at least half the time the polymerase diffuses over the stem-loop instead of displacing it (compare the intensity of the 2.5-kb product to 3.5-kb product). Pol III holoenzyme diffusion over a small loop is consistent with the ability of β to slide over duplex DNA containing a short stem-loop (shown in Fig. 1).

Increasing the size of the stem-loop to 12 bp resulted in both 2.5- and 3.5-kb extension products in the native gel (Fig. 5*D*). The 2.5-kb extension product indicates Pol III holoenzyme pauses or dissociates upon encounter with the 12-bp stem-loop. No 1.0-kb product is detected, indicating that the 2.5-kb product is the result of pausing or termination. This result is consistent with the finding that β cannot slide over this size of stem-loop (Fig. 1). The alkaline gel shows most of the radioactivity is present in the 3.5-kb full-length product, demonstrating that Pol III holoenzyme often displaces primer 2.

In the experiment of Fig. 5*E*, primer 2 contained either a 4- or 30-nucleotide 5′ flap. Replication of the template with the 4-nucleotide 5′ flap resulted in both 2.5- and 3.5-kb extension products of primer number 1 on the native gel. The 3.5-kb full-length product on the native gel mostly splits into the 2.5- and 1.0-kb fragments in the alkaline gel. This result indicates that Pol III holoenzyme, upon extending primer 1 and encountering primer 2, usually diffuses over the 4-nucleotide 5′ flap and then extends primer 2 to the end. This result is consistent with the earlier experiment showing that β slides over a 4-nucleotide 5′ flap (Fig. 3*A*). However, the native gel also shows some 2.5-kb product, which indicates that Pol III holoenzyme does not always slide across the 4-nucleotide 5′ flap but sometimes pauses or terminates replication at this position.

Replication of the template containing a 30-nucleotide 5′ flap also showed both 2.5- and 3.5-kb primer 1 extension products on the native agarose gel (Fig. 5*E*). Because β does not appear capable of sliding over a 30 nucleotide 5′ tail (Fig. 3), one would expect that the full-length products observed in this reaction were derived from displacement of primer 2 containing the 30-nucleotide 5′ tail. Consistent with this expectation, the presence of 3.5- and 2.5-kb fragments in the alkaline gel indicates that holoenzyme sometimes terminates extension of primer 1 at primer 2 and at other times strand displaces primer 2. A 1-kb product is also present in the alkaline gel, although this need not necessarily arise from sliding over primer 2 because Pol III holoenzyme appears fully capable of initially assembling onto the 30-mer tailed primer 2 as indicated by the 1-kb product of primer 2 extension in the native gel (unlike the largest loop, stem-loop, and bubble primer 2). However, we cannot rigorously exclude the possibility that Pol III holoenzyme sometimes slides over the 30-mer flap to produce full-length products composed of 2.5- and 1-kb fragments. Indeed, the rather low level of 3.5-kb product in the alkaline gel relative to full-length product in the native gel suggests that holoenzyme may sometimes slide over primer 2, producing 2.5- and 1.0-kb prod-

[3] N. Yao and M. O'Donnell, submitted for publication.

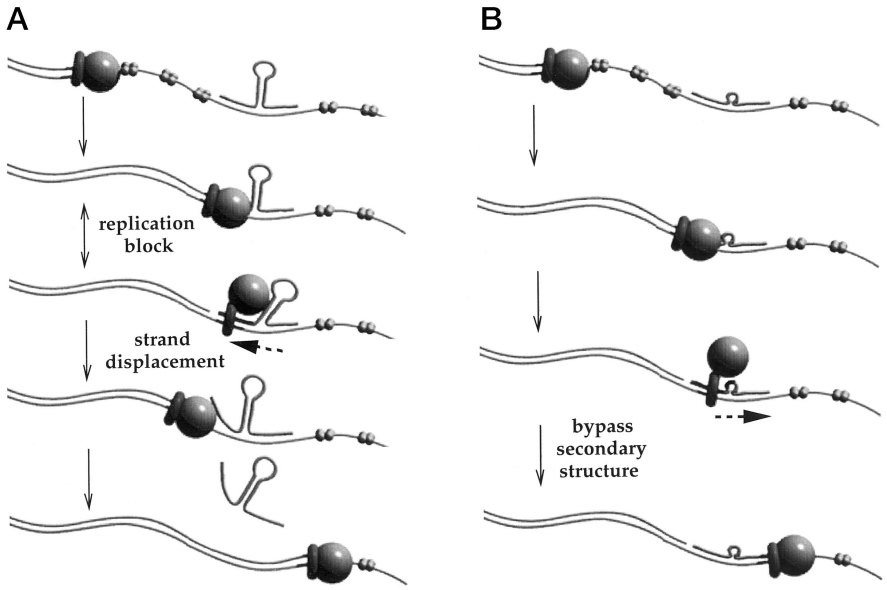


FIG. 6. **Model illustrating Pol III holoenzyme dynamics upon encounter with DNA secondary structures.** *A*, Pol III holoenzyme encounters a duplex section containing an internal DNA secondary structure that is too large for the β clamp to slide over. In this case, the polymerase presumably tries to slide across the duplex but is blocked. The enzyme then reestablishes contact with the original 3′ terminus and extends it, thereby displacing the duplex DNA containing the secondary structure obstacle and continues synthesis to the end of the template. *B*, Pol III holoenzyme simply slides over a duplex in its path that contains a secondary structure element that is small enough for the β clamp to slide over. The polymerase then resumes synthesis on the 3′ terminus of this oligonucleotide, extending it to the end of the template.

ucts in the alkaline gel. In conclusion, the experiments of Fig. 5 demonstrate that structures that β cannot slide over generally result in strand displacement of the oligonucleotide containing the blocking DNA structure, with pausing or termination at the blocking site occurring less frequently.

DISCUSSION

β and PCNA Slide Over Small DNA Secondary Structures—This study shows that β and PCNA are capable of sliding over a 4-bp stem-loop, a 13-nucleotide loop, a 4-nucleotide 5′ flap, and a 15-mer bubble, demonstrating that the inner diameter of these clamps is large enough to accommodate structures with cross-sectional widths larger than duplex DNA. Crystal structures of β and PCNA show inner diameters of ~35 and ~34 Å, respectively, (not including extended side chains), which should easily accommodate a B-form DNA duplex or A-form RNA-DNA heteroduplex (~18 and ~21 Å, respectively) (7, 36, 18). Why is the inner diameter of these clamps apparently larger than needed to encircle duplex DNA? Perhaps water, which occupies extra space between the inside of the ring and DNA, may aid the sliding motion of these clamps along the duplex. Another possibility, or at least a consequence of a ring that encircles DNA with room to spare, is the ability to slide over DNA secondary structures of a certain size. Possible *in vivo* functions of sliding over such structures are discussed below.

Pol III Holoenzyme Strand Displaces at DNA Structures β Cannot Slide Over—This study demonstrates that *E. coli* Pol III holoenzyme performs strand displacement synthesis upon encounter with a duplex containing a large secondary structure that β cannot slide across. Most of the past studies have not observed this strand displacement activity, apparently because of the use of primers that were fully annealed to the template. However, a few earlier studies have detected strand displacement by Pol III holoenzyme, particularly on DNA containing secondary structure. In a detailed earlier study, Stephens and McMacken (54) observed strand displacement activity by Pol III holoenzyme in a rolling circle replication assay (in the absence of DnaB helicase). The template used in their study was a circular nicked duplex DNA with a 758-nucleotide single-stranded DNA tail at the 5′ terminus of the nicked strand. The strand displacement activity of Pol III holoenzyme may be facilitated by the presence of a single-stranded 5′ tail in the strand that will be displaced. Strand displacement through a stem-loop structure was reported by Canceill and Ehrlich (55) in their study of copy choice recombination by Pol III holoenzyme. The template used in that study was a singly primed ssDNA circle containing a stem-loop (300-bp stem and 1370-nucleotide loop). Finally, Liu *et al.* (56) observed that a 41-mer oligonucleotide annealed inside a 42-mer single-stranded bubble on a double strand circular template (*i.e.* a D-loop) could be extended by Pol III holoenzyme to the full-length of the template in the absence of added helicase or other primosomal proteins, suggesting that Pol III holoenzyme could strand displace.

The present study shows strand displacement synthesis by Pol III holoenzyme is the result of the polymerase encountering a DNA secondary structure that the β clamp cannot traverse. In fact, the blocking secondary structure does not need to be at the 5′ end but can be set in from the 5′ end and still lead to strand displacement. The model shown in Fig. 6 proposes a relationship between the dynamics of polymerase/clamp sliding and strand displacement action upon encountering a duplex containing an internal DNA secondary structure.

Upon encountering the 5′ end of a duplex structure in the path of synthesis, the catalytic core releases the 3′ terminus, and the holoenzyme slides up onto the duplex remaining attached to DNA via the ring. This action underlies the ability of the holoenzyme to replicate multiprimed templates starting at a unique position without dissociating from DNA (37). Given 1 s of time, the Pol III* will depart from the β ring on DNA (13). Therefore, if the duplex to be traversed is sufficiently long, Pol III* will dissociate and β will need to slide by itself, and then Pol III* may reassociate with β upon arrival at the 3′ terminus. For short distances, however, Pol III* remains bound to the clamp as the clamp diffuses along the duplex.[4] If the duplex contains only a small secondary structure that the clamp can slide over, the holoenzyme presumably diffuses over it, as it would on a fully annealed duplex, to the next 3′ hydroxyl end and resumes elongation (as in Fig. 6*B*). However, if the secondary structure is too large for the clamp to slide over, the holoenzyme relocates back to the original 3′ end abutting the 5′ end of the duplex with the internal secondary structure (as in Fig. 6*A*, *third* and *fourth diagrams*). At this point, or after repeated trials, the polymerase succeeds in extending the 3′ terminus, resulting in lifting the 5′ end (*e.g.* perhaps simply taking advantage of thermal melting). It is possible that during

[4] J. Anjelkovic and M. O'Donnell, unpublished result.

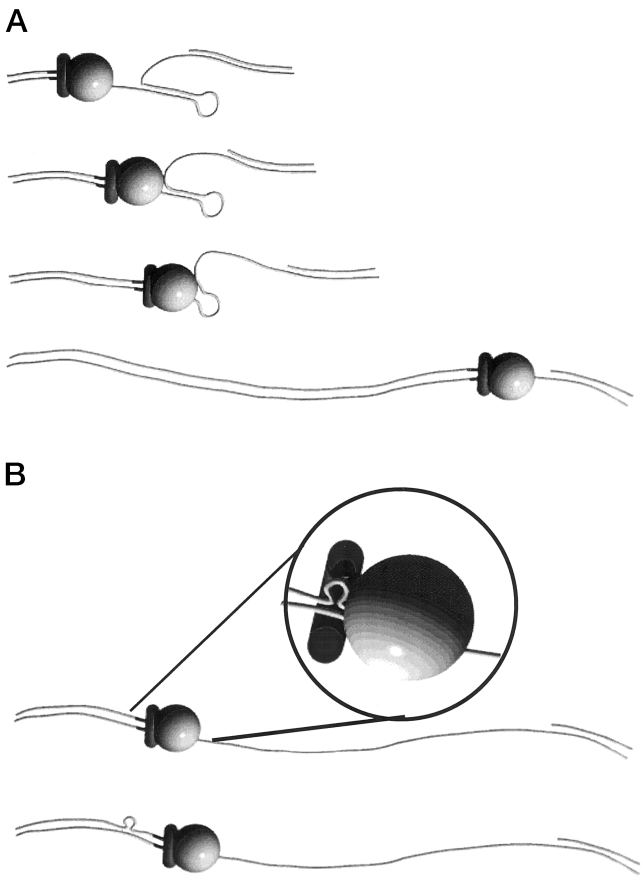


FIG. 7. **Possible *in vivo* consequence of Pol III holoenzyme dynamics.** *A*, the strand displacement activity of Pol III holoenzyme may be useful for removal of secondary structures (*e.g.* a stem-loop illustrated in the Fig.) on the lagging template strand during elongation that are not removed by SSB. *B*, ability or inability to slide over secondary structures made by strand slippage of the 3′ terminus may result in leaving an insertion or deletion (*e.g.* a an insertion in the figure) in the newly synthesized strand. A strand slippage event that produces a structure that is too large for the polymerase to traverse may result in inability of polymerase to proceed until the slipped strand is corrected.

the process of strand displacement, Pol III* repeatedly dissociates and reassociates with β. Although this may not occur during displacement of short stretches of DNA (*i.e.* <50 bp), this is especially likely for extensive strand displacement action, because in the absence of DnaB helicase Pol III* rapidly dissociates from β on templates with a 5′ tail (57).

When Would Limited Strand Displacement Synthesis by Chromosomal Replicases Be Needed in Vivo?—Limited strand displacement synthesis by Pol III holoenzyme could be a useful property in situations where replication is impeded by a DNA secondary structure, but a helicase is not present on the DNA to remove it. For example, some secondary structures on ssDNA may not be removed by SSB. Thus, during lagging strand synthesis on the *E. coli* chromosome, Pol III holoenzyme could remove hairpin or stem-loop structures that form on the ssDNA template strand in front of the elongating polymerase (Fig. 7*A*).

A particular defined case of this action may be found in phage G4 replication. The single-stranded chromosome of G4 phage can be replicated using only primase (DnaG), SSB, and Pol III holoenzyme (58). The G4 origin of replication is comprised of three potential stem-loop structures, resistant to SSB melting and important for primer synthesis by primase (59, 60). The primer is complementary to the most upstream stem-loop. Extension of this primer therefore requires Pol III holoenzyme to melt the two remaining downstream stem-loops (19 and 5 bp long) of the origin region.

Strand displacement activity has also been observed in eukaryotic and T4 replicases (61, 62). The removal of RNA primers during Okazaki fragment maturation in human cells has been proposed to involve strand displacement synthesis by DNA polymerase δ holoenzyme to produce a 5′ tail (*i.e.* flap) containing the RNA portion of the primer, and then FEN-1 endo/exonuclease cleaves these 5′ flap structures, thus removing the RNA primer (26). PCNA directly interacts with FEN-1 and stimulates the nuclease activity (63, 64). Therefore, the PCNA clamp, perhaps left on the template by Pol δ in analogy to the *E. coli* system, will be localized to the flap for action with FEN-1, by virtue of inability of PCNA to slide over the flap.

Replicative polymerases may also encounter secondary structure formed by strand slippage of the 3′ terminus during synthesis. Strand slippage that results from misalignment of the nascent 3′ terminus on the template strand during replication can lead to insertion and deletion mutations (65). The evolution of reiterative sequences such as mono-, di-, and trinucleotide repeats have been proposed to occur via strand slippage (66). The product of strand slippage that forms during synthesis is a loop (or possibly a stem-loop depending on the sequence) formed just behind the polymerase. If the clamp is unable to slide over the loop, the polymerase will be unable to continue elongation and may dissociate from the template prematurely, requiring the action of repair machinery to restart replication. In this case, the clamp that is left behind may pose as a convenient marker to signal certain repair proteins to the site of the mutagenic loop.

If the size of the loop generated by strand slippage is small enough for the clamp to slide over (≤15 nucleotides), then the holoenzyme may simply ignore the error and continue chain elongation (Fig. 7*B*). In this case, some form of post replication repair will be needed to correct the error. It has been previously shown that the human homologue of *E. coli* MutS, hMSH2, can bind to duplex oligonucleotides containing short (5–16 nucleotides) insertion/deletion loops (30). This repair protein has also been demonstrated to bind PCNA (29, 67). Perhaps the interaction between hMSH2 and PCNA can result in DNA polymerase δ, bound to the clamp, excising DNA back to the site of the mutagenic loop, and thus participate in its removal and repair. Further studies should reveal the role of sliding clamp dynamics over DNA secondary structure elements in replication fidelity, recombination, and repair.

Acknowledgments—We are grateful to Dr. Susan Taylor for the catalytic subunit of the cAMP-dependent protein kinase, Dr. Manju Hingorani for the reconstituted γ complex, and Maija Skangalis and Jeff Finkelstein for Pol III*.

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