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)† 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: δ, δ′, χ, and ϕ) 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 trimere with 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
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 hampered 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 loops, folds, 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), BioGel 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, θ (38), β, 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), δ’ (7), γ’ (7), and θ (41) were purified from E. coli whole cell extracts 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 0.15 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-Sepharose column (Bio-Rad) equilibrated in buffer F plus 150 mM NaCl. The SSB was eluted with a 1.5-liner 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 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 F from 10 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 5 mM MgCl2. Buffer C is 20 mM Tris-HCl (pH 7.5), 8 mM MgCl2, 4% (v/v) glycerol, 0.5 mM EDTA, 100 μg/ml BSA, 2 mM DTT, and 50 mM NaCl. BmHI 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.

Construction of M13EBNA1 Phage
A single 18-bp EBNA1-binding site sequence was inserted into a M13mp18 vector (Roche Molecular Biochemicals) by polymerase chain reaction amplification. The upstream primer 5’TACGATTTTTAAAAAGGCTATACCTCACCTTCTGAGGCGTACGGCAA-AAG-3’ has an EcoRI site (underlined) that is also present in the M13mp18 vector as well as a unique NdeI site (bold and underlined) located within the single EBNA1-binding site sequence (shown in bold). The downstream primer 5’ATTGAGAGATCTCAAAAGGCTATCAG-3’ is complementary to the M13mp18 template sequence and hybridizes over a BglII 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 EcoRI and BglIII. 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 BglII and gel purified. The construct 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 on ice and vortexed 13 × 100-mn times, then allowed to cool to room temperature (~32 °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 formed either 15 or 20-base ‘bubbles’ (i.e. continuous mismatches; shown in boldface in Table I) flanked by duplex regions 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 HincII site for linearization of the circular primed templates (Table I, underlined). Formation of Templates Hybridxed with “5’ Flag” Oligonucleotides—The 5’ flag template contains two oligonucleotides. The first has a 5’ tail comprised of either 4, 10, or 28 T residues (Table I, boldface).
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.

### Table I

<table>
<thead>
<tr>
<th>Oligonucleotides used in the study of β and PCNA sliding over DNA containing secondary structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Bubble-15</td>
</tr>
<tr>
<td>Bubble-20</td>
</tr>
</tbody>
</table>
| 5' Flap-4 nucleotide | 6191 | ScrFI | TTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Sliding Clamp Dynamics

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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Mag position (5′ end)</th>
<th>Sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>6190</td>
<td>CCGTCACAAATTCCACACAAAATCGACGCC</td>
</tr>
<tr>
<td>No 3 structure 2</td>
<td>3628</td>
<td>AAACAAATGTTACACATGGCTACTGCAAGACGCC</td>
</tr>
</tbody>
</table>
| Flap 2–4 nucleotide | 3628             | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
indicate that $\beta$ slowly slides off this DNA and that only half of the $\beta$ 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 $\beta$ to slide off the DNA (relative to a similar further incubation of the circular template). Hence, $\beta$ can slide over an 8-bp stem-loop, but slowly. The results using the 4-bp stem-loop, in the left panel of Fig. 4A, indicate that $\beta$ is capable of sliding over this 4-bp stem-loop structure (11.4% remained relative to the circular DNA control).

In Fig. 1B, 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 SbaI or SbaI storage buffer. Gel filtration analysis, in the right panel in Fig. 1B, indicates that 75% of $^{32}$P-PCNA was retained on the DNA, similar to the result using $^{32}$P-$\beta$. 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. 1B, 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 $\beta$. The result using a 4-bp stem-loop, in the left panel of Fig. 1B, 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, $\beta$ 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 $\beta$ 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 XbaI 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 $\beta$ can slide off the linearized DNA provides an assay for the ability of $\beta$ to slide over loops.

In the experiment shown in the right panel of Fig. 2A, $^{32}$P-$\beta$ was assembled onto the 25-nucleotide loop template using $\gamma$ 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 XbaI, whereas XbaI 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-$\beta$ that remained bound to the DNA. The results demonstrate that nearly 80% of the $^{32}$P-$\beta$ 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. 2A, respectively). The results show that a 20-nucleotide loop is still an effective barrier; approximately 80% of the $^{32}$P-$\beta$ remained on the linear DNA compared with the circular DNA control.

However, the $\beta$ clamp was capable of sliding over the 13-nucleotide loop; nearly 90% of the $\beta$ 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. 2B. The results demonstrate that $^{32}$P-PCNA is unable to slide over the 25-nucleotide loop; 64% of PCNA is retained on linear DNA.
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* II 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* II to generate a linear molecule 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 tagged 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 the 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. 3A, 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. 3A, 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. The results, shown in Fig. 3C, 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. 3B, 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 HindII 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. 4A, 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 heparinase/glucose and divided. HindII was added to one half, and HindII 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 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. 4A, 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).

In Fig. 4B 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 that β cannot traverse.
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 [α-³²P]dCTP (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).

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-³²P]dCTP (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). 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. 5A, 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. 5A, 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 holoenzyme to traverse duplex DNA containing secondary structure elements. In Fig. 5B, 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. 4A). 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. 4A), 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. 5C) and 12-45 stem-loop (Fig. 5D). 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. 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. 5C, 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. 2A). 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. 5D, 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. 5D). The 2.5-kb extension product indicates Pol III holoenzyme pauses or dissociates upon encountering 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. 5E, 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. 3A). 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. 5E). 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.
encounter with a duplex containing a large secondary structure. III holoenzyme performs strand displacement synthesis upon 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.

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.

**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. 6B). 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. 6A, 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.
May be useful for removal of secondary structures (a stem-loop, e.g., dynamics. A, is corrected. May result in inability of polymerase to proceed until the slipped strand produces a structure that is too large for the polymerase to traverse (figure) in the newly synthesized strand. A strand slippage event that result in leaving an insertion or deletion (secondary structures made by strand slippage of the 3′ terminus may not be removed by SSB.

Sliding Clamp Dynamics

A particular defined case of this action may be found in A particular defined case of this action may be found in human cells. 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 endonuclease 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 repetitive 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. 7B). 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 AMP-dependent protein kinase, Dr. Manju Hinogarani for the reconstituted γ complex, and Maija Skangalis and Jeff Finkelson for Pol III*. 

REFERENCES

NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

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

Nina Yao, Jerard Hurwitz and Mike O'Donnell
doi: 10.1074/jbc.275.2.1421

Access the most updated version of this article at http://www.jbc.org/content/275/2/1421

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 66 references, 41 of which can be accessed free at http://www.jbc.org/content/275/2/1421.full.html#ref-list-1