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An Explanation for Lagging Strand Replication: Polymerase Hopping among DNA Sliding Clamps

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Summary

The replicase of E. coli, DNA polymerase III holoenzyme, is tightly fastened to DNA by its ring-shaped β sliding clamp. However, despite being clamped to DNA, the polymerase must rapidly cycle on and off DNA to synthesize thousands of Okazaki fragments on the lagging strand. This study shows that DNA polymerase III holoenzyme cycles from one DNA to another by a novel mechanism of partial disassembly of its multisubunit structure and then reassembly. Upon completing a template, the polymerase disengages from its β clamp, hops off DNA, and reassociates with another β clamp at a new primed site. The original β clamp is left on DNA and may be harnessed by other machineries to coordinate their action with chromosome replication.

Introduction

The replicative polymerases of humans, yeast, Escherichia coli, and bacteriophage T4 are highly processive multiprotein machines (Kornberg and Baker, 1992). Their source of processivity is a sliding clamp subunit that completely encircles DNA and tethers the polymerase to the template (O'Donnell et al., 1992; Kuriyan and O'Donnell, 1993). The ten-subunit replicase of E. coli, DNA polymerase III holoenzyme (Pol III holoenzyme), contains two DNA polymerases, two sliding clamps, and a complex that assembles clamps around DNA (Kornberg and Baker, 1992). These activities have been resolved from the holoenzyme and studied individually. The core polymerase, a heterotrimer of α (DNA polymerase), ε (proofreading 3′–5′ exonuclease), and θ, only extends a primer a few nucleotides before dissociating from DNA (Fay et al., 1981). Core polymerase becomes remarkably rapid (>500 nt/s) and highly processive when associated with the β sliding clamp (Stukenberg et al., 1991). In the Pol III′ assembly, two core polymerases are held together through interaction with the two protomers of a τ dimer (McHenry, 1982; Studwell and O'Donnell, 1991). Two polymerases within one structure fits nicely with the hypothesis that polymerases act in pairs for coordinated synthesis of both strands of a chromosome (Sinha et al., 1980). The β clamp does not assemble around DNA by itself, for this it needs the ATP-dependent clamp-loading activity of the five-subunit γ complex ($\gamma\delta\delta'\chi\psi$) (Maki and Kornberg, 1988; O'Donnell, 1987). The nine-subunit Pol III* assembly contains one γ complex clamp loader connected to Pol III′ (Maki et al., 1988; Onrust, 1993).

The replicases of eukaryotes and phage T4 also utilize a sliding clamp protein and a clamp-loader complex for high processivity (Kuriyan and O'Donnell, 1993). The sliding clamp of human DNA polymerase δ is the proliferating cell nuclear antigen (PCNA) protein, which is assembled onto DNA by the five-subunit replication factor-C (RF-C) complex (Hurwitz et al., 1990). The T4 polymerase sliding clamp is the gene 45 protein (g45p) and is assembled onto DNA by the gene 44/62 protein complex (g44/62p) (Nossal and Alberts, 1983; Gogol et al., 1992).

A sliding protein ring to tether a polymerase to DNA may explain the high processivity needed during continuous extension of the leading strand. However, this tight grip on DNA would presumably hinder polymerase action on the lagging strand, where the DNA is synthesized discontinuously as thousands of Okazaki fragments. As there are only 10–20 molecules of Pol III holoenzyme in the cell, there must exist a mechanism to rapidly recycle Pol III holoenzyme for repeated action on multiple lagging strand fragments. However, Pol III holoenzyme appears to be slow in dissociating from replicated DNA for recycling to other primed templates (Burgers and Kornberg, 1983). This prompted the idea that Pol III holoenzyme may slide from one fragment to the next and thereby could be recycled without dissociating from the lagging strand. The test of this sliding hypothesis showed that Pol III holoenzyme could indeed slide on duplex DNA; however, it could not slide over single-stranded DNA (ssDNA), thus blocking the access of the polymerase to upstream primers (O'Donnell and Kornberg, 1985).

The mechanism of Pol III holoenzyme cycling from one DNA to another is the subject of this report. The study shows that Pol III holoenzyme remains firmly fixed to the primed template as long as there is still DNA to be replicated. But upon finishing a template to a nick, the Pol III* assembly rapidly disengages from the β sliding clamp and dissociates into solution, leaving the β clamp behind. Pol III* then rapidly reassociates with a new primed template, provided a β clamp has been preassembled onto the primed site by the γ complex clamp loader. This mechanism predicts that during replication Pol III* dissociates from its sliding clamp upon completing an Okazaki fragment and reassociates with a new sliding clamp at the upstream primer for the next fragment (Figure 1).

Results

Pol III Holoenzyme Cycles to a β Clamp on DNA

A model system to study polymerase cycling on the lagging strand utilizes two circular bacteriophage genomes of different sizes, each primed with a single DNA oligonu-

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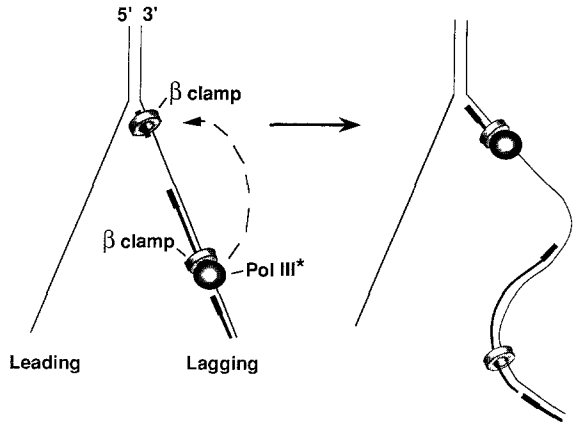


Figure 1. Scheme of Polymerase Cycling on the Lagging Strand

Pol III holoenzyme is held to DNA by the β sliding clamp for processive extension of the Okazaki fragment (left). After finishing the fragment, Pol III* disengages from its β clamp and cycles to a new clamp on an upstream RNA primer (right). The original β clamp is left behind on the finished Okazaki fragment.

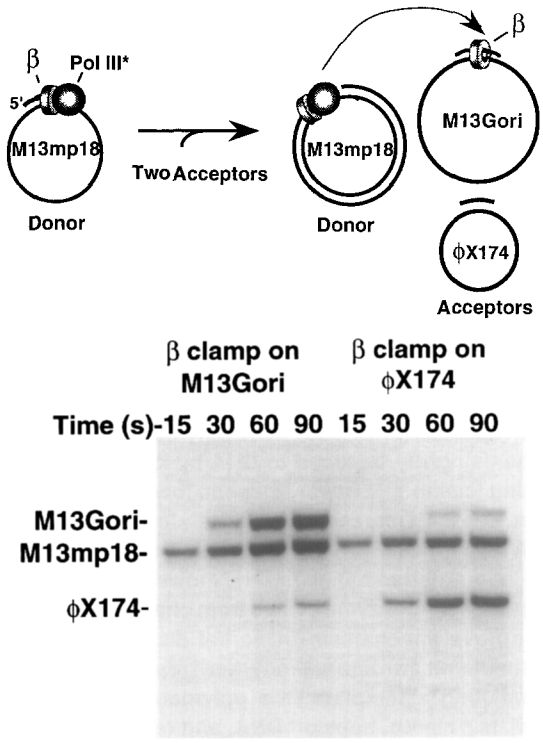


Figure 2. Pol III Holoenzyme Cycles Specifically to a Primed DNA Containing a β Clamp

Pol III holoenzyme was assembled on a primed M13mp18 donor and then mixed with two acceptor DNAs, only one of which contains a β clamp. The polymerase replicates the donor M13mp18 and then cycles to the acceptor that contains a β clamp (see scheme at the top). Lanes 1–4 show the time course of polymerase cycling from M13mp18 to an equal mixture of M13Gori with a β clamp and φX174 without a β clamp. Lanes 5–8 show polymerase cycling from M13mp18 to a mixture of φX174 with β and M13Gori without β. Reactions were at 30°C, and since two acceptors were used the volume was increased by 50%.

cleotide and "coated" with ssDNA-binding protein (SSB) (O'Donnell, 1987). Pol III holoenzyme is assembled onto one template (donor), mixed with another primed template of different size (acceptor), and replication is monitored in a neutral agarose gel that resolves the donor and acceptor DNAs. An earlier study exploiting this system showed that Pol III holoenzyme rapidly transfers from the completed donor to the acceptor DNA only in the presence of added accessory proteins, β and γ complex (O'Donnell, 1987). There are two general mechanisms by which these accessory proteins could mediate this polymerase transfer event. They may dissociate Pol III holoenzyme from the replicated donor DNA, or they may act on the acceptor template, by assembling a β clamp at the primed site to attract an independently dissociated Pol III holoenzyme.

To distinguish between these possibilities, Pol III holoenzyme was assembled onto a primed M13mp18 ssDNA (donor), and then two acceptors were added, only one of which had been pretreated with β and γ complex (e.g., to form a β clamp; see the scheme in Figure 2). If Pol III holoenzyme dissociates from the completed donor by itself and needs a preassembled β clamp on the acceptor, it should cycle preferentially to the acceptor containing the β clamp. On the other hand, if the accessory proteins act on the donor to extract Pol III holoenzyme from replicated DNA, then the polymerase may cycle to either acceptor with equal efficiency.

In the experiment of Figure 2, substoichiometric Pol III holoenzyme was assembled onto primed M13mp18 (donor), then replication was initiated and two acceptor templates of different sizes were added, M13Gori (large) and φX174 (small). The first four lanes show the time course of this reaction in which only the M13Gori acceptor was pretreated with accessory proteins. The donor M13mp18 is complete within 15 s, and the next product to appear is the M13Gori acceptor; very little φX174 acceptor is replicated. In the last four lanes of Figure 2, the experiment was repeated but with the small φX174 acceptor pretreated with β and γ complex, and this time Pol III holoenzyme preferred to cycle to it rather than to M13Gori. These results indicate that the accessory proteins accelerate cycling of Pol III holoenzyme to new templates by acting on the acceptor, presumably by forming a β clamp on it, rather than to extract Pol III holoenzyme from the replicated donor. However, this experiment does not exclude the possibility that accessory proteins also pry Pol III holoenzyme loose from the replicated donor. The next experiment shows that Pol III holoenzyme dissociates from replicated DNA without the help of added accessory proteins.

Pol III* Dissociates from the β Clamp upon Completing Replication

The fact that a β clamp must be preassembled on the acceptor for polymerase to transfer to it suggests that Pol III holoenzyme does not reuse its β clamp for successive rounds of synthesis. Presumably Pol III*, or a smaller subassembly such as Pol III′ or core, separates from its β clamp and cycles to the new clamp on the acceptor. To determine whether Pol III* and β dissociate from one an-

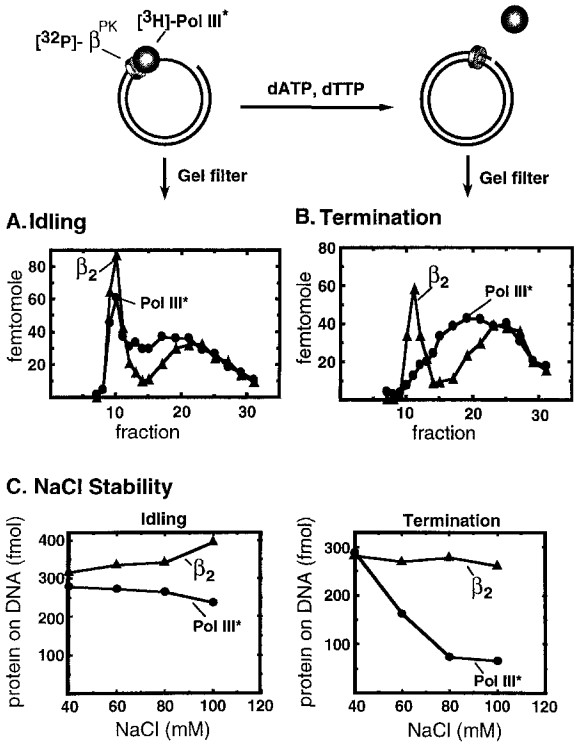


Figure 3. Pol III* Dissociates from the β Clamp upon Completing a Template

[^{3}H]Pol III* (labeled in θ) and [^{32}P]β^{PK} were assembled on gapped circular DNA under idling conditions (only two dNTPs present) (A), or with all four dNTPs to fill in the gap (B) as described in the Experimental Procedures. Reactions were gel filtered in column buffer containing 100 mM NaCl, and fractions were analyzed by dual channel scintillation. In (C), the reactions were gel filtered using different concentrations of NaCl in the column buffer. Triangles, [^{32}P]β^{PK}; circles, [^{3}H]Pol III*.

other after completing a template, we radiolabeled each to follow their fate. Pol III* was labeled in the core polymerase by reconstituting it from pure subunits using [^{3}H]θ. To follow the β clamp, we engineered a six-residue protein kinase recognition motif onto the C-terminus of β (referred to as β^{PK}) and labeled it with ^{32}P (β^{PK} retains full activity, Kelman et al., 1994). In the experiment of Figure 3, we followed the fate of these proteins while the polymerase was idling on DNA and upon complete replication of the template. In Figure 3A, [^{3}H]Pol III* was assembled with [^{32}P]β^{PK} on a plasmid with a ssDNA gap of approximately 500 nt. The reaction was then gel filtered in the presence of dCTP and dGTP to prevent DNA synthesis from closing the gap and exonuclease activity from removing the primer strand. Quantitation of the [^{32}P]β^{PK} and [^{3}H]Pol III* in the column fractions show they comigrate in stoichiometric amounts with the gapped DNA (fractions 9–11), excess protein over DNA elutes later (fractions 15–25). To observe binding of Pol III* to DNA, both ATP and β are needed and therefore the Pol III*–DNA interaction is specific to proper β clamp assembly (Table 1). In Figure 3B, the gap was closed by adding all four dNTPs before gel filtering the reaction. Most of the [^{3}H]Pol III* dissociated from the replicated DNA and eluted in the included fractions, but the [^{32}P]β^{PK} remained bound to DNA. Hence, the Pol III*, or at least the core polymerase within it, dissociates from DNA specifically upon completing replication of a template, while the β clamp is left behind on the DNA.

The [^{3}H]Pol III* in the experiment of Figure 3 was labeled in the θ subunit, and therefore it is possible that only core polymerase (or only [^{3}H]θ) dissociated upon closing the gap. Hence, the analysis was repeated using [^{3}H]Pol III* labeled in either the γ complex or the τ subunit. In each case, the [^{3}H]Pol III* remained bound to gapped DNA but dissociated from DNA upon closing the gap (Table 1). Hence, it appears that the entire Pol III* assembly dissociates from DNA upon completing replication and leaves the β clamp behind.

The DNA binding analysis of Pol III* and β in Figures 3A and 3B was performed using 100 mM NaCl in the gel filtration buffer. Figure 3C shows the result of using lower concentrations of NaCl during gel filtration. When the gel filtration buffer included 40 mM NaCl, the Pol III* remained bound even to replicated DNA (right panel). As the concentration of NaCl was increased, Pol III* dissociated after replicating the DNA (right panel) but not from the gapped, unreplicated DNA (left panel). Use of too little salt in earlier gel filtration studies probably led to conclusions that Pol III* remains bound to DNA even after replication of a circular template (Burgers and Kornberg, 1982; Studwell et al., 1990). Gel filtration in the presence of 160 mM potassium glutamate, the physiological osmolyte of E. coli (Richey

Table 1. Pol III* Separates from β after Replication

[^{3}H]Subunit in Pol III*	[^{3}H]Pol III* on Gapped DNA (fmol)	[^{3}H]Pol III on Replicated DNA (fmol)
Pol III* ([^{3}H]θ)	181	42
Pol III* ([^{3}H]τ)	102	27
Pol III* ([^{3}H]γ)	126	21
Pol III* ([^{3}H]θ), 160 mM glutamate	184	41
Pol III* ([^{3}H]θ), no ATP	34	42[a]
Pol III* ([^{3}H]θ), no β added	23	–[a]

The amount of [^{3}H]Pol III* bound to β while idling on gapped DNA, and after replicating the template, was determined by gel filtration in column buffer containing 100 mM NaCl (unless indicated otherwise) as described in the Experimental Procedures. The [^{3}H]Pol III* was constituted using either [^{3}H]θ, [^{3}H]τ, or [^{3}H]γ.

[a] Replication does not occur without β or ATP thereby precluding the measurement. However, to determine the extent to which Pol III* binds nonspecifically to the product of replication (i.e., a nick), Pol III* and β were added to a singly nicked M13mp18 plasmid in the absence of ATP and then gel filtered (42 fmol bound).

et al., 1987), also resulted in Pol III* dissociating from replicated DNA and therefore dissociation is not due to "chloride poisoning" (Table 1). The β clamp remained associated with both gapped and replicated DNA at all levels of NaCl tested (Figure 3C) and in 160 mM potassium glutamate (data not shown).

Fate of the β Clamp

When Pol III* cycles to a new β clamp on an acceptor, does the original β clamp remain bound to the donor DNA as the previous experiment implies? The following experiment was designed to directly answer this question. Pol III holoenzyme was assembled on a donor M13mp18 using [^{3}H]β, and in a separate tube an unlabeled β clamp was assembled on an acceptor φX174 using γ complex (see the scheme at the top of Figure 4). The donor and acceptor templates were mixed, and replication was allowed to proceed for 45 s at 37°C, sufficient time for Pol III holoenzyme to replicate the donor and cycle to and complete the acceptor. The reaction was then shifted to 4°C and gel filtered. Analysis of the column fractions (Figure 4A) shows that [^{3}H]β remains bound to DNA, presumably the donor, after Pol III* cycled to the unlabeled β clamp on the acceptor (the amount of [^{3}H]β in the excluded fractions is comparable to that in a control reaction performed using only two dNTPs to prevent full replication). To establish which DNA template the [^{3}H]β clamp was on after Pol III* cycled away, we took advantage of the fact that the β ring slides off linear DNA but not circular DNA (Stukenberg et al., 1991). Hence, after providing time for Pol III* to cycle to φX174, we linearized only the donor (Figure 4B) or only the acceptor (Figure 4C) with a 30 s restriction endonuclease treatment prior to gel filtration. Gel filtration after linearizing the donor, in Figure 4B, showed that the [^{3}H]β clamp had slid off DNA into solution and therefore must have been left on the donor as anticipated. Consistent with this interpretation, the [^{3}H]β clamp remained on DNA after linearizing the acceptor (Figure 4C).

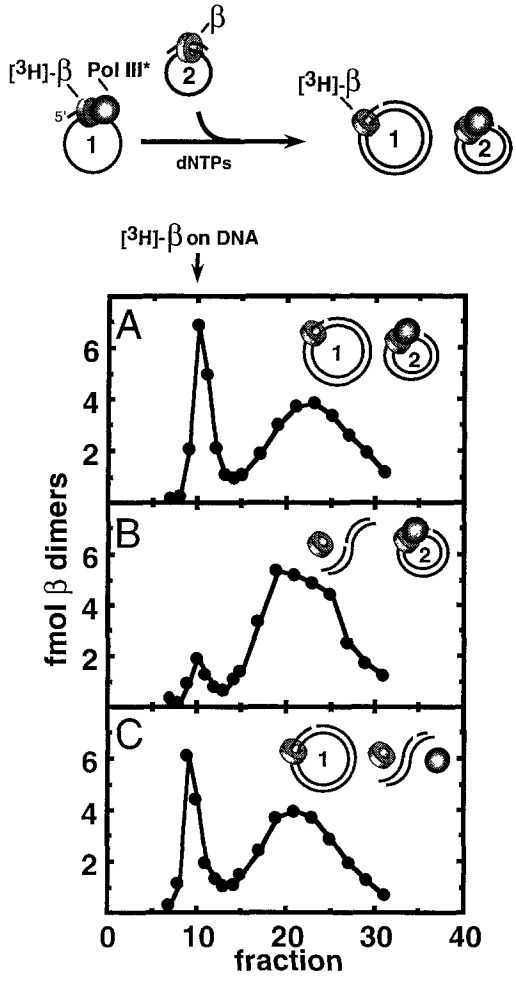


Figure 4. The β Clamp Remains on Donor DNA after Pol III* Cycles to the Acceptor

A [^{3}H]β clamp was assembled with Pol III* on a M13mp18 donor, and an unlabeled β clamp was assembled on the φX174 acceptor. After replication of the donor and cycling to the acceptor, the reaction was either gel filtered without further treatment (A), or treated with BamHI to linearize the donor before gel filtration (B), or with XhoI to linearize the acceptor before gel filtration (C).

A Kinase Protection Assay for Pol III* Dissociation from the β Clamp

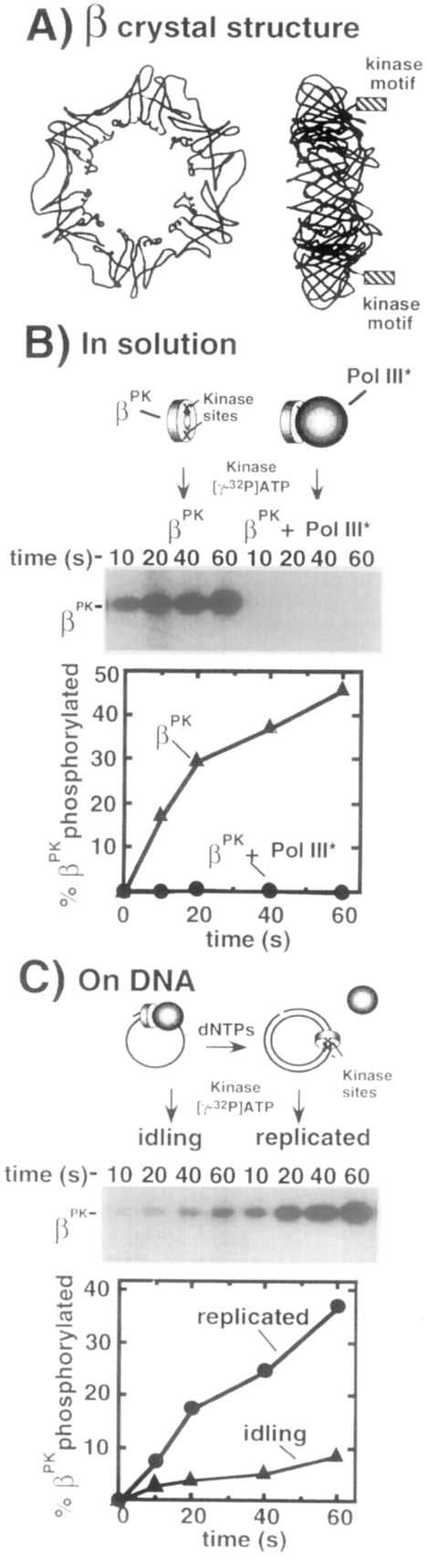
Upon completing a template, how rapidly does Pol III* dissociate from its β clamp? The time frame of gel filtration experiments is quite slow, approximately 20 min, and during this time the reaction is not at equilibrium. Perhaps the gel filtration technique causes Pol III* to dissociate from its β clamp. To examine the contact between β and Pol III* in real time and under equilibrium conditions, we developed a "kinase protection" assay. The structure of the β dimer is shown in Figure 5A. The two C-termini extrude into solution from the same face of the β ring, and it is on these termini that the kinase recognition sequence was placed in β^{PK} (stippled boxes in Figure 5A).

The time course in Figure 5B shows the phosphorylation of β^{PK} upon adding protein kinase and [γ-^{32}P]ATP. The extent of labeling of the C-terminal kinase motif is approximately 50% in 1 min (triangles, Figure 5B) (Kelman et al., 1994). However, upon addition of Pol III*, the kinase motif is completely protected from phosphorylation (circles, Figure 5B).

In Figure 5C, this kinase protection assay was used to probe the speed with which Pol III* dissociates from its β clamp upon completing DNA synthesis. The β^{PK} was first placed on primed M13mp18 ssDNA by Pol III* under idling conditions and then treated with kinase. As expected, the β^{PK} was protected from phosphorylation when associated in the Pol III holoenzyme structure (triangles). But upon replicating the template, β^{PK} was phosphorylated at a rate similar to β^{PK} in solution in the absence of Pol III* (compare the circles in Figure 5C with the triangles in Figure 5B), indicating that Pol III* rapidly dissociates from the β clamp upon completing replication.

DNA Is Replicated to the Last Nucleotide before Pol III* Cycles Away

The extent to which Pol III holoenzyme replicates the donor before it dissociates and cycles to the acceptor was deter-



mined by analyzing the product strand of a donor template in a sequencing gel. The analysis showed that Pol III* had extended the primer full circle until it was flush to the 5′ end of the starting oligonucleotide primer (Figure 6). This result implies that the signal for Pol III* to dissociate from DNA is produced upon or after replicating the DNA to a nick.

How Well Does This Model System Relate to In Vivo Conditions?

E. coli duplicates its 4.4 Mb chromosome in 30–40 min. Assuming this speed of 1 kb/s and lagging strand fragments of 1–2 kb, a new Okazaki fragment must be completed every 1–2 s. If Pol III* disengages from its β clamp upon completing one fragment and cycles to a new β clamp to start the next, as this study would suggest, then a new β clamp must be assembled every second or two, and Pol III* must also cycle to it within this time. The next two experiments were designed to test whether the assembly of β clamps and the pace of polymerase cycling are fast enough to account for the speed of lagging strand replication. In Figure 7, a two template cycling assay was performed in the presence of 160 mM potassium glutamate, and timepoints were analyzed in an agarose gel followed by autoradiography. Quantitation of the DNA products showed that Pol III holoenzyme replicated the ϕX174 donor and completed 4.5 copies of the M13mp18 acceptor within 90 s. Accounting for the time spent during DNA synthesis of these long ssDNA templates, each polymerase transfer event required approximately 1.1 s (calculated in the legend to Figure 7). This is faster than the 9 s reported in an earlier study and may be ascribed to more efficient dissociation of Pol III* from the β clamp (after replicating the DNA) in 160 mM potassium glutamate relative to buffer containing no added salt (O'Donnell, 1987). Further, we have shown previously that as the concentration of acceptor template is increased, Pol III holoenzyme cycles even faster (Studwell et al., 1990). On the lagging strand, multiple polymerase transfer events all occur on the same DNA molecule (i.e., it cycles from the end of one Okazaki fragment [donor] to a new RNA primer [acceptor] on the same strand). This unimolecular proxim-

Figure 5 Kinase Protection Assay of Pol III* Disengaging its β Clamp upon Completing Replication

The structure of the β dimer is shown in (A). The left view shows the central cavity and on the right the dimer is turned 90° to show the two C-termini that extrude from one side. The stippled boxes on the C-termini denote the six-residue kinase recognition sequence on β^{PK}.

(B) The β^{PK} was treated with protein kinase in the presence or absence of Pol III* (see scheme), and time points were analyzed in a SDS polyacrylamide gel followed by autoradiography. The first four lanes show the reaction lacking Pol III* and the last four lanes are with Pol III*. Quantitation of the autoradiogram is shown below. Triangles, absence of Pol III*; circles, presence of Pol III*.

(C) Pol III* and β^{PK} were assembled on primed ssDNA either in the presence of only two dNTPs (idling) or all four dNTPs (replicated) then kinase was added (see scheme) and timepoints were analyzed as in (B). The first four lanes are with only two dNTPs (idling) and the last four lanes are with all four dNTPs (replicated). Quantitation of the autoradiogram is shown below.

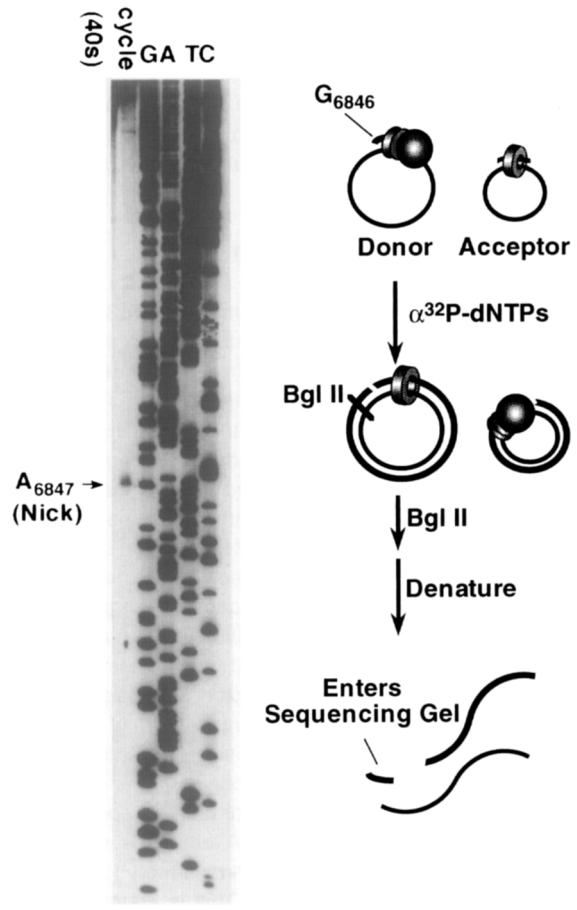


Figure 6. Pol III* Replicates a Primed Template to the Last Nucleotide before It Transfers to an Acceptor.

In the cycling assay, the donor template was M13mp18 ssDNA and the acceptor was ϕX174 ssDNA. After 40 s of replication using [^{32}P]TTP, the reaction was quenched with 1% SDS, 40 mM EDTA, the DNA was purified and digested with Bgl II, and the reaction was analyzed in a 0.6% sequencing gel (lane labeled "cycle"). The only fragment small enough to enter the gel is the fragment from the BglII site to the point at which Pol III* dissociates from its β clamp. The reference sizing ladder was produced using a synthetic DNA 20-mer annealed to M13mp18 at the BglII site.

ity of donor and acceptor on the lagging strand would increase their effective concentration and may result in a polymerase cycling time substantially less than 1 s.

Can β clamps be assembled fast enough, under conditions that mimic those inside the cell, to account for a new clamp for every Okazaki fragment (i.e., one clamp per second)? The β subunit is present at a level of 300 dimers per cell (Kornberg and Baker, 1992). Assuming a cell volume of 10^{-15} L, the intracellular concentration of β would be 500 nM. Likewise, the concentration of a single DNA molecule in E. coli, such as the chromosome, would be 1.7 nM. The intracellular level of the γ complex must exceed the 17–34 nM concentration of Pol III holoenzyme (10–20 molecules per cell) since in addition to the γ complex within Pol III holoenzyme, there is also an undefined amount of γ complex in free form (Maki and Kornberg, 1988). In Figure 8, we measured the speed of β clamp assembly on DNA in buffer containing 160 mM potassium glutamate, 500 nM [^{3}H]β, and 2 nM gapped DNA. Assembly reactions were performed at different concentrations of γ complex and allowed to proceed for no longer than 3 s. To terminate the reaction rapidly and gently without removing β clamps, sufficient hexokinase was added to consume the ATP within 0.1 s. The reaction was then gel filtered, and the amount of [^{3}H]β on DNA was quantitated by scintillation counting. The gel filtration analyses are shown in Figure 8A. In Figure 8B, this information has been reploted as a function of the number of β clamps assembled relative to the number of γ complex molecules

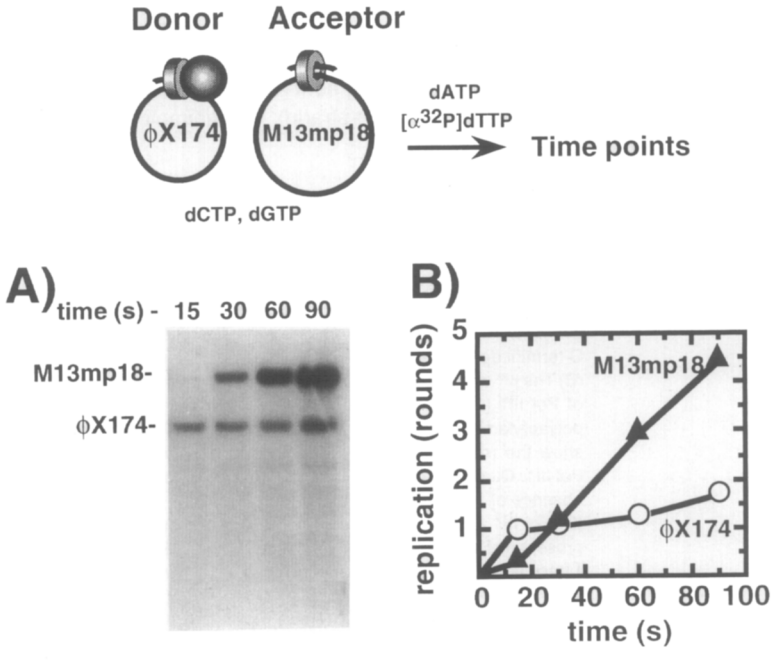


Figure 7. Time Course of Polymerase Cycling

The polymerase cycling assay was performed as described in the Experimental Procedures except 160 mM potassium glutamate (pH 7.5) and twice as much acceptor M13mp18 DNA was present and the temperature was 27°C. Positions of replicated ϕX174 and M13mp18 are indicated. In the plot at the right, the autoradiogram is quantitated in rounds of replication relative to the amount of ϕX174 produced in the first 15 s. The rate of DNA synthesis under these conditions is approximately 450 nt/s. Accounting for one ϕX174 circle (5386 nt) and 4.5 M13mp18 circles (4 × 7250 nt) at the 90 s timepoint, the enzyme spends approximately 85 s in DNA synthesis leaving approximately 1 s for each transfer event: (90s − 85s)/4.5 = 1.1.

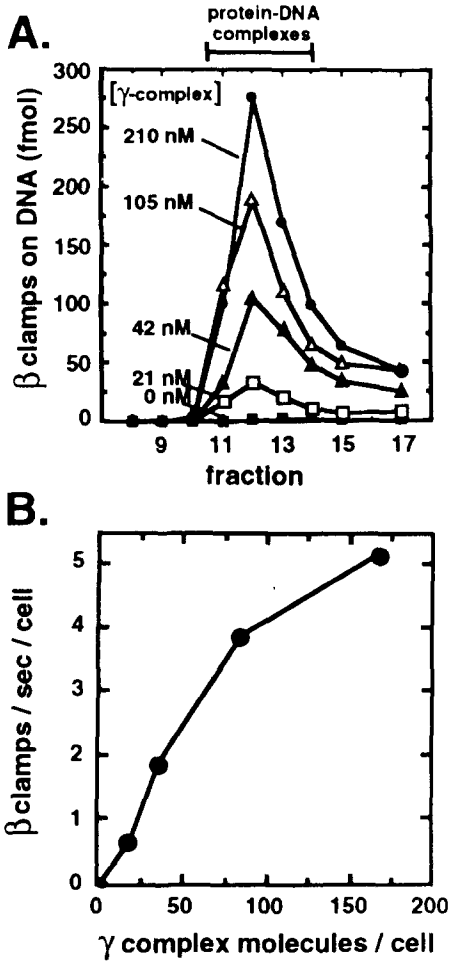


Figure 8. Assembly of β Clamps on DNA Is Rapid

The number of β clamps assembled on DNA within 3 s was measured near physiological concentrations of β, DNA, and salt. Assembly reactions were initiated upon adding γ complex (0–210 μM) to 25 μl final of reaction buffer at 37°C containing 500 nM β, 2 nM gapped DNA, 0.8 μg SSB, 160 mM potassium glutamate (pH 7.5), and 4 mM glucose. Within 3 s, reactions were quenched upon adding 1.25 U of hexokinase to remove ATP followed by gel filtration. In a mock experiment using 0.125 U hexokinase, 0.5 mM [γ-^{32}P]ATP is fully hydrolyzed within 1 s and therefore 1.25 U of hexokinase should hydrolyze the ATP within 0.1 s. (A) shows the gel filtration analysis of [^{3}H]β placed on DNA at different γ complex concentrations. (B) shows the number of β clamps assembled each second at the different concentrations of γ complex. To relate the results to the cell, the concentrations of β and γ complex have been converted to molecules per volume of an E. coli cell (10^{-15} L).

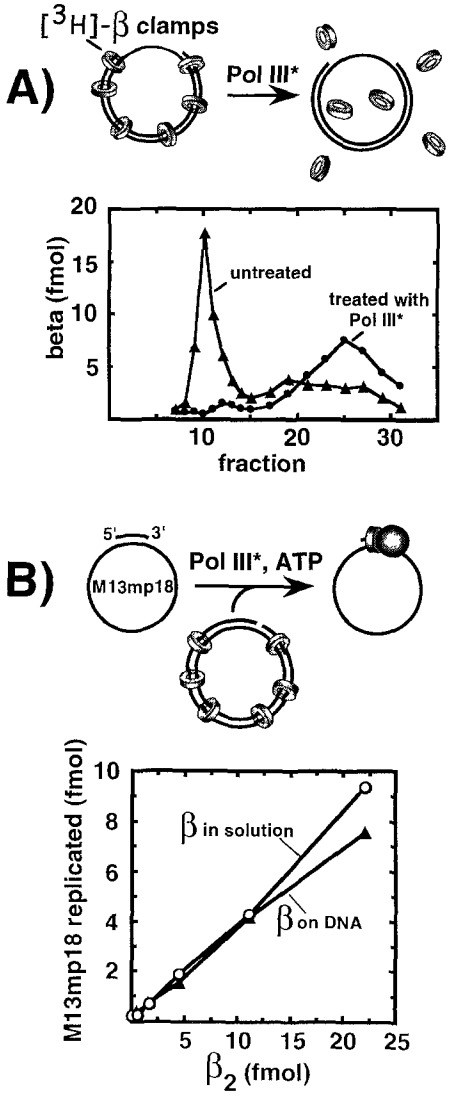


Figure 9. Pol III* Efficiently Recycles β Clamps on DNA

Multiple β clamps were assembled onto circular DNA and purified by gel filtration for use in studying the ability of Pol III* to remove them from DNA (A) and ability of Pol III* to reposition them onto primed M13mp18 ssDNA for replication (B) as described in the Experimental Procedures. In (A), the [^{3}H]β clamps on DNA were either gel filtered a second time without treatment (triangles) or after treatment with *Pol III** (circles). In (B), the [^{3}H]β clamps on DNA were titrated into a replication assay using Pol III* and primed M13mp18 ssDNA (triangles). The circles are a comparative titration using [^{3}H]β in solution.

present per 10^{-15} L of reaction, a volume comparable to an E. coli cell. The results show that the rate of β clamp assembly increases with the concentration of γ complex, and at 42 nM γ complex, one β clamp is assembled on DNA every ½ s. Hence, β clamp assembly appears rapid enough to account for use of a new β clamp for each Okazaki fragment, especially considering that the effective concentration of γ complex would be very high at a replication fork, since γ complex is held near the DNA by its presence in the Pol III holoenzyme structure.

Given that there are only 300 β dimers in a cell, if each of the 2000–4000 Okazaki fragments requires a β clamp, the available pool of β will soon be exhausted. Hence, orphaned β clamps will need to be removed from DNA and reused. The experiments of Figure 9 were designed to test the ability of Pol III* to remove β from DNA and reuse it. First, [^{3}H]β clamps were assembled onto gapped DNA by the γ complex followed by gel filtration to remove free [^{3}H]β and γ complex. The [^{3}H]β clamps encircling the DNA were then treated with Pol III* and gel filtered a second time (see scheme in Figure 9A). The results show that β clamps are stabile on DNA through the second gel

filtration column (triangles), but upon treatment with Pol III* most of the β clamps are removed from DNA (circles). Hence, Pol III* can release β clamps from DNA. Next, we asked whether Pol III* can reuse these β clamps. In Figure 9B, multiple [^{3}H]β clamps were placed on DNA and gel filtered as before, and then the β–DNA complex was titrated into an assay as the source of β for Pol III* in replicating primed M13mp18 ssDNA. The results show that Pol III* efficiently plucks β clamps from DNA for reuse on primed M13mp18, as the titration profile is similar to that of a titration using β in solution.

Discussion

A Model for Lagging Strand Replication

Using a two template model system, this study reveals a mechanism by which Pol III holoenzyme rapidly cycles from completed DNA to a new primed site. Upon replicating DNA to the last nucleotide, Pol III* loses its tight grip on the β sliding clamp and dissociates to solution leaving the clamp behind on DNA. Pol III* then rapidly associates with a new β clamp on another primed template. An important feature of the cycling reaction is that Pol III* remains tightly coupled to the β ring prior to completing DNA, such as when it is idling for lack of a complete set of dNTPs (O'Donnell, 1987). This feature provides a highly processive polymerase that could faithfully complete an Okazaki fragment before leaving for the next fragment.

In the model of Figure 10, these protein dynamics are shown in the context of a moving replication fork. Studies of the Pol III holoenzyme structure show that it is composed of two polymerase cores, two β clamps, and one γ complex (Maki et al., 1988; Studwell and O'Donnell, 1991; Onrust, 1993). The two core–β clamp units of Pol III holoenzyme are presumed to act concurrently for processive replication of both strands of DNA, and they are linked together by a τ dimer (McHenry, 1982; Studwell and O'Donnell, 1991), which also serves as the point of attachment for the γ complex (Onrust, 1993). The single γ complex imposes a structural asymmetry about the two core polymerases, and its catalytic clamp-loading activity is needed for the numerous reinitiation events on the lagging strand. In proceeding from diagram A to B, the γ complex assembles a β ring around a new primed site at the fork. Also in going from diagram A to B, the core polymerase on the lagging strand completes an Okazaki fragment to a nick thereby effecting its release from the β clamp and DNA. Loss of the β clamp results in a vacancy in the binding site for β on the core polymerase, a logical prerequisite for association of this core with a new β clamp on the upstream RNA primer. In proceeding from diagram B to C, the lagging strand core cycles to the new β clamp to initiate processive extension of the next Okazaki fragment.

This entire cycle of events must occur within a second or two. The studies of this report show that β clamps can be assembled onto DNA within 0.5 s under conditions as they exist in the cell and that Pol III* cycles within a second from a completed DNA to a new β clamp. An advantage to a polymerase with a separate clamp protein could be the ability to assemble a clamp at a new primed site while the polymerase is busy extending an Okazaki fragment as hypothesized in Figure 10 (diagrams A to B). This would save time if clamp assembly is rate limiting and may be reason enough to have evolved a separate processivity factor from the polymerase polypeptide chain. Whether Pol III holoenzyme can indeed assemble clamps while extending DNA is under investigation.

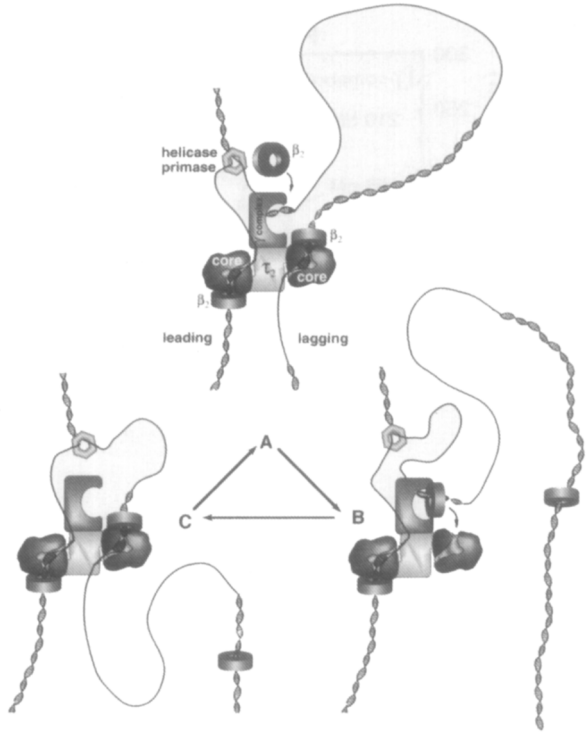


Figure 10. Model of DNA Polymerase III Holoenzyme at the Replication Fork

The helicase and primase are shown as a gray hexamer at the forked junction. The putative structure of Pol III holoenzyme has been placed at a replication fork such that the two core polymerases are duplicating the leading and lagging strands at the same time. The τ dimer holds together two core polymerases and one γ complex. The γ complex is positioned near the fork and is asymmetrically disposed relative to the two cores such that it points toward the lagging strand to repeatedly load β clamps on primers to initiate processive extension of Okazaki fragments. (A) As the lagging strand polymerase extends an Okazaki fragment, the γ complex assembles a β clamp onto an RNA primer. (B) Upon completing an Okazaki fragment the core disengages its β clamp creating a vacancy for the new β clamp. (C) The new β clamp falls into place with the lagging strand core polymerase to start the next Okazaki fragment.

The polymerase transfer mechanism outlined here entails stoichiometric use of β for each Okazaki fragment, consistent with the cellular abundance of β (300 molecules/cell) relative to Pol III holoenzyme (10–20 molecules/cell). However, there are approximately 10 times more Okazaki fragments produced during chromosome replication than there are β dimers in the cell. This report shows that Pol III* can remove and recycle β clamps from DNA.

Although this study provides an outline of lagging strand events, it raises many new questions. For example, what happens at a nick that signals Pol III* to dissociate from

its β clamp? Does Pol III* recognize the abutting 5′ terminus at a nick as a signal to disengage from β and DNA? Is this dissociation event the responsibility of one of the subunits? Is ATP involved in the process? Which subunits of Pol III* are responsible for recycling β clamps from replicated DNA? Are they the same or different as those that load β onto DNA? Do RNA primers confer different characteristics onto these protein dynamics than DNA primers? We observe similar results using donor and acceptor templates with the RNA primer at the G4 origin (unpublished data), but this RNA primer may not be representative of RNA primers on the lagging strand of a moving chromosome.

This mechanism of polymerase cycling using defined circular templates is consistent with results of a more complete system using helicase (DnaB–protein) and primase (DnaG–protein) with Pol III holoenzyme on a rolling circle template (Zechner et al., 1992). In those studies, the concentration of β could not be lowered without decreasing the efficiency of primer utilization by the lagging strand polymerase, consistent with stoichiometric consumption of one β clamp for each Okazaki fragment as suggested by the results of this report. Further, under some conditions, the final number of Okazaki fragments in the rolling circle system was greater than the total amount of β added to the assay consistent with the ability of Pol III holoenzyme to eventually reutilize β clamps.

Replication fork studies in the T4 and T7 systems also show rapid recycling of their replicative polymerase holoenzymes during lagging strand replication (Nossal and Alberts, 1983; Debyser et al., 1994). In fact, studies in the T4 system show the polymerase rapidly dissociates from DNA when it can not continue polymerization (Munn and Alberts, 1991), consistent with a hopping mechanism. The T7 polymerase holoenzyme is composed of two subunits, gene 5 protein (the polymerase) and thioredoxin (the processivity factor), it lacks a clamp-loader complex. Both subunits are required for high processivity. Perhaps polymerase cycling in the T7 system is achieved by separation of the two subunits upon completing an Okazaki fragment followed by reforming the holoenzyme at the next primed site.

Eukaryotic polymerase δ also uses both a sliding clamp (PCNA) and a clamp loader (RF-C) and therefore is more like E. coli and T4 than T7. Indeed, direct sequence homology has been noted among the clamp loaders of E. coli, humans, and T4 (O'Donnell et al., 1993). The structural similarity may indicate a synonymous mechanism of lagging strand polymerase transfer.

Are Sliding Clamps Used Downstream of Replication?

Beside their use by the replicative polymerase, the β clamp and γ complex also increase the processivity of DNA polymerase II, an enzyme implicated in repair of DNA (Hughes et al., 1991; Bonner et al., 1992). The fact that the β clamp can be harnessed by at least two different DNA polymerases suggests its use may generalize to yet other machineries. If β clamps are deposited on the lagging strand during replication as this study suggests, perhaps these clamps are used by other enzymes after finishing replication. For example, the clamp may be used by recombination or repair machineries, or as a signal to a protein that links DNA replication to cell cycle processes such as cell division.

The hypothesis that DNA polymerase clamps may be utilized by other enzymatic machineries is strengthened by the fact that the clamps of the other systems also interact with proteins besides the replicative polymerase. The PCNA clamp is utilized by two DNA polymerases, δ and ε (Hubscher and Thommes, 1992). In the T4 system, the g45p clamp is used not only by the DNA polymerase but also by RNA polymerase. In a series of elegant experiments, Peter Geiduscheck's group has shown that the g45p, along with the g44/62p complex (clamp loader), assembles a clamp at a nick that then tracks along DNA acting as a mobile enhancer to activate E. coli RNA polymerase (modified with g33p and g55p) specifically on late gene promoters (Herendeen et al., 1992). Late gene activation is tightly coordinated with T4 replication; if the T4 genome does not divide, the late genes do not switch on. This early-to-late gene switch is also prevalent in several viruses such as simian virus 40, adenovirus, baculovirus, and herpes simplex virus. In fact, the ETL protein of the insect baculovirus, which is required for late gene expression, is 42% identical to PCNA (O'Reilly et al., 1989), suggesting that the use of a clamp protein to produce the early-to-late switch may generalize to eukaryotic viruses.

The replication fork mechanics outlined in this report provides a ready explanation for the coordination of replication with the late gene switch. Sliding clamps preassembled on DNA would become available for transcription during ongoing replication by their repeated deposition, use, and then abandonment by the polymerase. Could these clamps also be used to coordinate replication with cell division or mitosis? Human PCNA binds an array of cyclins and their associated kinases (Zhang et al., 1993), specifically interacting with the p21 kinase inhibitor (Waga et al., 1994; Flores-Roxas et al., 1994). These interactions between PCNA and cell cycle machinery suggest a further involvement of these clamp proteins beyond replication of the chromosome.

Experimental Procedures

Materials

The sources of materials were as follows: radioactive nucleotides (Dupont–New England Nuclear), unlabeled nucleotides (Pharmacia–LKB), DNA modification enzymes (New England Biolabs), DNA oligonucleotides (Oligos etc.). Proteins were purified as described by the following: α, ε, γ and τ (Studwell and O'Donnell, 1991), β (Kong et al., 1992), δ and δ′ (Dong et al., 1993), χ and ψ (Xiao et al., 1993), θ (Studwell-Vaughan and O'Donnell, 1993), Pol III* (Maki et al., 1988), γ complex (Maki and Kornberg, 1988). The M13 gene protein II (gp II)-overproducing strain was a gift of Dr. P. Model and Dr K. Horiuchi (Rockefeller University) and was purified as described (Greenstein and Horiuchi, 1987). The catalytic subunit of cAMP-dependent protein kinase produced in E. coli was the gift of Dr Susan Tayler (University of California, San Diego). Protein concentrations were determined from their ε_{280} values except for Pol III* and γ complex, which were determined using Protein Stain (Bio-Rad) with bovine serum albumin (BSA) as a standard. M13mp18 ssDNA and φX174 ssDNA were phenol extracted from doubly banded phage and were uniquely primed with a DNA 30-mer (map positions 6817–6846 and 2794–2823, respectively) as described (Stukenberg et al., 1991). Gapped plasmid was made

by introducing a specific nick in 60% of M13mp18 plasmid DNA (376 μg) using M13 gp II protein (35 U) as described (Meyer and Geider, 1979) followed by treatment with 1000 U of exonuclease III for 1 min at 37°C to remove approximately 500 nucleotides. Buffer A is 20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA (pH 7.5), 2 mM DTT, and 20% glycerol. Column buffer is 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 50 μg/ml BSA, 60 μM dCTP, 60 μM dGTP, 40 mM NaCl, 5 mM DTT, and 5% glycerol. Reaction buffer is 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 40 μg/ml BSA, 0.5 mM ATP, 60 μM dCTP, 60 μM dGTP, 0.1 mM EDTA, and 5% glycerol.

Gel Filtration

Gel filtration was performed on 5 ml columns of agarose A15m (Bio-Rad) equilibrated in column buffer. Fractions of 200 μl were collected (0.33 ml/min) at 4°C, and ^3H and ^{32}P were quantitated by counting 150 μl aliquots (windows were: ^3H, 0–350; ^{32}P, 400–1000). The specific activity of [^{32}P]β and [^3H]subunits was comparable and therefore bleedover between the windows was less then 1%. Recovery of radioactive protein through gel filtration was 75%–95%.

Polymerase Cycling Assays

Donor reactions were assembled in 47 μl of reaction buffer containing 144 ng (80 fmol) primed φX174 ssDNA, 1.6 μg SSB, 22 ng (270 fmol) β, and 44 ng (62 fmol) Pol III* followed by a 5 min incubation at 37°C. Acceptor reactions were assembled in 47 μl of reaction buffer containing 720 ng (310 fmol) primed M13mp18 ssDNA, 4 μg SSB, 66 ng (815 fmol) β, and 10 ng (52 fmol) γ complex followed by a 5 min incubation at 37°C. Replication was initiated upon adding 6 μl of 1.5 mM dATP, 0.5 mM [α-^{32}P]dTTP to the donor reaction and then adding 47 μl of acceptor template within 3 s. Aliquots of 20 μl were removed and quenched at the indicated times upon addition to 20 μl of 1% SDS, 40 mM EDTA. Rapid manipulations were performed as described (O'Donnell and Kornberg, 1985). One half of each timepoint was analyzed for nucleotide incorporated by spotting onto DE81 paper as described (Studwell and O'Donnell, 1991). The other half was analyzed in a 0.8% native agarose gel followed by laser densitometry of the autoradiogram.

Radioactive Labeling of Proteins and Constitution of [^3H]Pol III*

Subunits were tritiated by reductive methylation as described (Kelman et al., 1994). The specific activities were the following: [^3H]θ, 15,000 cpm/pmol; [^3H]τ, 29,000 cpm/pmol; [^3H]γ, 29,000 cpm/pmol; and [^3H]β, 67,000 cpm/pmol. The [^3H]β retained 100% activity with Pol III*; the activity of the other [^3H]subunits was within 90% of the unlabeled subunit as determined by constituting them into the nine-subunit Pol III* (described below) followed by assaying replication activity with β as described (Maki et al., 1988).

Pol III* was assembled in stages by incubating 352 μg of γ (3.7 nmol as dimer) with 178 μg of τ (1.25 nmol as dimer), 145 μg of χ (8.73 nmol), 114 μg of ψ (7.5 nmol) in 789 μl of buffer A for 30 min at 15°C. To this was added 328 μg of δ (8.48 nmol) and 278 μg of δ′ (7.53 nmol) in 1394 μl of buffer A followed by further incubation for 30 min at 15°C. During this time, the core was constituted in a separate tube containing 200 μg of α (1.55 nmol as monomer), 154 μg of ε (5.6 nmol as monomer), and 76 μg of θ (8.84 nmol as monomer) in 283 μl of buffer A and incubated 30 min at 15°C. Then, the two tubes were mixed and incubated a further 30 min. Pol III* was separated from all free subunits except δ and δ′ on a 1 ml heparin–agarose column eluted with a 15 ml gradient of 0–325 mM NaCl in buffer A. Fractions containing Pol III* were pooled, dialyzed, and then chromatographed on a 1 ml Mono Q column eluted with a 19 ml gradient of 0–0.4 M NaCl in buffer A that cleanly resolved Pol III* from δ and δ′. The Pol III* was dialyzed against buffer A before storage at −70°C.

We constructed a derivative of β (β^{PK}) that contains an additional six amino acids at the C-terminus (NH_2–RRASVP–COOH). The β^{PK} is an efficient substrate for cAMP-dependent protein kinase and was labeled using [γ-^{32}P]ATP to a specific activity of 20,000–65,000 cpm/pmol as described (Kelman et al., 1994). The β^{PK} and [^{32}P]β^{PK} were as active as wild-type β in replication of primed ssDNA using Pol III*.

Interaction of [^3H]Pol III* and [^{32}P]β with DNA

Analysis of [^3H]Pol III* and [^{32}P]β^{PK} while idling on DNA was performed in 400 μl of reaction buffer containing 6 μg (1.34 pmol) gapped M13mp18 DNA, 12.8 μg SSB, 0.28 μg (3.6 pmol) [^{32}P]β^{PK}, and 1.1 μg (3.2 pmol) [^3H]Pol III*. After 3 min at 37°C, 6 μl of water was added, and after a further 30 s, the reaction was split into four tubes, placed on ice, and gel filtered on columns equilibrated with column buffer containing either 40, 60, 80, or 100 mM NaCl. Analysis of [^3H]Pol III* and [^{32}P]β^{PK} on replicated DNA was performed likewise except after the 3 min incubation, 6 μl of 1 mM dATP and dTTP was added instead of water. All the DNA was replicated to RFII as determined by agarose gel analysis.

Kinase Protection Assays

The β^{PK} (22 ng, 267 fmol) was incubated with 15 μM [γ-^{32}P]ATP and 1.1 μg (1.6 pmol) Pol III* (when added) in 100 μl of reaction buffer. After 30 s at 37°C, 5 ng of protein kinase was added and after 10, 20, 40, and 60 s of reaction, 20 μl was removed and quenched with 20 μl of 1% SDS, 40 mM EDTA. Kinase protection assays in the presence of DNA were performed in 100 μl of reaction buffer containing 1.2 μg (500 fmol) primed M13mp18 ssDNA, 11 μg SSB, 216 ng (2.6 pmol) β^{PK}, and 670 ng (1 pmol) Pol III*. After 2 min at 37°C, the reaction was gel filtered to remove free β^{PK} and ATP, and the peak excluded fraction, containing Pol III* with β^{PK} on DNA, was divided into two 100 μl portions. Then, 5 ng of protein kinase was added to each followed by adding 6 μl of 1 mM dATP and dTTP to one portion in order to initiate replication and 6 μl of water to the other portion. Timepoints were analyzed on a 12.5% SDS–polyacrylamide gel, and phosphorylation of β^{PK} was quantitated by densitometry of the autoradiogram. All values were normalized to the level of phosphorylation of β^{PK} in 60 s in the absence of DNA and Pol III* at which time 50% of the sites are phosphorylated (Kelman et al., 1994).

Location of the β Clamp after Pol III* Cycles Away

A [^3H]β clamp was assembled on the donor DNA in 142 μl of reaction buffer containing 0.42 μg (182 fmol) gapped M13mp18 DNA, 2.7 μg SSB, 20 ng (245 fmol) [^3H]β, and 133 ng (190 fmol) Pol III*. The acceptor template was primed φX174 ssDNA (2.1 μg, 133 fmol) incubated in 142 μl of reaction buffer containing 24 μg SSB, 0.20 μg (2.42 pmol) unlabeled β, and 64 ng (317 fmol) γ complex. The donor and acceptor were mixed, and after 15 s, three 90 μl aliquots were placed in tubes containing 10 μl of either 200 U BamHI, 200 U XhoI, or digestion buffer (10 mM Tris–HCl [pH 7.9], 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and were incubated a further 30 s, and then 75 μl of each reaction was analyzed by gel filtration. A 10 μl aliquot of each reaction was removed prior to gel filtration, quenched with SDS and EDTA, and analyzed on an agarose gel, which confirmed that all restriction digestions were complete within the level of detection (90%).

Pol III* Assays for Unloading β Clamps from DNA

An average of 13 β clamps were assembled on each circular DNA in a reaction containing 1 μg (13 pmol) [^3H]β, 0.17 μg (850 fmol) γ complex, 0.4 μg (100 fmol) gapped DNA in 100 μl of reaction buffer and incubated for 7 min at 37°C. The reaction was gel filtered to isolate [^3H]β clamps on DNA (excluded fractions). To test the ability of Pol III* to remove [^3H]β clamps from DNA, 100 fmol of [^3H]β on DNA was incubated 2 min at 37°C with either 35 μl of reaction buffer or 35 μl of reaction buffer containing 0.4 μg (566 fmol) Pol III* followed by gel filtration in the cold room. [^3H]β released from DNA eluted in the included volume. The relative efficiency of Pol III* in reusing [^3H]β clamps on DNA compared with [^3H]β in solution was tested by titrating [^3H]β (either β in solution or on nicked DNA isolated by gel filtration) into a reaction containing 72 ng primed M13mp18 ssDNA, 0.8 μg SSB, 60 μM dATP, 20 μM [α-^{32}P]TTP, and 44 ng (62 fmol) Pol III* in 25 μl of reaction buffer. After 2 min at 37°C, aliquots of 23 μl were analyzed for nucleotide incorporated.

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