THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 266, No. 17, Issue of June 15, pp. 11328-11334, 1991
Printed in U.S.A.

Mechanism of the Sliding β-Clamp of DNA Polymerase III Holoenzyme*

(Received for publication, January 2, 1991)

P. Todd Stukenberg, Patricia S. Studwell-Vaughan, and Mike O'Donnell

From the Howard Hughes Medical Institute, Hearst Microbiology Department, Cornell University Medical College, New York, New York 10021

DNA polymerase III holoenzyme (holoenzyme), the multiprotein replicase of *Escherichia coli*, is essentially unlimited in processive DNA synthesis. Processive activity can be reconstituted from two components. One component, the β preinitiation complex, is a β dimer clamped onto primed DNA. The β preinitiation complex is formed by the five-protein γ complex, which hydrolyzes ATP to chaperone β onto primed DNA. The other component is the αε polymerase. The αε polymerase itself is not processive, but is endowed with extremely high processive activity upon assembly with the β preinitiation complex. Here we examine the mechanism by which the β preinitiation complex confers processivity onto the αε polymerase. We find the β preinitiation complex to be mobile on DNA. Diffusion of β on DNA is specific to duplex DNA, is bidirectional, does not require ATP, and appears to diffuse linearly along the duplex. Furthermore, β directly binds the αε polymerase through contact with α, the DNA polymerase subunit. Hence, the high processivity of the holoenzyme is rooted in a "sliding clamp" of β on DNA that tethers the polymerase to the primed template. Implications for transcription and translation are discussed.

The high processivity of replicative DNA polymerases ensures the smooth and efficient replication of long chromosomes. Highly processive replicative polymerases, such as the phage T4 DNA polymerase (1), *Escherichia coli* DNA polymerase III holoenzyme (2–4), and mammalian DNA polymerase δ (5, 6), all require accessory proteins and ATP hydrolysis to initiate processive DNA synthesis. Here we explore the mechanism by which accessory proteins confer high processivity onto DNA synthesis using the *E. coli* DNA polymerase III system.

DNA polymerase III holoenzyme (holoenzyme)[1] is isolated as a complex of at least 10 subunits (α, ε, θ, τ, γ, δ, δ′, χ, ψ, and β) (7, 8). The holoenzyme hydrolyzes ATP to bind tightly to a primed template and thereafter is processive in DNA synthesis throughout the length of the DNA template (3, 4, 9). The highly processive polymerase activity of the holoenzyme can be reconstituted from isolated subunits in two stages (8, 10, 11).[2] In the first stage, the γ complex ($\gamma\delta\delta'\chi\psi$) hydrolyzes ATP to chaperone β onto primed DNA to form the preinitiation complex. The γ complex is only needed in catalytic amounts to chaperone β onto primed DNA. In the second stage, the αε polymerase (1:1 complex of α and ε, the DNA polymerase (12) and 3′-5′ exonuclease (13) subunits, respectively), which lacks processivity alone, becomes highly processive upon assembly with the preinitiation complex. Thus, the preinitiation complex is responsible for the high processivity of the holoenzyme.

In this report, we have studied the preinitiation complex for principles that underlie unlimited processivity. We find that β slides freely along duplex DNA once it has been transformed into a preinitiation complex by the γ complex and ATP. The β subunit is bifunctional in that besides binding DNA, it also binds the αε polymerase. Hence, β confers processivity onto the αε polymerase by leashing it to the template and sliding along the duplex in back of the polymerase during DNA synthesis.

MATERIALS AND METHODS

Sources—Radioactive chemicals were from Du Pont-New England Nuclear; unlabeled nucleotides were from Pharmacia LKB Biotechnology Inc.; Bio-Gel A-1.5 m was from Bio-Rad; and DNA modification enzymes were from New England BioLabs, Inc. pure proteins were prepared as described: holoenzyme (7), pol III* (7), α (12), ε (13), β (14), γ (15), τ (15), γ complex (8), αε polymerase (16), SSB (17), and ^{3}H-β (500,000 cpm/μg; prepared by reductive methylation using formaldehyde and B^3H_4 (74 Ci/mmol)) (18). Concentrations of SSB and β were determined by absorbance at 280 nm using ϵ_{280} values of 1.5 ml mg^{-1} cm^{-1} (19) and 17,900 M^{-1} cm^{-1} (14), respectively. Concentrations of α, ε, and αε polymerase were determined by absorbance at 280 nm using ϵ_{280} values of 95,440, 12,090, and 107,530 M^{-1} cm^{-1}, respectively, calculated from their tryptophan and tyrosine content predicted from their respective genes. The concentration of ^{3}H-β was determined by comparison with unlabeled β in the Bradford assay (20). Concentrations of the holoenzyme and pol III* were determined by the method of Bradford (20) using bovine serum albumin as standard. The concentration of the γ complex was the average of measurements by the Bradford assay (20) and by Western blot assay. In both assays, a standard curve was calibrated with pure γ analyzed by amino acid analysis (Protein Microchemistry Facility, Biological Chemistry Department, University of Michigan). The γ antibody was prepared in a rabbit by Poccono Rabbit Farms using 100 μg of pure γ in complete Freund's adjuvant subcutaneously followed by two boosters of 50 μg each at 2-week intervals.

DNA Substrates—Plasmid DNA was prepared by the alkaline lysis

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

[1] The abbreviations used are: holoenzyme, DNA polymerase III holoenzyme; pol III*, DNA polymerase III* (holoenzyme lacking β); RF, replicative form; SSB, *E. coli* single-strand DNA-binding protein; EBNA1, Epstein-Barr virus nuclear antigen 1; ssDNA, single-strand DNA; SDS, sodium dodecyl sulfate.

[2] In early reconstitution studies, only nine subunits were required for processive replication: the γ complex ($\gamma\delta\delta'\chi\psi$), β subunit, and core polymerase ($\alpha\epsilon\theta$) (8, 10, 11). Studies using pure single subunits showed that five proteins were essential: the β subunit; the γ and δ subunits, which substitute for the γ complex; and the α and ε subunits, which are both needed to substitute for the core polymerase (36). In this report, we used β and the γ complex to form the preinitiation complex, and the αε polymerase was used in place of the core polymerase.

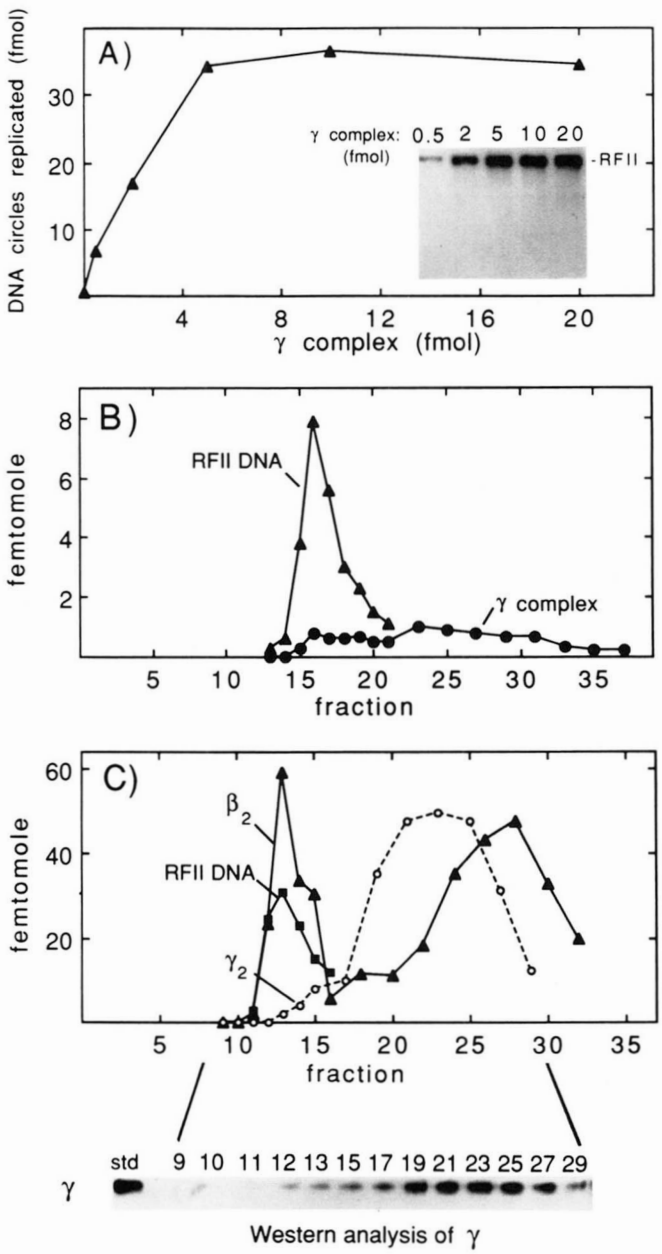

FIG. 1. **Stoichiometry of γ complex and β subunit in the preinitiation complex.** *A*, multiple polymerases are assembled by each γ complex. Reactions contained 140 ng of primed M13mp18 ssDNA, 1.8 μg of SSB, 129 ng of β, 550 ng of αε, and the indicated amount of the γ complex in 25 μl of 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 5 mM dithiothreitol, 4% glycerol, and 40 μg/ml bovine serum albumin (buffer A) containing 20 mM NaCl, 0.5 mM ATP, and 60 μM each dCTP and dGTP. After 5 min at 37 °C, a pulse of DNA synthesis was initiated with 1.5 μl of 1 mM dTTP, 0.33 mM [α-^{32}P]dATP and then was quenched after 20 s with 25 μl of 1% SDS, 40 mM EDTA. One half of the reaction was spotted on DE81 to quantitate DNA synthesis, and the other half was analyzed for full-length products (RFII) on a 0.8% neutral agarose gel (*inset*). *B*, the gel-filtered preinitiation complex is active with αε. The preinitiation complex was formed in 75 μl of buffer A containing 1.1 μg of primed M13mp18 ssDNA, 11 μg of SSB, 0.6 μg of β, 3 ng of the γ complex, and 0.5 mM ATP. After 12 min at 37 °C, the reaction was filtered over a 2.5-ml column of Bio-Gel A-1.5 m in buffer A containing 100 mM NaCl at 4 °C, and 90-μl fractions were collected. ▲, preinitiation complexes were quantitated from the number of DNA circles replicated (RFII products) in 30 s upon addition of 5 μl of column fraction to 0.66 μg of αε in 45 μl of buffer A containing 60 μM each dCTP, dGTP, and dATP and 20 μM [α-^{32}P]dTTP; ●, the γ complex was quantitated by the ability to reconstitute a processive polymerase within 10 min at 37 °C in 50 μl of buffer A containing 100 ng of αε, 150 ng of τ, 112 ng of β, 140 ng of primed φX174 ssDNA, 1.4 μg of SSB, 0.5 mM ATP, and 60 μM each dCTP and dGTP. A 30-s pulse of replication was performed as described for *A*. To correct the replication in the excluded fractions for the β preinitiation complex assembled on primed M13mp18 ssDNA before gel filtration, replication products were analyzed on a 0.8% neutral agarose gel to separate away the M13mp18 RFII, and the φX174 RFII products were quantitated by scanning the autoradiograph with a laser densitometer (Pharmacia LKB Biotechnology Ultrascan XL). The percentage of the γ complex in each fraction was calculated by dividing the amount of φX174 DNA replicated by that fraction by the sum total of φX174 RFII produced by all the fractions. The femtomoles of the γ complex in each fraction were calculated by multiplying the percent γ complex in the fraction times the total γ complex present before gel filtration. *C*, stoichiometry of the γ complex and β in the preinitiation complex.Primed M13mp18 ssDNA (1.4 μg) was incubated for 5 min at 37 °C with 12 μg of SSB, 73 ng of ^{3}H-β (975 fmol as dimer), 100 ng of the γ complex (480 fmol), and 0.5 mM ATP in 100 μl of buffer A and then gel-filtered over 5 ml of Bio-Gel A-1.5 m at 5 °C in buffer A containing 100 mM NaCl. Fractions of 180 μl were collected. ▲, ^{3}H-β was quantitated by liquid scintillation analysis of 20 μl of fraction; ■, DNA circles containing a preinitiation complex were quantitated from the amount of RFII produced upon incubating 10 μl of fraction with 100 ng of pol III*, 0.5 mM ATP, 60 μM dCTP, 60 μM dGTP for 1 min at 37 °C in 25 μl of buffer A followed by a 20-s pulse of synthesis as described for *A*; ○, Western blot analysis of the γ complex was performed upon electrophoresis of 130 μl of column fraction on a 12% SDS-polyacrylamide gel followed by transfer to nitrocellulose and probing using γ antiserum and ^{125}I-protein A. The γ subunit was quantitated by densitometry analysis of the autoradiograph of the Western blot (shown below the plot) and comparison to a standard (*std*) curve of γ in the same gel.

method followed by two bandings in cesium chloride equilibrium gradients (21). RFII DNA was prepared by treating 72 μg of supercoiled (RFI) plasmid DNA with 1 ng of DNase I in 250 μl of 50 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$ at 30 °C until 50% of the RFI DNA was converted to RFII DNA (20 min) and was then purified by phenol extraction. The linear DNA with recessed 3′-ssDNA ends was prepared by exonuclease III (25 units) digestion of *Sca*I-linearized pUC18 DNA (43 μg) for 30 min at 30 °C in 250 μl of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl followed by phenol extraction. Approximately 800 nucleotides were removed from each end determined by comparison with size standards on a 1% alkaline agarose gel.

M13mp18 and φX174 ssDNAs were prepared by banding the phage down and then up in two successive cesium chloride gradients as described (22) and were uniquely primed with a synthetic DNA 30-mer oligonucleotide (M13mp18, map positions 6816–6847; φX174, map positions 2794–2823) as described (16).

RESULTS

To understand the principles that underlie processive polymerization by the holoenzyme, we have studied the preinitiation complex structure (Fig. 1), its interaction with the αε polymerase (Fig. 2), and its motion on DNA (Fig. 3).

Preinitiation Complex Structure—In Fig. 1*A*, the γ complex was titrated into the assay for assembly of a processive polymerase. The assay contained excess αε polymerase, β subunit, and primed M13mp18 ssDNA "coated" with SSB. After addition of the γ complex and 5 min for polymerase assembly, the number of processive polymerases formed was determined by a 20-s pulse of DNA synthesis. Whereas the αε polymerase by itself shows no detectable synthesis in 20 s (16), the holoenzyme completes one M13mp18 DNA circle in 15 s (11). Hence, the number of DNA circles replicated equals the number of processive polymerases assembled. The three subsaturating amounts of the γ complex in the titration resulted, on average, in 10 processive polymerases assembled (10 circles replicated) per molecule of the γ complex. Thus, the γ complex is only needed to assemble the processive polymerase and is not required during processive elongation. The DNA products were analyzed on an agarose gel (Fig. 1*A*, *inset*) to

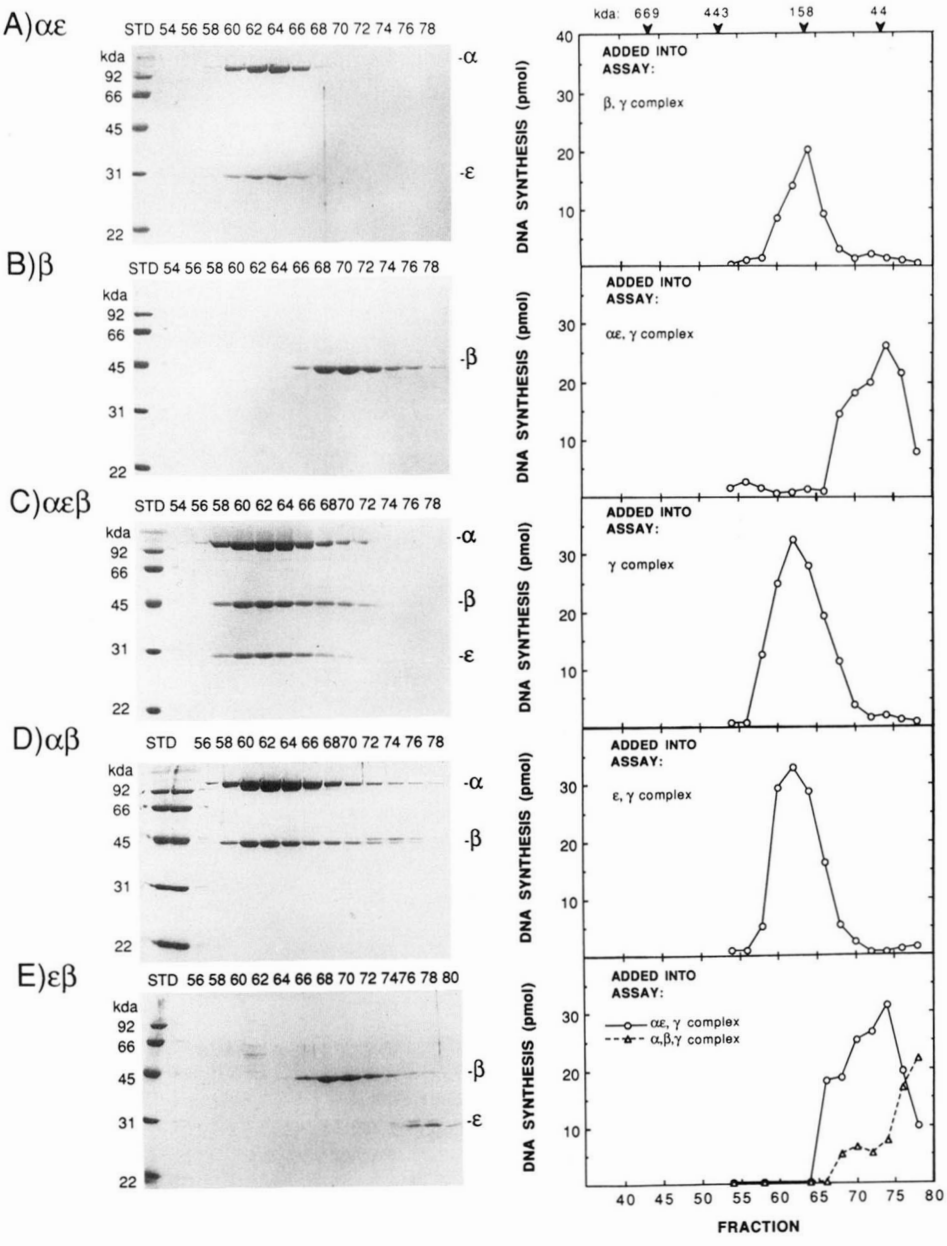

FIG. 2. **Gel filtration analysis of interaction between β and $\alpha\epsilon$.** Proteins were incubated for 1 h at 15 °C in 200 μl of 25 mM Tris-HCl (pH 7.5) 1 mM EDTA, 10% glycerol, and 100 mM NaCl and then injected onto a 30-ml fast protein liquid chromatography Superose 12 column equilibrated in the same buffer. Proteins analyzed were: $\alpha\epsilon$, 40 μg (*A*); β, 36 μg (*B*); $\alpha\epsilon$ and β, 140 and 36 μg, respectively (*C*); α and β, 153 and 36 μg, respectively (*D*); ϵ and β, 60 and 36 μg, respectively (*E*). Fractions of 180 μl were collected. *Left*, 15% SDS-polyacrylamide gel analysis of 100-μl aliquots of column fractions stained with Coomassie Brilliant Blue as described (23). Holoenzyme subunits are identified to the *right*, and molecular masses of protein standards are indicated to the *left*. *Right*, reconstitution assays of column fractions. Proteins added to reconstitute processive activity of the column fraction are indicated. Assays were performed by addition of 2 μl of column fraction to 72 ng of primed ϕX174 ssDNA, 720 ng of SSB, 0.5 mM ATP, 60 μM each dCTP and dGTP in 21.5 μl of buffer A. Other proteins added to reconstitute processive synthesis were as follows; *A*, assays of $\alpha\epsilon$ contained 12 ng of β and 4 ng of the γ complex; *B*, β assays contained 72 ng of $\alpha\epsilon$ and 4 ng of the γ complex; *C*, $\alpha\epsilon\beta$ activity required 4 ng of the γ complex; *D*, $\alpha\beta$ activity required 50 ng of ϵ and 4 ng of the γ complex; *E*, ϵ assays contained 140 ng of α, 12 ng of β, and 4 ng of the γ complex. Assays were incubated for 6 min at 37 °C, and then DNA synthesis was initiated upon addition of 1.5 μl of 1.5 mM dATP, 0.5 mM [α-^{32}P] dTTP. After 20 s, synthesis was stopped by spotting the reaction on DE81 paper, which was then washed and counted as described (24). Positions of standards analyzed separately are indicated at the *top*: thyroglobulin (669 kDa), apoferritin (443 kDa), IgG (158 kDa), and ovalbumin (44 kDa).

confirm that they were products of true processive replication (*i.e.* completed DNA circles). Furthermore, the preinitiation complex was gel-filtered in buffer containing 100 mM NaCl,[3] which resolved most of the γ complex activity in the included fractions (Fig. 1*B*, *circles*) from the preinitiation complex on primed DNA in the excluded fractions (Fig. 1*B*, *triangles*). The gel-filtered preinitiation complex was active in replication upon addition of the $\alpha\epsilon$ polymerase (Fig. 1*B*, *triangles*).

The stoichiometry of β within the preinitiation complex was measured using tritium-labeled β subunit. The preinitiation complex was formed on primed M13mp18 ssDNA using ^{3}H-β and unlabeled γ complex and then was gel-filtered to separate ^{3}H-β bound to DNA in the excluded fractions from ^{3}H-β free in solution in the included fractions (Fig. 1*C*, *triangles*). ^{3}H-β is expressed in Fig. 1*C* as femtomoles of β dimers since β appears to act as a dimer (18, 25). The number of preinitiation complexes on DNA was quantitated from the number of DNA circles replicated in 20 s upon addition of pol III* (holoenzyme lacking β) to reconstitute the holoenzyme (Fig. 1*C*, *squares*). The analysis yielded a stoichiometry of 1.4 β dimers in the preinitiation complex, consistent with ~1 β dimer by previous estimates (8, 18).

A molar excess of the γ complex over primed DNA circles was used in the experiment of Fig. 1*C* to determine whether a preinitiation complex containing the γ complex could be isolated. The stoichiometry of the γ subunit in the preinitiation complex was determined by Western blot analysis of column fractions using γ antiserum (Fig. 1*C*, *circles*). Western blot analysis showed only a small amount of the γ complex in the preinitiation complex, consistent with its catalytic role in the reaction. The γ complex activity was also followed by reconstitution assays, which agreed with Western blot analysis (data not shown).

α Binds β Directly—A simple mechanism by which β confers processivity onto $\alpha\epsilon$ is for β to bind both DNA and $\alpha\epsilon$, thereby

[3] By Western blot analysis using γ antiserum, we find 74, 35, and 4% nonspecific binding of the γ complex to unprimed SSB-coated ssDNA through gel filtration in buffer A (see legend to Fig. 1) containing no added NaCl, 40 mM NaCl, and 100 mM NaCl, respectively.

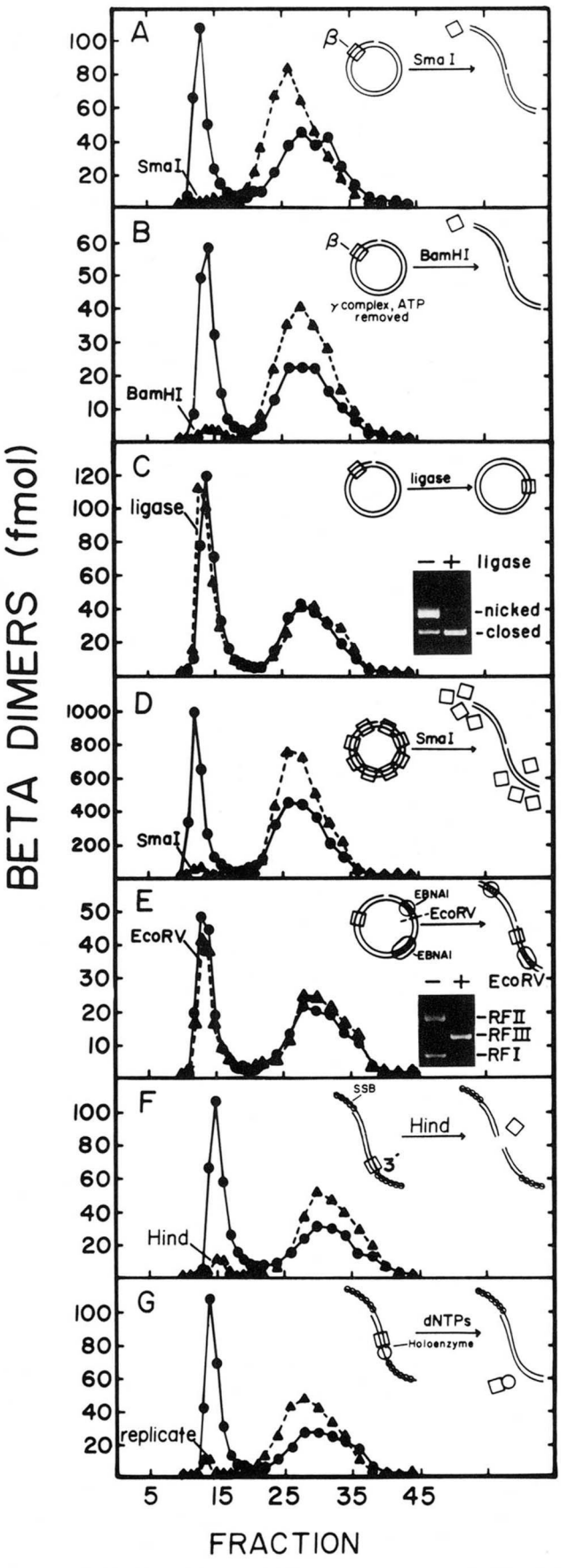

FIG. 3. **Mobility of the preinitiation complex on DNA.** *A*, the preinitiation complex forms on RFII DNA, but dissociates upon linearization. Preinitiation complexes were formed in 6 min at 37 °C in 200 μl of buffer A containing 2.2 μg of pGEMII RFII DNA, 105 ng of β (1442 fmol as dimer), 90 ng of the γ complex (450 fmol), and 0.5 mM ATP. After incubation, the reaction was divided, 30 units of *Sma*I were added to half (▲), and *Sma*I buffer was added to the other (●). After 2 min at 37 °C, 10 μl were removed for analysis on an agarose gel, and both halves were filtered over 5-ml columns of Bio-Gel A-1.5 m at 5 °C in 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 8 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, and 4% glycerol (buffer B). Fractions (140 μl) were collected and analyzed for ^{3}H-β by liquid scintillation. *B*, ATP and the γ complex are not required for ^{3}H-β to dissociate from linear nicked DNA. Preinitiation complexes were formed as described for *A* (except for use of 1.2 μg of β) and then gel-filtered in buffer B containing 100 mM NaCl to remove the γ complex and ATP. Excluded fractions (280 μl) were pooled and halved; *Bam*HI (80 units) was added to half (▲), and *Bam*HI buffer was added to the other half (●) and then incubated for 2 min at 37 °C and analyzed as described for *A*. *C*, ligase does not displace ^{3}H-β. Preinitiation complexes were formed as described for *A* (except for use of 130 ng of ^{3}H-β), divided in two, treated with 24 units of T4 DNA ligase (▲) or T4 DNA ligase buffer (●), and incubated for a further 2 min at 37 °C before analysis as described for *A*. *Inset*, agarose gel analysis (in ethidium bromide) of each half-reaction sampled prior to gel filtration. *D*, multiple β dimers bind one RFII DNA. Procedures were as described for *A* except for use of 430 ng of M13mp18 RFII DNA, 45 ng of the γ complex (225 fmol), and 1 μg of ^{3}H-β. ●, no *Sma*I: ▲, *Sma*I-treated. *E*, EBNA1 bound to the ends of linear DNA traps the preinitiation complex. Procedures were as described for *A* except that the reaction contained pGEMoriP7 RFII DNA (5.4 μg) (37), 6 μg (120 nmol) of EBNA1, 60 ng (800 fmol) of ^{3}H-β, and 58 ng (300 fmol) of the γ complex. ●, no *Eco*RV; ▲, 80 units of *Eco*RV. *Inset*, agarose gel analysis of each half-reaction prior to gel filtration. *F*, the preinitiation complex is retained on linear DNA with ssDNA ends. Procedures were as described for *A* except that 2.1 μg (1160 fmol) of exonuclease III-treated pUC18 were used, and 25 μg of *E. coli* SSB were added. ●, no *Hin*dIII; ▲, 30 units of *Hin*dIII. *G*, ^{3}H-β exits linear DNA upon replication. Procedures were as described for *F*. After the 6-min incubation, 1.2 μg of pol III* and 60 μM each dCTP and dGTP were added, and the reaction was divided. To one half was added 60 μM each dATP and dTTP (▲), and both halves were incubated for another 30 s and then gel-filtered in buffer B containing 60 μM each dCTP and dGTP (●) or 60 μM each deoxynucleoside triphosphate (*dNTP*) (▲).

tethering αε to the template. Gel filtration experiments demonstrating that β binds αε are shown in Fig. 2. The αε polymerase and β subunit were first analyzed separately and then together (Fig. 2, *A–C*, respectively). SDS-polyacrylamide gel analysis of the column fractions (Fig. 2, *left*) showed that the αε polymerase alone eluted as a monomer (*i.e.* a complex of one α and one ε, 158 kDa total) and that the β subunit alone eluted as a dimer (74 kDa). An αεβ complex was formed upon mixing αε and β, as indicated by the shift in the elution volume of β such that it coeluted with αε (Fig. 2*C*). Likewise, formation of an αβ complex was demonstrated by gel filtration analysis of a mixture of α and β subunits (Fig. 2*D*). A mixture of ε and β subunits did not yield a complex (Fig. 2*E*). Reconstitution assays showed that the αεβ and αβ complexes were active (Fig. 2, *C* and *D*, *right*, respectively). Advantage has been taken of the resolution of β bound to αε from β not bound to αε to determine a K_d value of ~250 nM for αεβ complex formation in solution.[4] That αε binds over 60-fold tighter to the β preinitiation complex on primed ssDNA is indicated by saturation of αε at 4.2 nM in replication of the β preinitiation complex on primed φX174 ssDNA (16).

The Preinitiation Complex Slides on Duplex DNA—We next examined the preinitiation complex for mobility on DNA, which would allow it to move with αε during processive

[4] Gel filtration analysis showed that a solution of 0.44 μM αε and 0.22 μM ^{3}H-β contained 56% ^{3}H-β bound as αεβ and 44% ^{3}H-β free, yielding a K_d of ~250 nM (*i.e.* ([αε][β]/[αεβ] = ((0.317 μM) (0.097 μM))/0.125 μM = 246 nM). Likewise, a solution of 0.88 μM αε and 0.44 μM ^{3}H-β contained 68% ^{3}H-β bound as αεβ and 32% ^{3}H-β free, yielding a K_d of 274 nM.

synthesis, and if so, whether β moves on ssDNA ahead of the polymerase or on duplex DNA in back of the polymerase. We first asked whether the preinitiation complex could form on circular duplex DNA with only a nick in one strand (RFII) as a primer terminus. The RFII DNA was incubated with ^{3}H-β, γ complex, and ATP and then gel-filtered. Approximately 1 ^{3}H-β dimer was present per RFII DNA in the excluded fractions (Fig. 3*A*, *circles*).[5] Hence, formation of the preinitiation complex does not require ssDNA. In control reactions, ^{3}H-β did not appear in the excluded fractions when ATP, γ complex, or DNA was omitted from the preinitiation reaction (data not shown).

The initial experiment that suggested the preinitiation complex slides on DNA is also shown in Fig. 3*A*. The preinitiation complex was formed on RFII DNA, the reaction was divided into two tubes, and the DNA in one half was linearized at the unique *Sma*I site within 2 min prior to gel filtration (Fig. 3*A*, *triangles*); the DNA in the other half was not linearized (Fig. 3*A*, *circles*). ^{3}H-β was not bound to the linear DNA in the excluded fractions and therefore must have dissociated from it, possibly by sliding off the end. Dissociation of ^{3}H-β was efficient from DNA with blunt ends (*Sma*I) or 4-base 5′-terminal overhangs (*Bam*HI, *Hin*dIII, *Eco*RV) and was not particular to the plasmid (pGEMII, M13mp18, pUC18, pGEMoriP7) (Fig. 3, explained below). To demonstrate that restriction enzymes did not participate directly in dissociation of the preinitiation complex from linear DNA, RFII DNA was first linearized using *Sma*I, *Pst*I, or *Hin*dIII and then purified by phenol extraction. A preinitiation complex on these linear nicked DNAs could not be isolated by gel filtration upon treating any of them with ^{3}H-β, γ complex, and ATP (data not shown). Presumably, the preinitiation complex formed on them, but dissociated from the linear nicked DNA faster than it formed. As another control, linear duplex DNA added *in trans* did not induce ^{3}H-β to dissociate from RFII DNA (data not shown).

Are either the γ complex or ATP required to dissociate ^{3}H-β from linear DNA? To test this, both ATP and the γ complex were gel-filtered away from the preinitiation complex on RFII DNA. The gel-filtered RFII DNA with ^{3}H-β bound to it was divided, and one half was linearized with *Bam*HI before gel filtration a second time. The second gel filtration showed that ^{3}H-β remained bound to the circular RFII DNA (Fig. 3*B*, *circles*)[6] but dissociated from the linear nicked DNA (Fig. 3*B*, *triangles*). Hence, neither ATP nor the γ complex is required for ^{3}H-β to dissociate from linear DNA.

The experiments of Fig. 3, *C–F* test the possibility that ^{3}H-β dissociates from linear DNA by diffusing along the duplex and falling off the end. A preinitiation complex that diffuses along the duplex predicts that the preinitiation complex does not adhere to the nick on the RFII DNA. In this case, ligase should seal the nick without displacing ^{3}H-β. To test this, the preinitiation complex was formed on RFII DNA, the reaction was divided, and one half was treated with T4 DNA ligase. Gel filtration analysis showed that ligase sealed the nick to form closed circular duplex DNA, but did not displace ^{3}H-β (Fig. 3*C*, *triangles*). Hence, β was not confined to the nick on RFII DNA. Ligation was efficient (*inset*) even though ~1 β dimer was bound per RFII DNA circle (Fig. 3*C*, *circles*).

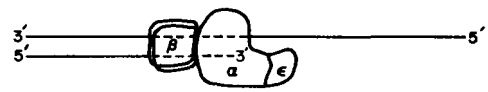

FIG. 4. **Predicted orientation of αεβ subunits of DNA polymerase III holoenzyme on a primed template.** See text for details.

A preinitiation complex that diffuses away from the nick and along the RFII DNA also predicts that multiple preinitiation complexes will form on each RFII DNA molecule. Indeed, >20 molecules of β (as dimer) were bound per molecule of RFII DNA by simply elevating the level of ^{3}H-β and decreasing the amount of RFII DNA in the assay (Fig. 3*D*, *circles*). It is noteworthy that each γ complex molecule chaperoned over 20 β dimers onto DNA in this experiment. The multiple β clamps dissociated from the DNA upon linearization using *Sma*I (Fig. 3*D*, *triangles*).

Provided β dissociates from linear DNA by sliding off the end, protein bound to both ends of the duplex may block β exit. To test this idea, we used materials from another project. EBNA1, a protein encoded by the Epstein-Barr virus, binds 24 sites in the latent replication origin of the Epstein-Barr virus, called *oriP* (26, 27). We recently overproduced EBNA1 in baculovirus and purified it to homogeneity (37). The EBNA1-binding sites in *oriP* are located in two elements, one of which contains 20 EBNA1-binding sites and the other has 4 EBNA1-binding sites. A preinitiation complex was formed on RFII DNA containing the *oriP* sequence followed by addition of sufficient EBNA1 to bind all 24 sites (37). The reaction was divided, and one half was treated with *Eco*RV, which cleaved the DNA between the two elements of *oriP*. Agarose gel analysis showed that EBNA1 did not prevent linearization by *Eco*RV (Fig. 3*E*, *inset*). Gel filtration analysis showed that nearly the same amount of ^{3}H-β was retained on linear nicked DNA (Fig. 3*E*, *triangles*) as on circular RFII DNA (Fig. 3*E*, *circles*). Hence, EBNA1 bound to the ends of linear DNA prevented the dissociation of ^{3}H-β, consistent with unidimensional diffusion of β along the duplex (*i.e.* sliding). In the absence of EBNA1, ^{3}H-β dissociated from the *oriP* RFII plasmid upon linearization with *Eco*RV (data not shown).

A second protein block experiment was performed using linear duplex DNA treated with exonuclease III to yield, at each end, a 3′-primer terminus recessed by ~800 nucleotides of ssDNA. The ssDNA ends of the linear template were first coated with SSB and then incubated with ^{3}H-β, γ complex, and ATP. Gel filtration analysis showed efficient binding of ^{3}H-β to the linear DNA (Fig. 3*F*, *circles*). Hence, the preinitiation complex appeared unable to slide across SSB-coated ssDNA. In fact, even in the absence of SSB, gel filtration of the preinitiation complex on the linear DNA with recessed 3′-ends showed 80% of the amount of ^{3}H-β bound relative to the presence of SSB (data not shown). Hence, β does not slide over a long stretch of naked ssDNA.

Unlike the RFII DNA, the linear DNA with recessed 3′-ends offered the opportunity to test the directionality of ^{3}H-β sliding. After the γ complex chaperoned ^{3}H-β onto the linear DNA at the recessed 3′-end, the reaction was divided; and one half was treated with *Hin*dIII, which cleaved the linear DNA in two (1.7 and 0.9 kilobases). For ^{3}H-β to slide off the cleaved DNA, β would need to slide backwards in the antielongation direction (see Fig. 3*F*, *diagram*).[7] Indeed, most

[5] Preinitiation reactions using RFI plasmid yielded ~30% of the level of ^{3}H-β bound to DNA through gel filtration relative to ^{3}H-β bound to the RFII DNA preparation. Inasmuch as some RFII DNA contaminates all RFI DNA preparations, ^{3}H-β was presumably bound to the RFII DNA, although preinitiation complex formation on RFI DNA cannot be ruled out.

[6] ^{3}H-β in the included fractions (Fig. 3*B*, *circles*) is presumably due to some dissociation of ^{3}H-β from RFII DNA in buffer A containing 100 mM NaCl during analysis of the first column and during the second incubation at 37 °C.

[7] It is possible that some β diffused back on the duplex (antielongation direction) away from the 3′-terminus before *Hin*dIII cleavage such that after cleavage it could exit the DNA in the elongation direction. The important point is that β must diffuse in the antielongation direction at some time to exit the cleaved linear DNA with recessed 3′-ends.

of the ^{3}H-β dissociated from the linear DNA upon cleavage of the duplex region, implying ability of β to slide in the antielongation direction (Fig. 3*F*, *triangles*).

The linear DNA with recessed 3′-ends was also used to show that ^{3}H-β could slide off duplex DNA in the forward (elongation) direction. The preinitiation complex was formed on the linear DNA using ^{3}H-β and the γ complex. pol III* was then added to reconstitute the holoenzyme on the linear template. ^{3}H-β dissociated from the linear DNA upon replication of the ssDNA end (Fig. 3*G*, *triangles*). In a control experiment, ^{3}H-β was efficiently retained on the linear DNA, while the holoenzyme "idled" on the DNA with only two deoxynucleoside triphosphates present (Fig. 3*G*, *circles*).

DISCUSSION

Although the β subunit alone does not bind DNA, it is transformed into a tight sliding clamp on DNA upon activation by the γ complex and ATP. The β subunit also binds the $\alpha\epsilon$ polymerase, thereby recruiting it for processive DNA synthesis. That β slides on DNA fits nicely with the simple notion that β tethers the polymerase to the template and confers processivity onto it by sliding along with the polymerase during DNA synthesis. It seems likely that this mechanism will generalize to other processive replicases. Indeed, DNA footprinting techniques locate the phage T4 DNA polymerase accessory proteins mainly on the duplex portion of the primer terminus and are thought of as a sliding clamp (1, 28). Likewise, mammalian polymerase δ requires primer binding accessory subunits for processivity (5, 6).

These studies suggest the orientation of α, ϵ, and β subunits of the holoenzyme on a primer terminus illustrated in Fig. 4. The DNA polymerase subunit, α, is placed on the ssDNA/double-stranded DNA-primed template junction, the substrate for a DNA polymerase. Since β and ϵ do not form a stable complex with each other yet both bind α, they are shown bound to separate sites on α. The β clamp slides on duplex DNA; hence, β is shown on the duplex in back of α. The ϵ subunit is drawn in front of α and not bound to DNA by analogy to the orientation of the polymerase and 3′-5′ exonuclease domains of the DNA polymerase I large fragment on a primer terminus inferred from the x-ray structure (29). This position for ϵ is also consistent with the 50-fold stimulation of ϵ exonuclease activity by α, suggesting that interaction of ϵ with DNA is mainly by virtue of binding α (30).

The specificity of the preinitiation complex for the primed ssDNA/double-stranded DNA junction is probably inherent in the γ complex since the γ complex is an ATPase that is stimulated by a primed template and is stimulated even further upon adding β (31).[8] Capacity for primer recognition by β is not ruled out by the experiments that show the β clamp can leave the 3′-primer terminus and slide on duplex DNA. The gel filtration experiments (Fig. 3) require >20 min and therefore would not have detected a few minutes visitation of the β clamp at the 3′-terminus before departing from it and sliding off linear duplex DNA.

In a previous study (32), the core polymerase activity was stimulated ~4-fold on uniquely primed M13Gori1 ssDNA by a 5000-fold molar excess of β in the absence of the γ complex and SSB. This stimulation of the core by β was the first evidence of a direct interaction between β and the core.

In a study of holoenzyme motion on multiprimed DNA, the holoenzyme did not require ATP to diffuse over duplex DNA and did not diffuse over a long stretch of ssDNA (33). Inasmuch as these holoenzyme dynamics are shared by the β preinitiation complex, it seems likely that the β clamp was responsible for the holoenzyme's motion. Mobility of the holoenzyme over duplex DNA was determined from the path of DNA synthesis, allowing study of motion in the elongation direction only. Here, the β preinitiation complex diffused on duplex DNA in both the antielongation and elongation directions (Fig. 3, *F* and *G*). Bidirectional diffusion is consistent with the lack of an ATP requirement for mobility. Although the β preinitiation complex does not diffuse over a long stretch of naked ssDNA, it can press across the 4-base overhang left by *Bam*HI. Perhaps a long stretch of ssDNA forms secondary structures (*e.g.* hairpins with loops at their tips) that β cannot pass. Another possibility is that the β dimer needs both "rails" of duplex DNA to diffuse a long distance.

At first glance, it seems appropriate for a "sliding clamp" protein to slide off the end of a linear molecule. However, if β is bound to DNA by hydrogen bonding, ionic interaction, and/or hydrophobic forces, then β should remain on DNA even at an end rather than dissociate into solution. We are not aware of another protein with such strong affinity to nicked circular DNA, but which loses its grip upon linearizing the DNA. The state of DNA in both cases is relaxed; their only difference is the geometry of the DNA molecule. Possibly, the β clamp is in a high energy state (since its formation requires ATP); and upon encountering a double-strand break, its energy is discharged, allowing β to dissociate from the DNA. Alternatively, the β clamp may not bind DNA by chemical contact but rather by virtue of topology on the DNA, for example, by encircling the duplex like a doughnut. In this "topological binding" mode, the β clamp would freely slide off the end of DNA since it would not be bound to DNA by chemical contact.

Highly processive polymerization, besides a characteristic of DNA replication, is also common to transcription and translation. RNA and DNA polymerases are quite similar in the reactions they catalyze. Furthermore, mammalian RNA polymerase II requires ATP hydrolysis and accessory factors to initiate processive synthesis (34). Likewise, the ribosome requires GTP hydrolysis and catalytic initiation factors to start processive synthesis. The eukaryotic ribosome even slides along messenger RNA to locate the first AUG codon (35). These similarities among processive machines that polymerize DNA, RNA, and protein tempt speculation of a sliding clamp protein underlying processivity in other systems besides DNA polymerase III holoenzyme.

Acknowledgment—We are indebted to Maija Skangalis for cell lysis and initial ammonium sulfate cuts of the many kilograms of *E. coli* that made these studies possible.

REFERENCES

1. Nossal, N. G., and Alberts, B. M. (1984) in *Bacteriophage T4* (Mathews, C., Kulter, E., Mosig, G., and Berget, P., eds) pp. 71–81, American Society for Microbiology, Washington, D. C.
2. Kornberg, A. (1982) *Supplement to DNA Replication*, pp. 532–535, W. H. Freeman & Co., New York
3. Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1981) *J. Biol. Chem.* **256,** 976–983
4. Burgers, P. M. J., and Kornberg, A. (1982) *J. Biol. Chem.* **257,** 11468–11473
5. Tsurimoto, T., and Stillman B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1023–1027
6. Lee, S.-H., and Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5672–5676
7. McHenry, C., and Kornberg, A. (1977) *J. Biol. Chem.* **252,** 6478–6484
8. Maki, S., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 6561–6569
9. Mok, M., and Marians, K. J. (1987) *J. Biol. Chem.* **262,** 16644–16654
10. Wickner, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3511–3515

[8] R. Onrust and M. O'Donnell, manuscript in preparation.

11. O'Donnell, M. (1987) *J. Biol. Chem.* **262,** 16558–16565
12. Maki, H., and Kornberg, A. (1985) *J. Biol. Chem.* **260,** 12987–12992
13. Scheuermann, R. H., and Echols, H. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7747–7751
14. Johanson, K. O., Haynes, T. E., and McHenry, C. S. (1986) *J. Biol. Chem.* **261,** 11460–11465
15. Maki, S., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 6547–6554
16. Studwell, P. S., and O'Donnell, M. (1990) *J. Biol. Chem.* **265,** 1171–1178
17. Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1975) *J. Biol. Chem.* **250,** 1972–1980
18. Burgers, P. M. J., and Kornberg, A. (1982) *J. Biol. Chem.* **257,** 11474–11478
19. Lohman, T. M., and Overman, L. B. (1985) *J. Biol. Chem.* **260,** 3594–3603
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp. 90–91, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Ray, D. S. (1969) *J. Mol. Biol.* **43,** 631–643
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Rowen, L., and Kornberg, A. (1978) *J. Biol. Chem.* **253,** 758–764
25. Johanson, K. O., and McHenry, C. S. (1980) *J. Biol. Chem.* **255,** 10984–10990
26. Yates, J. L., Warren, N., and Sugden, B. (1985) *Nature* **313,** 812–815
27. Rawlins, D. R., Milman, G., Hayward, S. D., and Hayward, G. S. (1985) *Cell* **42,** 859–868
28. Munn, M. (1986) *Analysis of the Bacteriophage T4 DNA Replication Complex.* Ph.D. thesis, University of California, San Francisco
29. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) *Nature* **313,** 762–766
30. Maki, H., and Kornberg, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4389–4392
31. Studwell, P. S., Stukenberg, P. T., Onrust, R., Skangalis, M., and O'Donnell, M. (1990) *UCLA Symp. Mol. Cell. Biol. New Ser.* **127,** 153–164
32. LaDuca, R. J., Crute, J. J., McHenry, C. S., and Bambara, R. A. (1986) *J. Biol. Chem.* **261,** 7550–7557
33. O'Donnell, M., and Kornberg, A. (1985) *J. Biol. Chem.* **260,** 12875–12883
34. Bunick, D., Zandomeni, R., Ackerman, S., and Weinmann, R. (1982) *Cell* **29,** 877–886
35. Kozak, M. (1983) *Microbiol. Rev.* **47,** 1–45
36. O'Donnell, M., and Studwell, P. S. (1990) *J. Biol. Chem.* **265,** 1179–1187
37. Frappier, L., and O'Donnell, M. (1991) *J. Biol. Chem.* **266,** 7819–7826