DNA Replicases from a Bacterial Perspective

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Annu. Rev. Biochem. 2011. 80:403–36

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-061208-091655

0066-4154/11/0707-0403$20.00

Keywords

DNA polymerase III, DnaX complex, processivity, error-prone polymerase, PHP exonuclease

Abstract

Bacterial replicases are complex, tripartite replicative machines. They contain a polymerase, polymerase III (Pol III), a β_2 processivity factor, and a DnaX complex ATPase that loads β_2 onto DNA and chaperones Pol III onto the newly loaded β_2. Bacterial replicases are highly processive, yet cycle rapidly during Okazaki fragment synthesis in a regulated way. Many bacteria encode both a full-length τ and a shorter γ form of DnaX by a variety of mechanisms. γ appears to be uniquely placed in a single position relative to two τ protomers in a pentameric ring. The polymerase catalytic subunit of Pol III, α, contains a PHP domain that not only binds to a prototypical ε Mg^{2+}-dependent exonuclease, but also contains a second Zn^{2+}-dependent proofreading exonuclease, at least in some bacteria. This review focuses on a critical evaluation of recent literature and concepts pertaining to the above issues and suggests specific areas that require further investigation.

Contents

1. INTRODUCTION

Cellular chromosomal replicases from all branches of life are tripartite. They contain a polymerase, DNA polymerase III (Pol III) in bacteria and Pol δ and ε in eukaryotes, a sliding clamp processivity factor (β_2 in bacteria and proliferating cell nuclear antigen in eukaryotes), and a clamp loader (DnaX complex in

bacteria and replication factor C in eukaryotes). By themselves, replicative polymerases do not exhibit special properties that distinguish them from other polymerases, but together with the sliding clamp and clamp loader, they become highly processive (1). Early functional studies revealed β_2 as the key processivity factor (2), and an ensuing crystal structure elegantly showed the structural basis for its function (3). β_2 forms a ring that surrounds the DNA template and tethers the polymerase to it, much like a carabiner surrounds a rope and tethers a rock climber so that any transient dissociation is reversible, enabling processive replication.

Other features of bacterial replication (**Figure 1**) are similarly conserved among all life forms. A hexameric helicase precedes other replication functions and separates two strands at the replication fork. In bacteria, the helicase ($DnaB_6$ in *Escherichia coli*) translocates 5′→3′ along the lagging-strand template (4). The exposed single-stranded DNA (ssDNA) is coated on the lagging strand by a ssDNA-binding protein (SSB in bacteria or a heterotrimeric replication protein A in eukaryotes). Priming is catalyzed by a dedicated primase in all systems, generating short RNA primers that are elongated by a DNA polymerase (**Figure 1*a***).

SSB: single-stranded DNA-binding protein

Pol III HE: DNA polymerase III holoenzyme

In this review, issues pertaining to bacterial replicases are examined using the *E. coli* DNA polymerase III holoenzyme (Pol III HE) as a prototype, except where differences are understood and viewed as important. Topics were chosen for inclusion either because they are relatively new and not covered by other reviews or because new ways of looking at some old problems are considered. I have taken the approach of reevaluating concepts that lack full support and, where alternatives exist, that have not been fully considered. My intent is

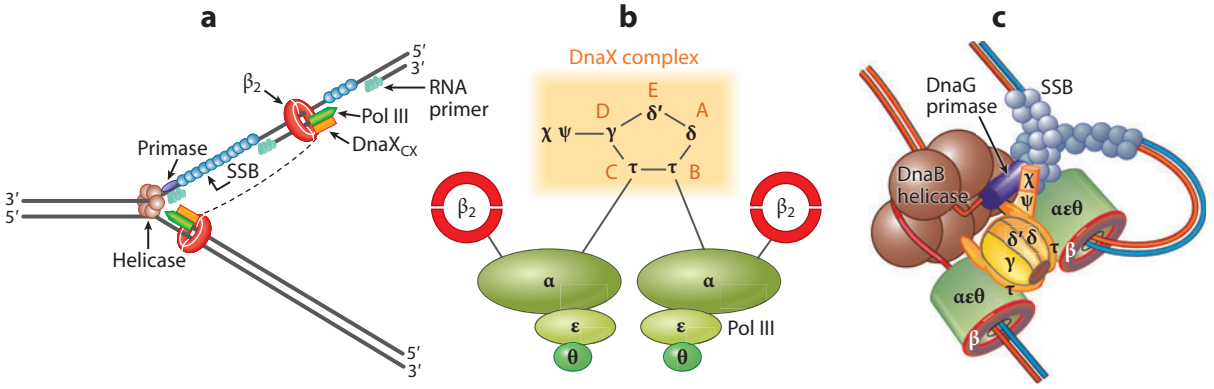

Figure 1

DNA polymerase III holoenzyme (Pol III HE) contacts at the replication fork. (*a*) A hexameric helicase (DnaB in *E. coli*) uses the energy of ATP hydrolysis to translocate down the lagging-strand template, splitting two strands apart in advance of the leading-strand replicase, Pol III HE. Single-stranded regions of the lagging-strand template are coated by ssDNA-binding protein (SSB). Primase interacts with the helicase and synthesizes short RNA primers for Okazaki fragment synthesis; these primers are extended by the Pol III HE until a signal is received to recycle to the next primer synthesized at the replication fork (Section 4). Gaps between Okazaki fragments are filled, RNA primers are excised by DNA polymerase I, and the resulting nicks are sealed by a DNA ligase (not shown). For clarity, this view is drawn with discrete $DnaX_{cx}$ on each Pol III; they are actually shared between the leading- and lagging-strand polymerase (*dashed line*). (*b*) Details of known subunit interactions within the Pol III HE. ψ threads through all three DnaX protomers (45) and is shown here to signify the unique cross-link observed with γ (46) (Section 2.2). In addition, there is a transient interaction between δ and, perhaps, additional $DnaX_{cx}$ subunits with β_2 during the clamp-loading reaction. The subunit positions (A, B, C, D, and E) are indicated using the scheme suggested by M. O'Donnell. (*c*) A cartoon of the replication fork showing relevant protein-protein interactions, including dimerization of the leading- and lagging-strand polymerases through contact of domain V of τ with α (24). A contact between domain IV of two τs and two DnaB protomers anchors the replicase to the helicase, placing all replication fork components into one replisome (25, 70).

to stimulate experimentation that will provide a firm foundation for correct concepts or lead to consideration of new paradigms. There are excellent reviews that cover historical and other issues more completely than I do here (5–10).

2. INITIATION COMPLEX FORMATION

2.1. The β_2 Clamp-Loading Reaction and the DnaX Complex

Before DNA elongation begins, the Pol III HE forms an initiation complex in an ATP-dependent reaction (11–14). This reaction is often artificially divided into an ATP-dependent clamp-loading reaction in which a β_2 ring is loaded around DNA and a subsequent association of DNA Pol III occurs with the loaded clamp (13–15). In cells, the reaction likely occurs in a tightly coupled series of reactions in which Pol III is chaperoned onto the newly loaded clamp by τ-containing DNA complexes (Section 2.3) (16). Under physiological protein concentrations, sliding clamps require ATP-powered clamp loaders for their assembly around DNA (13–15, 17). The *E. coli* clamp loader is composed of seven subunits, encoded by five genes (18, 19). The ATP-binding subunit that drives the assembly process is encoded by *dnaX*, which directs the synthesis of two proteins, τ and γ, by programmed ribosomal frameshifting (20–22). The shorter γ contains three domains (23); τ contains two additional domains (termed IV and V, respectively) that bind the replicative helicase $DnaB_6$ and Pol III (24, 25). Thus, τ serves as a central organizer for the replisome, holding all of the catalytic activities together (**Figure 1**). Three copies of DnaX form a ring with single copies of subunits δ and δ′ (23, 26). All five proteins contain the same three domain folds (23, 27). The five core subunits of the DnaX complex bind to one another by domain III. Deleting the other domains impairs function but not the thermodynamic stability of the complexes formed (28–31). This presumably allows significant conformational plasticity during the clamp-loading reaction.

The clamp loader was initially named after the DnaX γ-subunit (32) before τ was discovered (33) and later shown to also be a product of the *dnaX* gene (34). The literature often refers to the clamp loader as the γ complex (γ_{cx}), a term that can be confusing because clamp loaders can be constituted that do not contain γ, only τ. We use the general term DnaX complex ($DnaX_{cx}$) to refer to the clamp loader and use the term τ complex (τ_{cx}) when we are specifically referring to $DnaX_{cx}$ that lacks γ.

2.2. Mechanism of β_2 Loading on Primed DNA

The basic models of the first step of initiation complex formation, clamp loading, have been worked out by the O'Donnell laboratory in a long-standing productive collaboration with the Kuriyan lab. The δ-subunit of the $DnaX_{cx}$ binds β_2 by interaction of hydrophobic residues within δ domain I with a cleft between β-domains I and II (35). In addition, δ interacts with a helical segment that is thought to destabilize the dimer interface (35). Current models suggest this interaction cracks open one β_2 interface in an initial step. δ is sequestered or is in an alternative conformation in the unliganded state of the $DnaX_{cx}$ (23, 35). Binding of ATP changes the conformation, revealing δ to enable clamp binding and opening (36).

Rigorous presteady-state kinetic studies of the β_2-loading reaction have been performed by Bloom and colleagues (37). ATP serves as an allosteric effector, increasing the affinity of the $DnaX_{cx}$ for β_2 and DNA, bringing all reaction participants together (37, 38). The $DnaX_{cx}$, upon binding a primer template, rapidly hydrolyzes bound ATP, even in the absence of β_2, resulting in a futile cycle (39). In a study investigating the rate of binding of β_2 and primed DNA to γ_{cx}, it was found that β_2 bound to γ_{cx}-ATP at a diffusion-controlled rate, whether γ_{cx} had been preincubated with ATP or not. In contrast, primed DNA bound slowly (limited by the slow ATP-induced conformational change within the γ_{cx} of 3.3 s^{-1}) unless the γ_{cx} had been preincubated with ATP, overcoming a

rate-limiting conformational change (37). This suggests a mechanism for minimizing the futile cycle that would result from γ_{cx}-ATP-binding DNA in the absence of β_2. At replication forks, an ordered mechanism may be further enforced by the availability of primers for DNA replication that are only synthesized about every 2 s.

Another investigation addressed the relative order of β_2 release, primer-template release, and ATP hydrolysis in the final stages of the γ_{cx}-catalyzed loading reaction (40). A fluorescence resonance energy transfer pair was used to measure γ_{cx}-β_2 dissociation. Anisotropy, using fluorescently tagged DNA, was used to monitor DNA release. Once a γ_{cx}-β_2-ATP complex bound DNA, hydrolysis of three ATPs was triggered, followed by DNA release, then β_2 release. It is not yet known when β_2 becomes closed in this process. It was suggested that β_2 closing might have preceded DNA release, but the β_2 closing state was not experimentally determined. Thus, it is not yet known if ATP hydrolysis is coupled to or follows the closing of β_2. Recognizing that sliding away and loss of the DNA substrate does not likely represent the physiological reaction, it was suggested that a χ-SSB interaction might normally prevent DNA release (40). Electrostatic interactions between the basic interior of the β-ring and DNA may be important for β_2 closing and other properties of the reaction.

The χ- and ψ-subunits of the $DnaX_{cx}$ perform several interesting functions, including stabilizing the interactions between DnaX, δ and δ' so that they assemble at physiological protein concentrations (41). The ψ-subunit binds to domain III of DnaX (28) and serves as a bridge to the χ-subunit that binds to SSB (42, 43). Recently, the Bloom laboratory (44) demonstrated important functional consequences that correlate nicely with a structure determined by Kuriyan and colleagues (45) that reveals conformational changes induced by the interaction of an N-terminal peptide of ψ with γ_3 in a complex with $\delta\delta'$. In the absence of $\chi\psi$, $\gamma_3\delta\delta'$ binds DNA poorly in the presence of ATP, a defect partially compensated by the presence of β_2. It was concluded that ψ stabilizes an ATP-induced conformational state that binds DNA (44).

This structure also reveals how binding is restricted to a single copy of $\chi\psi$. The N terminus of ψ snakes through a collar of three DnaX domain IIIs in an asymmetric way with different contacts with each, but in a way that precludes further ψ association (45). The ψ peptide enters between DnaX subunits B and C (likely both τs in **Figure 1*b***) and contacts the DnaX subunit D (γ) with its N terminus. Interestingly, the only residue within the peptide that is capable of participating in an imidoester cross-link is in the N terminus, which contacts the DnaX subunit D, the position proposed to form a cross-link with the unique copy of γ within the Pol III HE (46). It was proposed that tethering ψ to the collar of the $DnaX_{cx}$ would allow the bound χ to contact SSB bound to ssDNA exiting the $DnaX_{cx}$ as β_2 is loaded onto primed DNA (45). Testing that hypothesis and learning if there are additional protein-protein contacts between $\chi\psi$ and components of the $DnaX_{cx}$ awaits additional experimentation.

Binding of the ψ peptide stabilizes a conformation that binds the ATP analog, ADP-BeF_3, in all three DnaX sites (45). DNA binding favors a similar conformational change. This contrasts with structures in the absence of ψ, where the DnaX subunit C (**Figure 1*c***) is in a conformation that cannot bind ATP (47). The DNA-ADP-BeF_3-$\gamma_3\delta\delta'$–ψ peptide structure adopts a "notched screw cap" conformation in which the ATPase subunits are symmetrically oriented in a spiral arrangement and contacts are made with the phosphates of the template strand through basic residues and α-helical dipoles. No contacts are made with the primer, other than a stack between δ Tyr316 and the terminal base of the primer. An interesting conjecture was made that this arrangement would allow for accommodating both RNA and DNA primers (45). The residues surrounding the ADP-BeF_3 binding site appear to be in a conformation in which all three are poised to catalyze hydrolysis, presumably leading to β_2 closing and loss of affinity of the $DnaX_{cx}$ for β_2 and DNA. It will be interesting to see, in future studies, whether β_2 is

open when bound to this assembly and whether the ATP sites remain equivalent.

The *E. coli* χ- and ψ-subunits play a critical role in clamp loader function, yet homologs are not recognizable in most bacteria. Even in *Pseudomonas aeruginosa*, closely related to *E. coli*, a ψ-subunit could not be found using standard bioinformatics approaches. Yet, *E. coli* χψ, if added to the minimal *Pseudomonas* Pol III HE subunits, stimulated activity (48). This gave us confidence to isolate the native *Pseudomonas* Pol III HE, permitting identification of ψ from *Pseudomonas* by a proteomics approach, cloning, and expression and demonstration of function (49). Thus, other minimal Pol III HE systems, such as those reconstituted from expressed *Thermus thermophilus* (50) or *Bacillus subtilis* (51, 52) subunits, might also profit from χψ and perhaps additional missing components.

2.3. Chaperoning of Polymerase III onto Newly Loaded β_2 by the DnaX Complex

Recently, it has been demonstrated that τ-containing DnaX complexes serve another role in initiation complex formation: They chaperone the associated polymerase onto the β_2 just loaded by the same complex (16). Chaperoning significantly increases the rate of initiation complex formation and drops the Pol III concentration required for efficient assembly. Both of these features are likely critical for the cell to sustain a rate of initiation complex formation required to support the rate of Okazaki synthesis needed for chromosomal replication. The τ-mediated chaperoning pathway reveals another important characteristic—it can proceed without ATP hydrolysis if ATPγS is present as an allosteric effector to increase the affinity of the $DnaX_{cx}$ for β_2 and primed DNA (16). This is consistent with the notion that ATP hydrolysis plays a downstream role (40) but is not a participant in the same mechanistic step where β_2 is closed around DNA. A working model incorporated the observation that ATPγS serves as an allosteric effector, converting the $DnaX_{cx}$ from a low-affinity state to one with high affinity for primed DNA and β_2 (16). Within the ternary β_2-$DnaX_{cx}$-DNA complex, a portion of β_2 is proposed to be closed around the DNA, likely driven by the positively charged inner surface of the β_2 ring. Pol III, presumably, has a higher affinity for the closed form of β_2 and may be able to attach to it, driving the closing reaction to completion (**Figure 2**). $DnaX_{cx}$ is then displaced from DNA by competition with Pol III, a process that is likely accelerated by ATP hydrolysis, increasing the dissociation rate of $DnaX_{cx}$ from DNA. Indeed, we have demonstrated in recent presteady-state kinetic experiments that the rate of functional initiation complex formation is 100-fold faster in the presence of ATP (C.D. Downey & C.S. McHenry, in preparation). Although ATPγS serves as a valuable tool for studying initiation complex formation, the results need to be interpreted with caution because its allosteric effects are not precisely those of ATP, perhaps because of interference with the interaction of the Arg finger or other residues with the γ-phosphate of ATP. β_2 binding to $DnaX_{cx}$ is weaker in the presence of ATPγS, and DNA binding is slower (37, 44).

The stimulation of initiation complex formation by SSB with τ-containing $DnaX_{cx}$ is even greater in ATPγS-supported reactions (**Figure 2*c***) than with ATP (**Figure 2*b***), suggesting an additional positive role for SSB interaction with a component of the Pol III HE beyond its protective role described above. The only known SSB-interacting protein within Pol III HE is χ (42, 43). The χ-SSB interaction has been shown to be important for allowing Pol III to replicate SSB-coated DNA (43) and for stabilizing Pol III on DNA in strand displacement reactions (53). Yet, the stimulation by SSB occurs in the absence of χ, revealing that an additional, undiscovered site for SSB interaction exists (16). SSB stimulation requires its C-terminal tail, which is involved in interactions with a large number of proteins that participate in transactions on ssDNA, but it is not required for DNA binding (42, 54, 55). An interaction between Pol III HE and the C-terminal

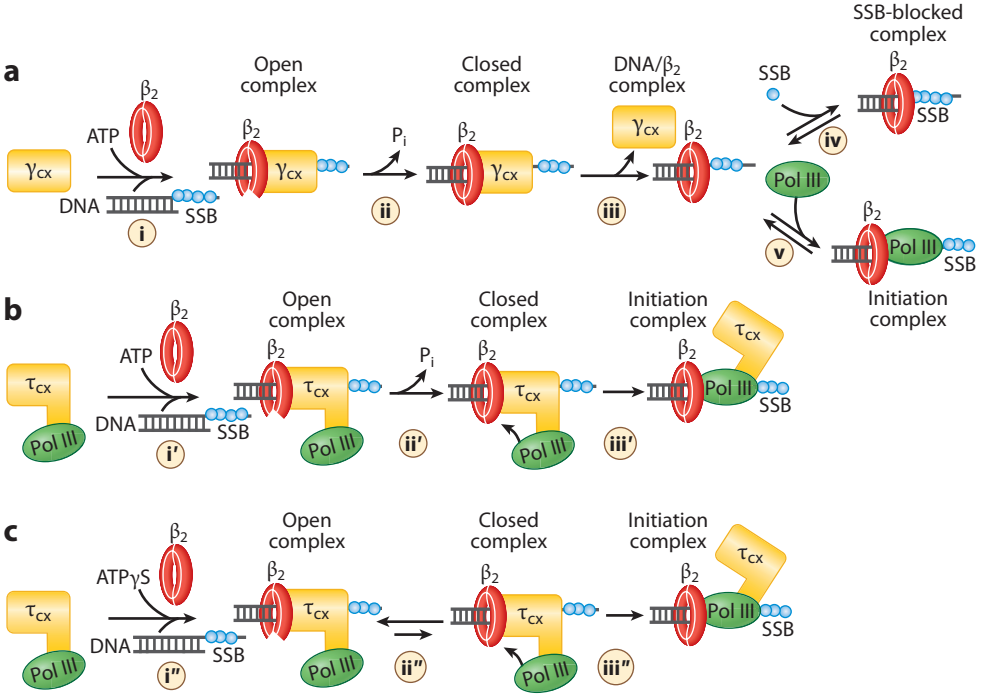

Figure 2

Models for unchaperoned and chaperoned initiation complex formation. (*a*) Model for unchaperoned initiation complex formation catalyzed by the γ complex. (*b*) Model for chaperoned initiation complex formation catalyzed by the τ complex. Steps i′ and ii′ of this reaction are the same as the analogous steps for γ complex in panel *a* except that polymerase III (Pol III) is associated with the $DnaX_{cx}$. In step iii′, Pol III binds and traps the newly closed β_2, permitting concerted Pol III loading. (*c*) Model for chaperoned initiation complex formation in the absence of ATP hydrolysis. The mechanism is the same as in panel *b* except that in i″ ATPγS substitutes for the allosteric effects of ATP and in step ii″ the closing of β_2 is presumably energetically unfavorable in the absence of ATP hydrolysis. The coupled equilibria of steps ii″ and iii″ drive the reaction to form an initiation complex, competent for extension upon the addition of deoxynucleoside triphospates (dNTPs). An early report using DNA polymerase III holoenzyme (Pol III HE) purified from wild-type cells related that ATPγS was hydrolyzed (131). Enzymes reconstituted from purified components appear not to hydrolyze ATPγS (60, 175). The higher rate measured with chromosomally encoded Pol III HE may have been due to helicase II (UvrD), which is tightly associated in vivo and was only removed by purifying Pol III HE from strains that contain a *uvrD* deletion (136). However, the issue of ATPγS hydrolysis requires a rigorous quantitative reexamination.

tail of SSB could either increase the rate of formation of the ternary β_2-$DnaX_{cx}$-DNA complex or increase its lifetime, permitting time for productive β_2 loading (steps i and ii in **Figure 2**). We note that such an interaction could stimulate the γ_{cx}-mediated loading of β_2 onto DNA, but the downstream inhibition of Pol III binding or elongation by SSB could have caused such stimulation to be missed in our assays.

In two-stage reactions, in which all reaction components except deoxynucleoside triphospates (dNTPs) are present in the first stage, the γ complex-driven reaction is markedly inhibited by SSB, but the τ_{cx}-driven reaction is modestly stimulated (16). Inhibition by SSB might arise from competition between Pol III and SSB for binding to the template after the dissociation of γ_{cx}, which presumably protects an SSB-free "landing pad" for Pol III

(**Figure 2*a***, steps iii–v). In τ_{cx}-mediated reactions, the putative landing pad is protected until Pol III is chaperoned into place, preventing inhibition by SSB (**Figure 2*b***, steps ii′ and iii′). Other possibilities exist, and the proposed mechanism of inhibition remains to be soundly established.

A Pol III HE-SSB interaction could also perform a remodeling function, opening up an SSB-free landing pad for association of $DnaX_{cx}$ and, subsequently, Pol III. Binding of proteins to the C-terminal tail of SSB has been shown to shift the internal equilibrium between $(SSB_4)_{65}$ and $(SSB_4)_{35}$ [forms of SSB_4 that bind 65 and 35 nucleotides (nt), respectively] in favor of the latter (56). Such an interaction could cause a 30-nt stretch of ssDNA to be released immediately after the primer terminus. A structure of the $DnaX_{cx}$ shows a contact between δ and at least the first 2 nt of the template that follow the primer (45). This contact could be responsible for protecting the region downstream of the primer terminus from SSB binding until Pol III is chaperoned into place.

2.4. Hydrolysis of an ATP by Each DnaX Protomer May Not Be Required for Initiation Complex Formation

Current models propose that the *E. coli* $DnaX_{cx}$ binds and hydrolyzes three ATPs in a synchronous wave during the clamp-loading process (57). And, the various DnaX protomers within the $DnaX_{cx}$ have been proposed to have specific functions in β_2 and DNA interactions (58). Recently, both the γ and τ forms of DnaX, with the critical Lys in the Walker A motif changed to a Glu, have been purified (59). Together with their wild-type counterparts, these mutants have been assembled into DnaX complexes, and the 10 possible resulting complexes have been purified and characterized. Surprisingly, complexes containing only one active ATP-binding DnaX protomer function efficiently in initiation complex formation.

Our working model is that DnaX exists in two states, a high-affinity state (for both β_2 and primed DNA) and a low-affinity state, and that a single ATP is adequate to shift the equilibrium between the two states. Three bound ATPs might be more effective in shifting the equilibrium to the high-affinity conformer. Indeed, three bound ATPs are more effective than one in increasing the affinity of the $DnaX_{cx}$ for β_2 (59). This could be due to local conformational changes, leading to additional contacts with β_2, as proposed in Reference 45. And, hydrolysis of a single ATP is adequate to shift the complex to a low-affinity state and efficiently drive β_2 loading or facilitate $DnaX_{cx}$ release. Further studies, including a presteady-state determination of the rate of initiation complex formation by mutant complexes and an investigation of their ability to sustain the required rate of Okazaki fragment initiation on authentic replication forks, are required. However, current findings strongly suggest that the pathway is likely different when the Pol III HE follows the natural course of initiation complex formation as opposed to the β_2 loading reaction in the absence of Pol III.

2.5. Does the DnaX Complex Really Open the β_2 Clamp?

The textbook view for *E. coli* clamp loading assumes that ATP binds to γ_{cx}-β_2 and drives the opening of β_2 at one interface (60). However, an equally plausible model has not been fully considered for *E. coli*, though it has been largely established for bacteriophage T4. Open β_2 could exist in equilibrium with closed β_2 in solution, and the allosteric state of the $DnaX_{cx}$ favored by a bound ATP effector could merely bind the open form and pull the equilibrium. The crystal structure of β_2 shows it closed, but that conformation could be favored by crystal packing forces. An exploration of the conformational states of β_2 in solution needs to be performed, perhaps using hydrodynamic and small-angle X-ray scattering techniques. A significant population of the T4 gp45 clamp is open in solution (42 Å) (61), even though crystal structures show it closed (62, 63).

Indeed, some evidence for an alternative β_2 conformation exists from gel filtration studies

in which the observed apparent Stokes' radius decreased in the presence of Mg^{2+} (64). Because of a β_2 concentration-dependent change in the Stokes' radius, we initially misinterpreted this change to be a dissociation of both interfaces of β_2. This elementary mistake could have been avoided if we had performed velocity sedimentation studies in parallel, which would have pointed to a shape change rather than a molecular weight change. We now know through equilibrium sedimentation that the second interface in a β_2 dimer does not dissociate readily (H.G. Dallmann & C.S. McHenry, unpublished). A possible explanation is that β_2 opens and closes at one interface, with low Mg^{2+} favoring the open form. The β_2 concentration dependency of the apparent Stokes' radius may have derived from the nonideal solution behavior of clamps, such as that observed for T4 gp45 (65).

3. ELONGATION

3.1. Interactions that Contribute to Polymerase III Processivity

The primary determinant of processivity of the *E. coli* replicase is the interaction of Pol III with β_2 (2, 3). Pol III alone exhibits a very low processivity (~10 nt) (1). Complete Pol III HE is able to replicate an entire 5-kb circle without dissociating (1) and, judging from the static stability of initiation complexes, could have the processivity to replicate an entire chromosome without dissociating (66).

However, interactions beyond β_2 stabilize the interaction of Pol III with the replication fork and, thereby, increase its processivity. The presence of the ε-subunit increases the apparent affinity of α for the primer template and the k_{cat} for the polymerization reaction (67) and also increases the processivity of α (68). The presence of τ also increases the processivity of Pol III and permits stimulation by spermidine, which decreases the processivity of Pol III (69). The presence of all additional subunits, except β_2, increases processivity further and renders the polymerase susceptible to stimulation by SSB, a protein that inhibits Pol III and Pol III-τ-τ-Pol III (69). Pol III alone is unable to replicate ssDNA coated by SSB. To accomplish this feat, τ, ψ, and χ must be present if β_2 is absent (43). τ does not serve its prototypical role as the clamp loader in this minimal system, but apparently only functions as a bridge, tethering χψ in the same complex with Pol III, enabling an otherwise weak Pol III–template interaction to be stabilized by a χ–SSB contact (43). Two τ protomers bind the DnaB helicase, further stabilizing the replicase at the fork (70, 71). Pol III HE acting in concert with DnaB exhibits a processivity of >150 kb (72, 73). These multiple interactions that contribute to processivity appear to offer redundancy. For example, in the presence of the DnaB-τ-Pol III interaction, ε is not required for high processivity (74).

During processive replication of long single-stranded templates, Pol III HE typically stops synthesis upon encountering a duplex (75). However, a strand displacement activity of the DNA Pol III HE has been observed under a variety of conditions (53). Interaction of the leading-strand polymerase with the lagging-strand template, mediated by a Pol III-τ-ψ-χ-SSB bridge, is essential for efficient strand displacement (53). Extrapolating these findings to natural replication forks suggests that the leading-strand polymerase is stabilized by interactions with the lagging strand coated with SSB, mediated through a τ-ψ-χ link.

τ serves in the additional role of protecting β within elongating complexes from removal catalyzed by an exogenous γ complex (76). It is not known whether this protective mechanism involves contact between the unique C-terminal sequences of τ (C-τ) and β or if it arises from τ sequences common with γ that prevent contact of β by the exogenous γ complex. If the latter mechanism is operational, then the γ portion of the τ attached to elongating Pol III must be "turned off" so that it cannot remove β. We note that photocross-linking of authentic Pol III HE, isolated from wild-type cells, to primers showed that γ (present in initiation complexes because of being present in mixed natural τ/γ-containing DnaX complexes)

interdigitated between Pol III and β_2 in part of the initiation complex (77), perhaps filling the gap present in models constructed using merged structures of β_2 and Pol III α complexed with DNA (Section 3.2.4.4) (78). These important issues pertain to the structure of and communication within the replicating complex and are worthy of further investigation. The $\delta\delta'$ subunits of the $DnaX_{cx}$, best known for their roles in β_2 loading, are also required for optimal processivity (79). It is not understood whether their role in processivity protects β_2 from removal, in concert with τ, or is something else.

The extremely high rate of elongation and high processivity of the Pol III HE suggests this enzyme may provide a superior tool for demanding polymerase chain reaction–based and other amplification strategies for very large regions of DNA. The availability of reconstituted bacterial replicases from thermophiles, such as *T. thermophilus*, which reproduce many of the properties of the *E. coli* replicase, provides promise for further development of such tools (50).

3.2. DNA Polymerase III Structure

3.2.1. Modular organization of the catalytic subunit of polymerase III. The α-subunit of Pol III has been classified as a class C polymerase, distinct from eukaryotic polymerases and the other polymerases found in *E. coli*. Functional and genetic experiments have demonstrated the modular nature of Pol III α, and recent structures have refined the definition of its domain boundaries and provided valuable insight into its function (**Figure 3**). The extra domains, appended to the polymerase, confer special properties that include the ability to bind to and communicate with other replication proteins.

3.2.2. Polymerase domains. Regions of α with distinct biochemical activities initially helped delineate the domain organization. Systematic mutagenesis of conserved acidic residues permitted the identification of the three acidic side chains (*E. coli* D401, D403, and D555) that coordinate two Mg^{2+} ions, facilitating catalysis (80). Antimutator and nucleotide selection mutants, presumably associated with

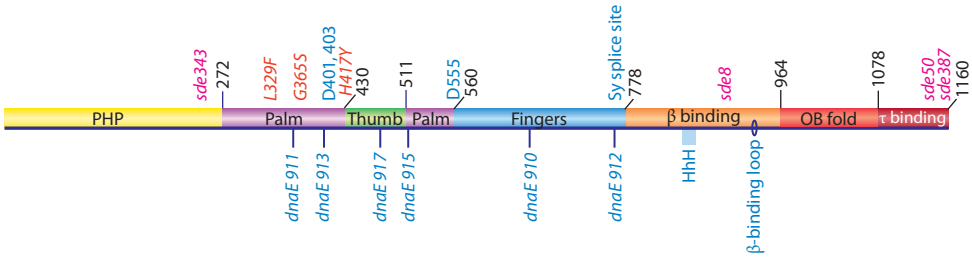

Figure 3

Modular organization of polymerase III (Pol III) α. The names and colors of the domains shown are from Bailey et al. (81) except that their C-terminal domain was further divided in the OB-fold and τ-binding domains. The residue numbers that define domain borders in *E. coli* α are shown (*above the bar* in *black*). The position of antimutator mutations for the *dnaE* gene (shown in *blue below the bar*) and the mutations selected to discriminate dideoxynucleotides (shown in *red above the bar*) are indicated (168, 176, 177, 178). It is likely that these influence either the rate of polymerization or base selection and reside within the polymerase active site. *sde* mutations that likely interfere with initiation complex formation (Section 3.2.4.3) are shown in magenta above the bar. Mutator mutations (not shown) in *dnaE* (168, 179, 180) also map within the polymerase domain (palm, thumb, fingers) with the exception of two temperature-sensitive alleles (74 and 486) that exhibit a slight mutator phenotype at the permissive temperature (168). *dnaE*74 maps to position 134 within the PHP domain and *dnaE*486 maps to position 885 between the β_2 binding site and the HhH element within the β_2 binding domain. A presumed template slippage mutant maps to residue 133 (181). D401, D403, and D555 are the three active-site acidic residues. Sy splice site designates the site of protein splicing in *Synechocystis*.

polymerase function, helped further define the limits of the polymerase domain (**Figure 3**).

The recently published apoenzyme structures of the full-length *Thermus aquaticus* α-subunit provided significant insight (81). A big surprise emerging from this study was that the palm domain has the basic fold of the X family of DNA polymerases that includes the slow, nonprocessive Pol βs, placing bacterial replicases as a special class within that family. A structure of a truncated version of *E. coli* α also exhibited a Pol β-like fold with perturbations in the active site, which are presumably corrected upon substrate binding (82).

Like all polymerases, Pol III α contains palm, thumb, and fingers domains, in the shape of a cupped right hand. Superposition of the Pol III α palm with that of mammalian Pol β aligns the three identified catalytic residues of α with those of Pol β (81). The palm also contains a universally conserved lysine (*E. coli* K552) that forms a salt bridge with the last phosphate of the primer. The fingers contain most of their conserved residues at the interface with the palm domain, including four arginines that appear to form a preinsertion nucleotide-binding site that binds the incoming dNTP before transfer to the actual catalytic binding site (81).

A ternary complex of a dideoxy-terminated primer template, incoming dNTP, and full-length *T. aquaticus* α provided significant insight into the function of class C polymerases (78). Among the primer template–induced conformational changes are movement of the thumb domain toward the DNA, bound by the palm, driven by interaction of two thumb α-helices in parallel with the DNA to make contacts with the sugar-phosphate backbone in the minor groove. The fingers also move, and a portion of the fingers domain rotates ∼15°, together with the palm and the 3′ terminus of the primer, forms a pocket that positions the incoming dNTP. The incoming dNTP is positioned above the three essential catalytic aspartates. The γ-phosphate contacts the Gly-Ser motif (*E. coli* 363–364), found in all polymerases, and an additional arginine (78). The polymerase contacts the template from its terminus to a position 12 nt behind the primer terminus, which is in excellent agreement with photocross-linking experiments (77). The fingers domain creates a wall at the end of the primer terminus, which forces a sharp kink in the emerging template strand. Finally, a 30° bend is induced 2 nt behind the primer terminus, induced by loops that connect the palm and thumb domains (78).

In the ternary complex structure of a Gram-positive Pol III, two novel elements not present in *E. coli* or *T. aquaticus* α were identified (83). The four-Cys Zn^{2+}-binding motif, discovered by Brown and colleagues (84) and shown as a requirement for activity, serves an apparent structural function and is not part of the catalytic site (83). DNA binding through the thumbs domain comes primarily from two β-strands that interact with the minor groove. Packing appears tighter around the template primer in this enzyme than in other polymerases (83). Proposals were made that this packing made unique contributions to preserving fidelity (83). However, no support was provided for that hypothesis. The discrimination against RNA primers made uniquely by PolC Gram-positive polymerases is a more likely consequence of the tight packing observed (51). This should be explored experimentally. The wider diameter of the A form RNA-DNA duplex appears not to fit well into the DNA-binding channel. Thus, an RNA-DNA duplex might not bind strongly, or conformational changes that are coupled to template-primer binding might not occur completely, leading to improper formation of the catalytic site. The presentation of the Gram-positive PolC structure concluded that the DNA template primer was bound in a significantly different orientation relative to that observed in *T. aquaticus* α (83). However, as pointed out by Wing (85), there really is no significant difference if one aligns the structures using the invariant catalytic acidic residues as a reference.

3.2.3. PHP domain. Aravind & Koonin (86) observed homology between the N-terminal region of bacterial Pol IIIs (called the PHP

domain) and a subclass of phosphoesterases. This domain is found in a wide variety of bacterial polymerases, including bacterial Pol βs. Initially, it was proposed that this region might be involved in pyrophosphate hydrolysis (86), but such an activity has not been found (82). Recently, this domain has been ascribed a second proofreading activity that is Zn^{2+} dependent (87) and also has been identified as the domain that binds the classical Mg^{2+}-based proofreading activity, ε (88). Deletion experiments initially restricted the domain to residues 1-(255–320), which means that the C-terminal boundary was only generally localized to somewhere between positions 255 and 320. Recent crystal structures provided further precision (**Figure 3**) (81, 82, 88). The structure of *T. aquaticus* α revealed a cluster of nine residues in the PHP domain; these included eight of the ligands predicted from informatics approaches (88) to chelate three metal ions (81), as shown directly for the *E. coli* YcdX homolog (89). A structure of a Gram-positive PolC PHP domain binds three metals, using the same nine ligands expected from the homologous *T. aquaticus* PHP structure.

Kuriyan and colleagues (82), from the structure of the *E. coli* α, pointed out a channel between the polymerase active site and the proposed PHP active site. The PHP domain contains a long loop on top of the domain (*E. coli* 107–116) that interacts extensively with the thumb. There may also be contacts between the PHP domain and DNA (78). This would explain the dependency of polymerase activity on the integrity of the PHP domain. Deletion of 60 N-terminal PHP residues or a D43A mutation within the proposed active site abolishes polymerase activity (90). In bacterial Pol βs that contain associated active PHP domains (91, 92), deletion of the polymerase domain abolishes PHP activity, an indication of the reciprocal nature of the interaction (93).

3.2.4. C-terminal domains. Analysis of α deletion mutants revealed that the C-terminal region is responsible for interactions with both τ and β (94, 95). An essential β_2 interaction site (*E. coli* 920–924) (96) was verified by mutagenesis, coupled with functional, genetic, and biophysical experiments (97). Deletion of residues from the C terminus abolished τ binding, but N-terminal deletions extending into the fingers domain also diminished τ binding, suggesting either extensive τ interactions or structural perturbations (95). More-detailed mutagenesis studies (97) have identified the C terminus as critical for τ binding, but the binding site has not been firmly identified.

The C-terminal region of α contains additional domains identified by their similarity to elements found in other DNA-binding proteins. These include a helix-hairpin-helix (HhH) motif (*E. coli* 836–854) (81, 98) and an OB fold (*E. coli* 964–1078) (81, 99). In yet another variation, *Synechocystis* encodes the N-terminal two-thirds and the C-terminal one-third of α as two distinct proteins that are spliced posttranslationally by an intein-mediated reaction (**Figure 3**) (100).

3.2.4.1. β_2 Binding domain. A structure of *T. aquaticus* α revealed a well-organized β_2 binding domain with double-stranded (ds) DNA-binding capability. DNA binding occurs through a HhH motif and its flanking loops (78). The β_2 binding consensus sequence is presented in a loop that is oriented adjacent to dsDNA as it exits the polymerase in the correct position to bind β_2 as it surrounds DNA. The β_2 binding domain rotates 20° and swings down into position as the enzyme binds DNA (78), a reorientation that is apparently driven energetically by the HhH motif binding to DNA and likely coupled to conformational changes of the thumb, palm, OB fold, and PHP domains. A later structure of a Gram-positive Pol III showed a domain similar to the β_2 binding domain containing HhH motifs that bind dsDNA and a β_2 binding consensus sequence (83). The domains C-terminal to the β_2 binding domain in *E. coli* and *T. aquaticus* are either absent in the Gram-positive PolC or moved forward in front of the polymerase domain.

3.2.4.2. OB-fold domain. The structure of the ternary complex of *T. aquaticus* α with a primer template and incoming dNTP reveals a striking conformational change that includes the OB fold moving to a position near the single-strand template distal to the primer (78). The path of the emerging template, which can be traced from electron density of the ribose-phosphate backbone, appears to come close to the OB fold. The element of the OB fold that comes closest to the ssDNA template, the β1–β2 loop, often contributes to ssDNA binding (99). However, the β1–β2–β3 face that commonly interacts with ssDNA (99) appears to face away from the emerging template and to face the τ-binding domain. So, binding of the OB fold, if it occurs, either occurs in a nonstandard way, or there are further rearrangements as the template strand becomes longer or when additional protein subunits are present. A ternary complex structure of a Gram-positive Pol III includes an OB fold, but it shows no interactions with the 5-nt single-stranded portion of the template. Whether it interacts with a longer template awaits experimental verification. Binding (101) and kinetic data (P.R. Dohrmann & C.S. McHenry, in preparation) suggest that the "sensor" for completion of an Okazaki fragment can distinguish a single-nucleotide gap from a nick. It is not clear if the OB fold can bind a single-nucleotide gap strongly. However, there is at least one example of an OB fold binding to a nick (102). Future research should consider whether the signal for collision-induced dissociation might involve the OB fold binding the resulting nick. The role of the OB fold is further discussed in Section 4.3.

3.2.4.3. τ-Binding domain. The second half of the C-terminal domain in the *T. aquaticus* α structure revealed a domain containing an incompletely conserved sequence that binds weakly to β_2 but is not required for processive replication in vitro or function in vivo. This domain is loosely packed against the OB fold, with many polar residues in the interface (81). Mutational studies support the importance of this subdomain in binding τ (97). Further information regarding possible sites of interaction of this extreme C-terminal domain with τ was derived from a genetic screen for suppression of a dominant-lethal phenotype of an extrachromosomally expressed *dnaE* (sde) that formed initiation complexes but was unable to elongate because of a mutation in a critical catalytic aspartate (D403E) (J.C. Lindow & C.S. McHenry, unpublished). Suppression could result from any defect that decreased the ability of *dnaE* D403 to form initiation complexes and compete with wild-type Pol III. Among the full-length, properly folded proteins obtained from this screen (**Figure 4**), it would be expected that most *sde* mutants would interfere with interaction with partners required for initiation complex formation or slow the relative rate of initiation complex formation. Mutants were analyzed for specific interaction defects. Two mutations (W1134C and L1157Q) appeared to severely diminish the interaction with τ, consistent with

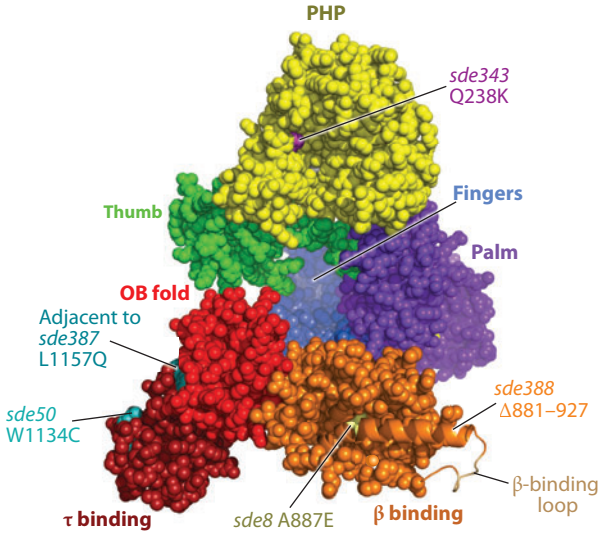

Figure 4

sde mutants mapped onto the polymerase III (Pol III) α structure. Mutations that suppress the dominant-negative phenotype of *E. coli dnaE* (D403E) are mapped onto the position of the corresponding residues in the *T. aquaticus* α-subunit structure (81). Labels specify the *E. coli* position number and residue. A deletion mutation that included the β_2 binding loop is shown in cartoon form (helix and loop not in space fill). Point mutations are shown as contrasting colors. Domains are shown using the convention of **Figure 3**: PHP (*yellow*), palm (*purple*), thumb (*green*), fingers (*blue*), β-binding (*orange*), OB fold (*bright red*), and τ-binding (*dark red*).

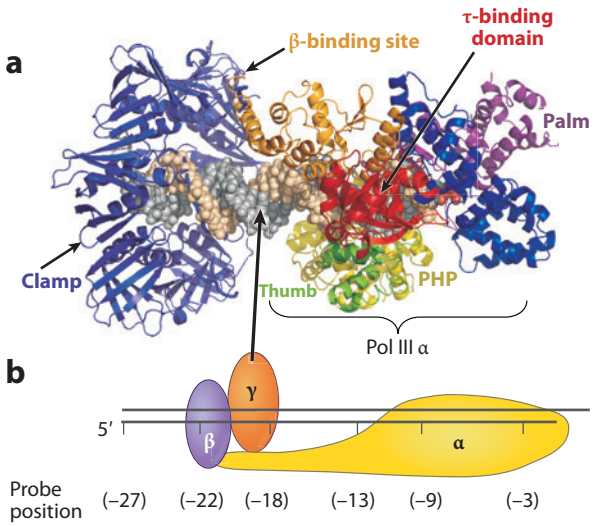

Figure 5

Models of β_2 binding to polymerase III (Pol III) α from Reference 78 with a proposed position for the binding of the γ-subunit of the $DnaX_{cx}$. (*a*) The β_2 binding site in Pol III α is indicated, docked to one of two polymerase-binding sites within the β_2 clamp (indicated by arrow to the *blue-purple* β_2 structure). The remaining protein components represent Pol III α, colored as in **Figure 3**. The structure in panel *a* was prepared from PyMOL files provided by R. Wing and rotated so that the PHP domain faces away from the plane of the paper and the τ-binding domain projects outward. (*b*) Positions of primer contacts with DNA polymerase III holoenzyme subunits determined by photocross-linking (77). These results suggest the γ-subunit of the $DnaX_{cx}$ fits into the open gap as indicated, contacting the primer strand (*gray*). Because domain V of the τ-subunit is believed to contact the upper portion of the C-terminal domain (shown in *dark red* in panel *a*, Section 3.2.4.3), γ could be drawn into position partially by that interaction. With a subunit sitting between the polymerase and the β_2 clamp, the $DnaX_{cx}$ could be in a position to modulate polymerase switching, perhaps using its chaperoning activity (Section 2.3), and to facilitate polymerase release and recycling during Okazaki fragment synthesis (Section 4.6).

a role of the C-terminal domain in an interaction with τ (**Figure 4**).

3.2.4.4. A model for polymerase III α bound to β_2 on the clamp. The availability of a structure of β_2 on dsDNA (103), and a knowledge of the β_2 binding site for polymerases (104, 105), permitted construction of a model of these proteins interacting on DNA (78). The model places β_2 approximately 20 nt behind the primer terminus (**Figure 5**), which is in agreement with footprinting, fluorescence resonance energy transfer, and photocross-linking studies (77, 106, 107). A proposal was made that the two polymerase-binding sites on β_2 could be used as an entry point for polymerase exchange at the replication fork (104). The model accommodates such an interaction. The same photocross-linking experiments that correctly assigned the contacts of β_2 and Pol III α with DNA also showed a clear cross-link of γ when photoreactive probes were placed on nucleotide −18 of the primer (77). We note that the open cleft might accommodate γ, which could be sequestered in mixed τ/γ DnaX complexes by interaction of τ with the τ-binding C-terminal domain (**Figure 5*b***).

3.3. Structure of Proteins Associated with Polymerase III

Structures of $DnaX_{cx}$ contained a truncated DnaX subunit, which excluded domains IV and V of τ. Subsequent structures of these domains (108, 109) show that domain V has a fold that is unique to τ-subunits. Available information suggests that domain V binds to α through a largely unstructured C terminus (108, 109). Deletion of seven amino acids from the C terminus of τ abolishes its ability to bind to α (109).

Structures for χ bound to ψ are also available (110), and the structure of the ψ peptide bound to $\gamma_3\delta\delta'\chi$ permitted models to be formulated regarding its placement (45), but additional structural information is required to reveal if there are other protein-protein interactions that orient χψ. Structural information regarding the proofreading subunit, ε, that binds to Pol III α and its associated θ-subunit is reviewed in Section 6.

4. CYCLING OF THE LAGGING-STRAND REPLICASE DURING OKAZAKI FRAGMENT SYNTHESIS

4.1. Competing Models for Replicase Cycling

The Pol III HE has the processivity required to replicate >150 kb (72, 73), and perhaps the

entire *E. coli* chromosome, without dissociation, yet it must be able to efficiently cycle to the next primer synthesized at the replication fork upon the completion of each Okazaki fragment at a rate faster than Okazaki fragment production. The rate of replication fork progression in *E. coli* is about 600 nt/s at 30°C (111), approximately the rate of replicase progression on single-stranded templates (14). Thus, most of the time in an Okazaki fragment cycle is spent on elongation, and little time (~ 0.1 s) remains for the holoenzyme to release, bind the next primer, and begin synthesis. A processivity switch must be present to increase the off rate of the lagging-strand polymerase by several orders of magnitude.

There are two competing but nonexclusive models for the signal that throws the processivity switch. The first (the signaling model) proposes that a signal is provided by synthesis of a new primer at the replication fork that induces the lagging-strand polymerase to dissociate, even if the Okazaki fragment has not been completed (112). The second (the collision model) was originally proposed for T4 (113) and then extended to the *E. coli* system (101). The collision model posits that the lagging-strand polymerase replicates to the last nucleotide (101) or until the Okazaki fragment is nearly complete (114). A communication circuit that proceeds through the τ-subunit has been proposed to sense the conversion of a gap to a nick, signaling release. Experiments designed to test which mechanism is dominant have yielded equivocal results (115).

Key issues remain pertaining to the mechanisms used for cycling in *E. coli*. Significant kinetic problems exist with the collision model (Section 4.5), and the mechanism and interaction links used to signal cycling upon new primer synthesis remain largely unexplored. The initial single-molecule exploration of *E. coli* fork dynamics suggested that primer synthesis caused the leading-strand polymerase to dissociate (116), but this issue needs to be reexamined using a $DnaX_{cx}$ that contains two or more τs (increasing helicase association) and in the presence of χψ and SSB to increase the affinity of the replicase for its substrate (42, 43, 53) and modulate the activity and affinity of the primase. Supporting that notion, more recent studies showed increased processivity of the *E. coli* replicase when a complete system was employed (117) that largely agreed with the rates and processivities observed in early ensemble experiments (1, 14, 73, 118). Multiple primase subunits (most likely three) needed to be bound to helicase to induce polymerase dissociation in the simple system, giving insight into the stoichiometry required for primer synthesis in a more complete system (116).

4.2. Cycling Models Provided by Bacteriophages T4 and T7

In the more fully characterized systems provided by the replication apparatus of bacteriophages T4 and T7, signaling through synthesis or the availability of a new primer appears to play an important role, with the collision pathway playing an apparent backup role (119). A handoff of the nascent tetraribonucleotide primer and the T7 polymerase is effected by direct primase-polymerase interaction (120). It takes time for synthesis of a new primer, release of the lagging-strand polymerase, and initiation of synthesis on a new primer. Differing views have been presented on how T7 overcomes this delay on the lagging strand to allow leading- and lagging-strand replication to remain coordinated (121, 122). In one model, it is proposed that the helicase, and thus leading-strand synthesis, is halted during slow primer synthesis (121). In the other model, it is proposed that the lagging-strand primer is synthesized before needed and held in a priming loop, close to the replication fork, facilitating handoff to the DNA polymerase (122). This model, if generally applicable, could explain the double loops sometimes observed at replication forks by electron microscopy (123). In the latter model, it is proposed that the reactions remain coordinated because the lagging-strand polymerase elongates slightly faster than the leading-strand polymerase (122).

In T4, primer synthesis does not halt progression of the leading-strand polymerase. Primer synthesis occurs by two mechanisms: (*a*) the dissociative, with the primase releasing from its helicase association during primer synthesis; or (*b*) the processive, whereby the primase remains associated, and a second loop is formed on the lagging-strand DNA (124, 125). The clamp and clamp loader increase the processive/looping mechanism, and it was suggested that the gp32 (T4 SSB) might further increase the processive looping mechanism in the natural system (124). A handoff of the nascent pentaribonucleotide primer occurs in a reaction that is facilitated by gp32 and by increased concentrations of the clamp and the clamp loader (124, 125). A proposal was made that the clamp loader/clamp interaction with a new primer might be the key signal required for release of the lagging-strand polymerase (125).

4.3. Does the OB Fold Provide the Processivity Switch for Cycling?

In a structure of Pol III α complexed to DNA, an OB fold was located close to the primer terminus (78). Because OB folds commonly bind to ssDNA, a proposal was made that the OB fold could be part of the sensing network (78, 81). Consistent with this hypothesis, the ssDNA-binding portion of Pol III was localized to a C-terminal region of α that contains the OB-fold element (126). A test of the importance of the OB-fold motif was made using a mutant in which three basic residues located in the β1–β2 loop were changed to serine (114). No ssDNA binding was observed in the mutant, indicating diminution in affinity. However, even the wild-type polymerase bound ssDNA extremely weakly, near the limit of detection in the assays used (K_D ~8 μM).

The processivity of the mutant polymerase was decreased by the β1–β2 loop mutations, an effect that was rescued by the presence of the τ_{cx} (114). The latter observation would seem to suggest that, although the OB fold contributes to ssDNA affinity and processivity, it is not the processivity sensor, or at least, the residues mutated are not the key interactors. The OB fold might bind to the nick generated by completion of an Okazaki fragment, as seen in human ligase 1 (102), inducing a nonprocessive conformation. Alternatively, the OB fold might act in concert with other binding changes as part of a more complex signaling network.

In my view, the entire polymerase active site is likely the processivity switch. Steitz and colleagues (78) have elegantly demonstrated a conformational change induced by substrate binding in which several elements move that include placement of the β_2 binding domain in a position where it can productively interact with the β_2 clamp on DNA. Follow-on studies with a Gram-positive polymerase suggest this observation is general (83, 85). The geometry and spatial constraints around the active site when the exiting template is double-stranded might make insertion of the last nucleotide energetically unfavorable. Upon insertion, the product might lose affinity for the active site, triggering a reversal of the conformational changes that occurred upon primer-template and dNTP binding, causing the β_2 binding domain to be pulled away, switching the polymerase to a low-processivity mode. The presence of an unliganded polymerase domain serves to decrease the affinity of the C-terminal domains of Pol III α for β_2 (94), which is consistent with this view. The genetic screen for mutants, which led to a loss of the dominant-negative phenotype of D403E α (Section 3.2.4.3), revealed a mutant at a conserved alanine in the interior of the β_2 binding domain (A887E), which no longer binds β_2 tightly. Future studies should address whether the communication circuit that modulates β_2 affinity flows through this region. Perhaps a bulky residue changes the presentation of the β_2 binding loop or β_2 binding surface, decreasing affinity, and, if relevant to cycling, the dissociation rate.

4.4. What Is τ's Role in Cycling?

It has been proposed that τ acts as a sensor for conversion of a gap to a nick upon completion of Okazaki fragment synthesis (101, 127,

128). When bound to ssDNA, τ was proposed to lose contact with the C terminus of α, leaving the C terminus free to contact β. When the τ-ssDNA contact is lost, τ was proposed to bind the C terminus of α, displacing β and allowing the polymerase to cycle to the next Okazaki fragment (101, 127, 128). This model did not consider earlier data that showed that τ and β_2 did not compete significantly for polymerase binding and that the critical binding site for β_2 was internal and not at the extreme C terminus of α (94, 96, 129). Follow-up work rigorously confirmed that the internal β_2 site is the one required for processive replication (97). Interestingly, replacement of the internal β_2 binding site with the consensus sequence identified by informatics (96) increased the affinity 120-fold, whereas the same change at the C terminus had no effect on β_2 binding but caused a 2,700-fold decrease in τ binding (97). This suggests that either the internal site provides the β-interaction sequence in a unique conformation or additional local contacts dictate the specificity of binding. Although it is possible that the C terminus of α could interact with β_2 for some undiscovered ancillary purpose, all current mutational effects can be attributed to defects in τ interaction or minor structural perturbations. Furthermore, a consensus β_2 binding motif is not found at the C terminus of many replicative DnaEs, whereas the internal sequence is conserved.

The function of τ in enabling rapid cycling remains unclear. The earliest observation pertaining to a possible role of τ in cycling was made by Marians and colleagues (118). They saw a τ-dependent acceleration that was codependent upon primase and, along with an observed lessening of the proportion of short Okazaki fragments, proposed that τ increased the transit rate from an old Okazaki fragment to a new one (118). Proposals for τ being the sensor for a gap-to-nick conversion derived from equilibrium measurements in which τ decreased the affinity for a nick, relative to a gap (101). Previous photocross-linking experiments failed to detect any cross-linking to τ when phenyl azides were placed near the primer terminus (77). However, τ could have been missed if its binding was restricted just to the single-stranded region or if it did not present any residues that reacted with a somewhat chemoselective agent. We have placed diazirine side chains at positions 4 and 8 nt ahead of the primer terminus and only observe α cross-links (C.M. Manhart & C.S. McHenry, in preparation). Because irradiation of diazirines generates carbenes that will insert into any amino acid, we can interpret not seeing τ cross-links with confidence, eliminating the possibility that τ directly senses gap-to-nick conversion.

4.5. The Rate of Polymerase Dissociation upon Collision with the Preceding Primer Is Too Slow to Support Okazaki Fragment Synthesis

Most of the measurements assessing pathways to trigger polymerase release and cycling have been performed using equilibrium measurements. Although a decreased affinity might be consistent with a role in accelerating release, the real issue is whether the lagging strand can be triggered to release in ~0.1 s or less upon collision with the preceding Okazaki fragment's 5′ end. One kinetic experiment presented reported release with a half-life of ~1 s (101). However, reanalysis of the published data yielded a fit to a single exponential exhibiting a half-life of 14 s, too slow to support the rate of in vivo Okazaki fragment synthesis (P.R. Dohrmann & C.S. McHenry, unpublished).

We are currently pursuing this issue and have set up a surface plasmon resonance assay in which immobilized primers are placed a set distance from an oligonucleotide that models the 5′ end of the preceding Okazaki fragment. We find that filling in a gap to the final nucleotide accelerates release, but the rate is far too slow to support the physiological rate of Okazaki fragment synthesis (P.R. Dohrmann & C.S. McHenry, in preparation). The differences with published results might be that they were derived from equilibrium measurements and ours from kinetic determinations.

4.6. ATP Hydrolysis by an Associated τ-Containing DnaX Complex Might Be Part of the Circuit for Polymerase Cycling

In a search for factors that might accelerate collision-induced dissociation, we found that the presence of the τ complex, ATP, and an exogenous template primer accelerates dissociation fourfold. ATPγS will not substitute in this reaction, suggesting that hydrolysis is required. The rate enhancement by these factors is only achieved when a gap has been completely filled. β_2 is not required. These findings might reconcile previous models and observations pertaining to the requirement for gap completion (101), the availability of a new template primer (112), and the requirement for τ (101). A series of switches may exist, all of which must be accommodated for maximal cycling. Gap completion may put the Pol III HE in a state in which a signal can be received from the associated τ-containing $DnaX_{cx}$ that involves binding of a new primer template and ATP hydrolysis. Additional acceleration might be derived from other factors or their arrangement/interaction at the fork. Or, the collision mechanism might only be a backup in *E. coli*, with the primary signal provided by new primer generation or clamp loading. If polymerase kinetics evolved so that most Okazaki fragment synthesis is normally complete before a new primer-dependent signal is generated, separating the two mechanisms would be difficult using methods previously employed. However, specifically perturbing the rate of lagging-strand synthesis, as has been done in the T4 (125) and T7 (119) systems, might provide a promising route to unveil a signaling pathway in *E. coli* lagging-strand cycling.

4.7. Primase-Polymerase III Handoff

A three-point switch model has been proposed (130) whereby Pol III HE does not have access to primers synthesized by primase unless the χ-subunit of the Pol III HE first contacts the SSB subunit to which the primase is bound, permitting displacement of primase from the nascent primer. This model was based largely on the behavior of the protein encoded by the temperature-sensitive *ssb*-113 mutant. In the presence of SSB-113, χ was necessary for synthesis on templates only when primers were synthesized by primase—not on templates where synthetic primers were provided. The report generalizes the finding to the replication fork by showing that lagging-strand synthesis is lost without χ, perhaps because of its inability to use primers synthesized by primase. However, it does not demonstrate directly one obvious prediction of the model—that replication proceeding from primase-synthesized primers on single-stranded templates in the presence of wild-type SSB requires χ. The model needs to be reexamined to resolve an inconsistency with experimental observations: Replication of primase-primed M13Gori does not require χ provided adequate δ-δ′ is present to saturate DnaX and salt is maintained at moderate levels (**Figure 1** at low potassium glutamate concentrations as in Reference 43). (Salt concentrations are not directly relevant to the primase issue, but χψ becomes essential at high salt concentrations because χψ-SSB interactions stabilize holoenzyme-DNA complexes at high salt regardless of the source of the primer.) The reported experimental observations are undoubtedly valid, but some of the effects may reflect a gain-of-function defect in SSB-113. That is, SSB-113 causes a problem that requires χ to overcome it.

4.8. Is the Asymmetry of the Polymerase III Holoenzyme Relevant to the Recycling Issue?

The asymmetry of the $DnaX_{cx}$ imposes asymmetry on an otherwise symmetric dimeric replicase (**Figure 1**). Early hints of a possible functional asymmetry derived from the observations that ATPγS could support initiation complex formation by only half of the Pol III HE and that if initiation complexes were completely formed in the presence of ATPγS, curiously, one-half dissociated in the presence

of ATPγS (131, 132). We now know that ATPγS-dependent initiation complex formation requires the τ-subunit (133) in a reaction where Pol III is chaperoned onto newly loaded β_2 by the $DnaX_{cx}$ (16). ATPγS only supported initiation complex formation in one-half of a functionally asymmetric Pol III HE, and once an initiation complex was formed in the second half in the presence of ATP, the complex remained asymmetric, revealed by the enzyme "remembering" the identity of the second primed template assembled (134). If ATPγS is added to a dimeric initiation complex, it is the second primer template loaded that dissociates. Although we still do not understand the mechanism of this dissociative reaction, these results indicate that the function of the $DnaX_{cx}$ is asymmetric and may function only to cycle the lagging-strand polymerase. This would be especially important if the $DnaX_{cx}$-dependent ATPase activity proves to be important in the cycling mechanism (Section 4.6).

5. DNAX COMPLEX COMPOSITION AND ASSEMBLY WITHIN CELLS

5.1. τ and γ Composition of Authentic DnaX Complex

All published preparations of Pol III HE contain both τ- and γ-subunits (32, 33, 135, 136). Transient questions have arisen regarding the origin of the shorter γ form of DnaX within authentic Pol III HE, isolated from wild-type cells, because τ can be proteolyzed to a protein of nearly the same size as γ (137, 138). This issue appeared to be largely eliminated when it was discovered that cleavage occurred between two lysines at the site of frameshifting, resulting in a protein two amino acids shorter than authentic γ. Recognizing that cleavage between two basic residues is a characteristic of the OmpT (outer membrane protein T) protease, *ompT* mutant strains were examined and found to eliminate this cleavage (138). Even in $ompT^+$ strains, proteolysis occurred only after lysis. As expected for a protease that does not exist in its active form in the cytoplasm, no cleavage occurs within cells. Proper handling of proteins post-lysis (avoiding prolonged steps such as dialysis at low ionic strength) avoided proteolysis (138).

A suggestion has been made that an additional protease may exist that cleaves τ to a γ-sized protein to explain the presence of γ within strains that overproduce the $DnaX_{cx}$ (137). However, this proposal overlooked the published observation that a $DnaX_{cx}$ overproducer that contained a *dnaX* gene mutated so it would not frameshift produced only τ-containing $DnaX_{cx}$, eliminating this possibility (138). More recently, we have demonstrated the Pol III HE purified from *ompT* mutants contains the same level of γ purified from protease-proficient cells (J. Chen & C.S. McHenry, unpublished). Further evidence for the presence of γ within authentic Pol III HE was derived from demonstration that γ resided in a unique position relative to τ protomers within DnaX complexes (**Figure 1**) (see Section 2.2 for further discussion) (46).

Two recent reports have suggested that Pol III HE lacks γ (137, 139). In one, a trimeric Pol III HE containing only τ was constructed in vitro and was shown to function in reconstituted rolling circle reactions (137). No experiments addressed the forms of Pol III HE present within cells. In another, slimfield fluorescence microscopy, a technique that can quantitate proteins within foci of live cells, was applied (139). A study of live cells with replication proteins tagged with YPet suggested that three Pol IIIs are present per replisome. The error was large enough to make this determination ambiguous (3.1 ± 1.1 αs, for example), but the assigned stoichiometry was supported by observing photobleaching within foci. One decay pattern was published that was consistent with a stoichiometry of three, and a second showed half the expected steps (139). No statistics were presented regarding the number of foci that yielded photobleaching profiles consistent with expectations, so it is not clear how reliable this support is.

The slimfield fluorescence microscopy experiments yielded a τ stoichiometry of

3.1 ± 0.8 per replication fork. These data were interpreted in terms of a replisome that contained three τs bound to three Pol IIIs without consideration of alternatives or the composition of Pol III HE isolated from cells. One Pol III HE containing both τ and γ might be present directly at the replication fork with another nearby that is participating in mismatch repair, a process associated with scanning of nascent hemimethylated DNA (140), or with one recruited to fill in gaps within Okazaki fragments if the signaling mechanism is dominant for cycling of the lagging-strand polymerase. Consistent with these alternative interpretations, mutation of *dnaX* to eliminate γ increased the τ stoichiometry 30% (139)—the amount expected with a Pol III HE dominated by a $\tau_2\gamma$ stoichiometry. Furthermore, expression of γ-YPet in *trans* yields fluorescent replisome foci. It was argued that association is peripheral because deletion of χψ led to a loss of γ association (139). However, omission of χψ probably decreased the affinity of $DnaX_{cx}$ components so that complexes disassembled at physiological protein concentrations (41). This approach has been used to dissociate δδ′ from elongating Pol III HE (79). If $DnaX_{cx}$ dissociated, only τ, which is tethered by association with Pol III and DnaB helicase, would be expected to remain associated with the replication fork.

The lack of accurate biochemical quantification of the various species present in cells represents a critical deficit in our understanding. The exciting new technique of slimfield microscopy provides an important technical advance, but additional studies that attempt to reconcile the results with extant data are needed. Certainly, the preponderance of evidence suggests that the majority of Pol III HE within cells has a mixed τ/γ composition.

What is the function of γ within Pol III HE? Even though some textbooks still present the formerly popular view that γ complex is the clamp loader, we now know that τ possesses important properties that are essential for DNA replication (16, 25, 33, 43, 53, 70, 76, 101). This begs the question regarding the function of γ: Why would insertion of a protein lacking two functional domains be important? γ is found in organisms as diverse as *E. coli* and *T. thermophilus* and is synthesized by at least two distinct mechanisms (20–22, 141). J. Helmann's lab at Cornell (personal communication) has demonstrated the presence of a γ DnaX form in *B. subtilis* (Bsu), even though examination of Bsu *dnaX* does not reveal the translational frameshifting or transcriptional slippage sequences found in *E. coli* and *T. thermophilus*. Such widespread conservation is testimony to the utility of γ. Yet, the function of γ has been a topic that has not been carefully investigated. *E. coli* mutants that apparently do not frameshift and produce only the τ DnaX form are viable (142), but the fitness of these mutants has not been examined. To understand the approaches suggested to address this issue, a brief digression into the role of error-prone polymerases is required.

Over the past decade, the presence of error-prone polymerases has been shown to be important for generation of mutations for evolution and fitness (143). In *E. coli*, the error-prone polymerases Pol IV and Pol V are thought to transiently exchange with Pol III by interaction with an unoccupied site on the β_2 (104, 143). Yet, the interaction of such polymerases with β_2 is weak (μM) (94, 97). It would appear that the presence of an extra Pol III at the replication fork, created by association with τ in τ_3-containing replicases, would likely outcompete the other polymerases. An experiment that could test this hypothesis would compare the rates of spontaneous mutagenesis that occur in the τ-only *dnaX* and *dnaX*$^+$ strains. If correct, this hypothesis could explain why replication appears to proceed at a near normal rate in τ-only *dnaX* mutants even though expression of γ and τ from *dnaX* is widespread in bacteria. Mutants that lack even one error-prone polymerase, in spite of appearing to grow normally in culture, become extinct when subjected to completion experiments with wild-type isogenic strains (144).

Pol IV and Pol V are both required for cells to acquire a growth advantage in stationary phase (GASP) phenotype (145). If

wild-type cells are incubated in a stationary phase for prolonged periods, 99% of the cells die, but a low number acquire mutations that make them more fit to compete with other cells in this stressful environment. If aged stationary phase cultures are mixed with growing cultures that have not been aged, even with an initial inoculum of a 10^{-3} minority, they take over the culture within the stationary phase (145). A corollary to the hypothesis pursued in the preceding paragraph would predict that τ-only *dnaX* cells would be at a selective disadvantage in a stationary phase because of the inability of Pol IV or V to effectively compete with the extra Pol III, thereby creating mutations that lead to a GASP phenotype. To test this notion, the relative ability of τ-only *dnaX* and *dnaX*$^+$ strains to acquire the GASP phenotype needs to be determined.

5.2. DnaX Complex Assembly

The Pol III HE complex can be readily reconstituted from individual components in solution. Because functional reconstitution is so straightforward and relatively easy to perform, it is routinely used as an assay for monitoring the purification of the components. Although the holoenzyme reconstitutes readily, it is difficult to assemble both γ and τ into the same complex (26, 146, 147). When all holoenzyme subunits are coincubated at high concentration in solution, the holoenzyme that forms has a $DnaX_3$ component with three τ-subunits and excludes the γ-subunit (148, 149). In contrast, γ and τ coassemble into the $DnaX_{cx}$ in vivo when overproduced from an artificial operon expressing all five *dnaX* genes on a single transcript (26).

To pursue the mechanism of the assembly reaction in vitro, biotin-tagged τ was generated and used in an assay to measure how much γ is assembled into complexes that contain τ (147). Assembly reactions were carried out with different combinations of components; products containing biotinylated τ were trapped on streptavidin beads. The remaining DnaX components were added to quench γ-τ coassembly and to stabilize the complexes, effectively stopping any subunit exchange. γ was found to be assembled into complexes with τ in a time-dependent manner if δ′ and either δ or χ ψ were omitted from the first stage of the coassembly reaction. Initial insight into a possible mechanism to guide τ into an assembly with γ came from the observation that Pol III stimulates the exchange of γ and τ in DnaX assemblies (147). In simple mixing experiments, the stimulation is modest, reducing the half-life for exchange of γ into complexes with τ severalfold. However, this stimulation may be centrally important for two reasons: (*a*) Pol III, upon binding τ or τ_4, converts it to τ_2, and (*b*) δ-δ′ likely does not assemble stably with τ_2. However, kinetic studies of assembly (147) indicate that τ_2 within Pol III-τ-τ-Pol III can bind free monomeric γ in solution and that the $\tau_2\gamma$ intermediate can be trapped by δδ′ and χ-ψ. The $\tau_2\gamma$ intermediate has not been isolated, but its time-dependent formation has been detected in kinetic assembly experiments (147).

I believe all previous studies, including our own, have been misguided by performing them at high protein concentrations that are biochemically convenient but not physiological (26, 146, 147, 150). This becomes important because τ exists in a monomer-tetramer equilibrium with a K_D of 170 nM (148). The concentration of DnaX that is not stably associated with δδ′χψ in DnaX complexes is likely considerably less than 30 nM. Under those conditions, DnaX will be completely dissociated (>99%), making studies that focus on disrupting a tetrameric DnaX irrelevant.

I propose here a new model for mixed γ/τ $DnaX_{cx}$ formation that is dependent upon assembly occurring at low, physiological concentrations of DnaX in the presence of protein partners to steer the reaction (**Figure 6**). The assembly reaction takes advantage of the "transformer" properties of DnaX, which can assume different stable stoichiometries depending on its protein partners. Unassembled DnaX in the cell starts out as a monomer. Upon association with Pol III, the reaction will be driven to Pol III-τ-τ-Pol III formation (K_D = 70 pM) (95). We know that this assembly can

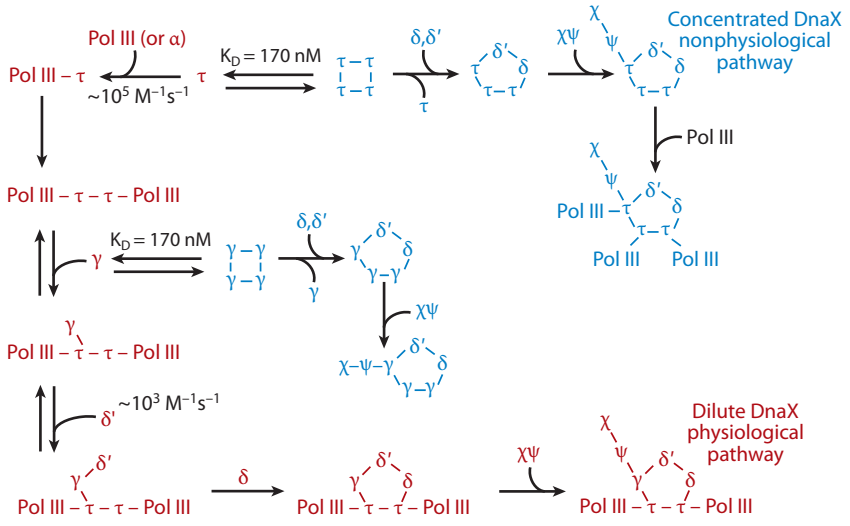

Figure 6

Model for assembly of the DNA polymerase III holoenzyme (Pol III HE) containing a unique copy of the γ DnaX protein next to δ′. This model shows a proposed pathway for holoenzyme assembly at different concentrations of DnaX. A key point of this model is that appropriate assembly only occurs in dilute solution. Starting at high concentrations (*blue*), DnaX is present primarily in the oligomeric (τ_4,γ_4 or mixed tetramers) form. When starting with τ_4 or γ_4 and adding δδ′χψ, mixed τ/γ forms are not obtained (148, 149). This is explained if δδ′ and χψ react directly with the oligomeric forms without reequilibration between DnaX species. In the dilute pathway (*red*), >99% of DnaX is monomeric. Monomeric τ reacts rapidly with Pol III (or α) to form Pol III-τ-τ-Pol III in a rapid assembly reaction (95). If stoichiometric Pol III is present, the free τ monomer is depleted by this pathway, leaving only free monomeric γ, which does not bind Pol III.

interact with γ monomers because a time-dependent entry of γ into complexes with τ in the presence of Pol III has been observed (147). A structure of the ψ N terminus complexed with γ_3δδ′χψ shows that ψ interacts asymmetrically with the three DnaX protomers (45). The contact shown in **Figure 6** designates the cross-link observed with the extreme N terminus of ψ (Section 2.2). Previous studies have shown that δ′ by itself, but not δ by itself, can interact with DnaX assemblies (31). The association reaction is ~100-fold slower than association of χψ with DnaX (28), ensuring ordered assembly. δ will bind in the presence of δ′ in a highly cooperative reaction, leading to closure of the pentameric $DnaX_{cx}$ ring (28, 30). The factors that cause ψ to interact uniquely in the orientation shown with three DnaX protomers and δ′ uniquely with DnaX subunit D (γ) (**Figure 1**) are not known but have been shown to occur in vivo through cross-linking studies of authentic Pol III HE (46).

6. PROOFREADING WITHIN A REPLICASE THAT MAY CONTAIN TWO DISTINCT EDITING EXONUCLEASES

6.1. Structure, Function, and Interactions of ε, the Major Proofreading Subunit

During DNA replication, a high level of fidelity is attained by the action of a proofreading exonuclease that removes nucleotides misincorporated by an associated polymerase. The proofreading exonucleases of most eukaryotic, bacterial, and viral DNA replicases contain acidic residues that chelate two Mg^{2+} ions that participate directly in catalysis (151). In

E. coli and other bacteria that use only one Pol III replicase, the proofreading exonuclease exists as a separate polypeptide chain, ε (152), which binds to the Pol III α-subunit (**Figure 1**) through α's N-terminal PHP domain (88).

The structure of the catalytic domain of ε has been determined and is consistent with two-Mg^{2+} catalysis (153–155). ε binds a nonessential (156) auxiliary subunit, θ, whose only apparent function is to stabilize ε (157). However, bacteriophage P1, which uses all other *E. coli* Pol III HE subunits for its replication, encodes a θ homolog, HOT, that presumably replaces θ in P1-infected cells (158). This implies more important, albeit nonessential, roles for θ that remain to be discovered. θ binds ε's catalytic domain (159, 160). ε has a C-terminal domain that binds α (161). A direct interaction of the exonuclease catalytic domain and Pol III α has not been detected. However, in other polymerases, the relationship of the polymerase active site and proofreading exonuclease is more rigidly fixed, and a channel connects the two sites (162). It is possible that weaker or regulated interactions between the ε catalytic domain and Pol III α exist that permit a direct coordination of elongation with proofreading (Section 6.2).

Kinetic studies indicate that ε has a high catalytic capacity (280 nt removed/s) and, by itself, acts distributively (163). However, when part of the replicative complex, it can processively digest primers to a limit of 6 nt, perhaps determined by instability of a limited primer-template duplex (164). In *E. coli* DNA polymerase I, at least four base pairs between the primer and the template need to be broken to permit transfer of the single-stranded primer terminus to the 3′→5′ exonuclease site 30 Å away (162, 165). If that were the case in Pol III HE, only a 2-nt duplex would remain in the 6-nt product when the primer terminus is in the exonuclease site. That exonuclease action within the Pol III HE is processive indicates that β_2 and other processivity factors are making similar contributions to both proofreading and polymerization. The kinetics of nucleotide removal by the proofreading exonuclease appear to be slower within full Pol III HE replicative complexes, suggesting that the catalytic capacity of the exonuclease may not be the rate-limiting step (166).

6.2. The PHP Domain of Polymerase III α, a Second Proofreading Activity?

The PHP domain was first identified by its sequence similarity to histidinol phosphatase, and the proposal was made that it might have a pyrophosphatase activity (86). However, such an activity is not present (82). The structure of YcdX, a protein more closely related to the Pol III PHP domain and whose function is unknown, revealed a Zn^{2+} trinuclear center with characteristics similar to several phosphoesterases (89).

This information prompted a search for intrinsic hydrolytic activity in α in the absence of ε that led to a discovery of a second proofreading activity within Pol III (87). The second activity follows the classical criteria for proofreading initially established for *E. coli* DNA polymerase I (167). The second 3′→5′ exonuclease exhibits higher activity on mispaired termini, and removal of a mispair precedes elongation by the associated polymerase (87). The activity was distinguished from the prototypical proofreading exonuclease by being dependent on an endogenous metal ion that is not Mg^{2+}, but likely Zn^{2+}. Addition of a Zn^{2+} chelator in the presence of excess Mg^{2+} destroys activity (87). The newly discovered activity in pure recombinant *T. thermophilus* α was distinguished from mesophilic exonuclease contaminants by high thermal stability that decayed in parallel with polymerase activity (87).

Follow-up studies showed that purified *T. thermophilus* α contained Zn^{2+} but that addition of Mn^{2+} was required for optimal activity (N.M. Stano & C.S. McHenry, unpublished). The structure of a Gram-positive PolC (83) showed that the PHP domain could chelate a mixture of Mn^{2+} and Zn^{2+}. The identity of the three metal ions in the PHP domain required for function within cells requires further investigation.

Whether PHP domains constitute a broadly distributed coproofreading activity or whether they are an evolutionary vestige of an ancient proofreader that was replaced by a Mg^{2+}-dependent activity, but preserved as a binding site for the new exonuclease subunit, remains to be determined. That a PHP domain remains, even in Gram-positive PolCs that contain a Mg^{2+}-dependent proofreader, as part of the polypeptide chain suggests a more important role. Evans et al. (83) concluded that the PHP domain in a Gram-positive PolC was not active, but they checked activity on a protein from which the ε-like exonuclease domain had been deleted from the middle of a two-part PHP domain. These experiments should be repeated with point mutations in the ε catalytic acidic residues.

We have observed an exonuclease activity in preparations of recombinant *E. coli* Pol III α from strains that contained a *holE* deletion (structural gene for θ), which permitted rigorous removal of any contaminating ε by passing proteins over columns that contain immobilized θ to which ε tightly binds (N.M. Stano & C.S. McHenry, unpublished). Conclusive evidence must come from showing that the activity discovered is part of Pol III α and not a highly active trace contaminant. This could be accomplished by showing the activity shifts in molecular weight when τ is added. We also observed that the exonuclease within the *T. thermophilus* α cannot cleave a phosphorothioate link (N.M. Stano & C.S. McHenry, unpublished). ε can (166), although Stano observed it only goes to 50% completion, likely revealing activity on only one of two stereoisomers in the mix. These activity differences could also be used to distinguish the PHP exonuclease from ε.

Novel proofreading exonuclease activities have been observed in bacterial DNA polymerases that resemble eukaryotic Pol β but contain an extra PHP domain on their C terminus (91, 92). In the case of Pol X from *B. subtilis*, the activity has been linked to PHP by point mutation and deletion analysis (92). Such domains may function as independent proofreaders or may bind a separate ε proofreading subunit.

If a coproofreading activity exists within bacterial replicases, what might its function be? It is clear that the PHP domain provides the binding site for the standard Mg^{2+}-dependent ε (88). Kuriyan and colleagues (82) observed a narrow channel that may serve as a conduit for moving a mispaired primer terminus to the metal-binding site within the PHP domain. If the active site of ε faced the metal-binding site of PHP, such a channel could feed the 3′ terminus to both the PHP and ε active sites. The PHP nuclease has been characterized as a standard proofreader yet could function on a richer variety of substrates. N.M. Stano (unpublished) has shown that *T. thermophilus* PHP can remove a 3′-phosphate-terminated nucleotide. Other possibilities include the substrates inherited from incomplete repair processes, such as 3′ phosphoglycolates or 3′ α,β-unsaturated aldehydes. If both PHP and ε are active, perhaps they could work in concert exploiting a complementarity in specificity. An understanding of these processes must include a survey of possible substrates and identification and mutagenesis of essential PHP active-site residues that do not perturb polymerase function or ε activity, coupled with an evaluation of the in vivo phenotype. In that regard, it is intriguing that *dnaE*74, which exhibits a mutator phenotype (168), maps to the active site of PHP [which corresponds to the conserved G144 in the *T. aquaticus* Pol III α structure (81)].

7. EMERGING AREAS OF INTEREST: BACTERIA WITH MULTIPLE POLYMERASE IIIS AND THE CHEMICAL BIOLOGY OF DNA REPLICATION

New areas pertinent to Pol III and bacterial replicases warrant attention, but space restrictions require that they be covered in a separate review (182). In short, low-GC Gram-positive and related bacteria employ two Pol IIIs (PolC and DnaE) to replicate their chromosomes (169). PolC apparently serves as the

major replicase, and DnaE serves a role similar to eukaryotic Pol α in elongating RNA primers before handoff to the replicase (51). In other bacteria, a second Pol III serves to replace the error-prone Pol Y polymerase (Pol V in *E. coli*) that is involved in induced mutagenesis (170, 171). Chemical biology approaches promise tools to block specific replication functions; these tools will likely prove useful in sorting out the functions of multiple polymerases and other replication proteins (52, 172–174).

SUMMARY POINTS

1. Bacterial replicases are tripartite. They contain a replicative polymerase (Pol III); a sliding clamp processivity factor (β_2), which tethers the polymerase to DNA; and a clamp loader ($DnaX_{cx}$), which contains a multiprotein ATPase that assembles the sliding clamp onto DNA.
2. Binding of ATP by the $DnaX_{cx}$ places it in a form with high affinity for β_2 and primer termini. The loading reaction is accelerated by ATP hydrolysis, but this step appears to be downstream, after β_2 is assembled onto DNA. If Pol III is present during the assembly reaction, $DnaX_{cx}$ chaperones it onto the newly loaded β_2.
3. In addition to the key interaction between β_2 and Pol III α, other molecular interactions contribute to the stability of the polymerase at the replication fork. These include interaction of τ with α, the presence of δδ′ in the attached $DnaX_{cx}$, the presence of the proofreading exonuclease ε, interactions of τ with the DnaB helicase, and interaction of χ with SSB.
4. Pol III α is organized in modules that confer its special replicative properties. A Pol β-like polymerase domain contains the standard palm, fingers, and thumb domains. A PHP domain provides a binding site for an ε proofreading exonuclease. In at least some organisms, the PHP domain also provides a second Zn^{2+}-dependent proofreading activity. A β-binding domain binds the duplex template and β_2, an OB-fold domain contributes to binding the ssDNA template, and the most C-terminal domain binds τ.
5. The Pol III HE functions as an asymmetric dimer that remains highly processive on the leading strand but appears to have a regulated affinity on the lagging strand that permits rapid release and recycling between primers during Okazaki fragment synthesis.
6. Many cells produce both a five-domain τ DnaX subunit and a shorter three-domain γ-subunit by a variety of mechanisms, including programmed ribosomal frameshifting or transcriptional slippage. Both subunits have β_2 loading activity, but only τ binds Pol III α and the DnaB helicase, serving as the central organizer for the replisome.

FUTURE ISSUES

1. What molecular interactions are required for the $DnaX_{cx}$ to chaperone β_2 onto DNA? Is τ simply increasing the local concentration of Pol III α, or are additional interactions between the clamp loader and the polymerase required? Where is the site with which the C-terminal tail of SSB interacts, stimulating the chaperoning reaction? Are the ATP requirements and relative steps changed when one monitors initiation complex formation rather than only loading of β_2?

2. What is the function of the exonuclease activity associated with the PHP domain and what are its physiological substrates? How widespread is this activity? Do PHP and the ε proofreading activity cooperate in processing errors or damaged DNA?
3. What is the dominant mechanism for cycling of the lagging-strand polymerase upon completion of an Okazaki fragment—collision with the primer for the preceding fragment or a signal generated by synthesis of a new primer at the fork? What is the communication circuit for polymerase release and recycling? Does the polymerase removal activity share features with the chaperoning mechanism associated with initiation complex formation?
4. Are the functions of the $DnaX_{cx}$ the same for both the leading and lagging strands or is its activity in loading β_2 and triggering polymerase release uniquely directed toward the lagging strand?
5. What is the composition and distribution of forms of DnaX within Pol III HE isolated from cells? If the presumed $\tau_2\ \gamma$ stoichiometry within natural Pol III HE is correct, what is the mechanism used to assemble γ in a unique location? What is the function of the γ-subunit within Pol III HE? Does the presence of γ influence the ability of error-prone polymerases to interchange with Pol III α?
6. A wealth of structural information exists for most components and domains of replisomal components. However, important questions remain pertaining to the structures formed when these components assemble and to the differing conformers assumed during reaction stages.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

I thank Laurie Kaguni, Ken Marians, Steve Benkovic, Richard Wing, Michelle Spiering, and members of my laboratory for critically reading this manuscript and for helpful comments. I also thank Diane Hager for preparation of the figures and assembling the bibliography and Melissa Stauffer, PhD, of Scientific Editing Solutions, for editing the manuscript. I offer a special acknowledgment to four scientists who enabled my career and contributions: Daniel V. Santi, Arthur Kornberg, I. Robert Lehman, and Brian J. McCarthy. Work from the author's laboratory was supported by Grant R01 GM060273 from National Institutes of General Medical Sciences.

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