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Bacterial DNA Replicases

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Synopsis

Bacterial replicases are complex, tripartite replicative machines. They contain a polymerase, Pol III; a processivity factor, β_2; and an ATPase, DnaX complex, which loads β_2 onto DNA and chaperones Pol III onto the 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 is uniquely placed relative to two τ protomers in a pentameric ring. The catalytic subunit of Pol III, α, contains a PHP domain that not only binds to prototypical ε, a Mg^{++}-dependent exonuclease but also contains a second Zn^{++}-containing proofreading exonuclease, at least in some bacteria. Replication of the chromosomes of low-GC Gram-positive bacteria requires two Pol IIIs, one of which, DnaE, appears to only extend RNA primers a short distance before handing the product off to the major replicase, PolC. Other bacteria encode a second Pol III (ImuC) that apparently replaces Pol V, which is required for induced mutagenesis in *E. coli*.

Introduction

All cells contain multiple DNA polymerases that function in repair, replication, and even the creation of mutations. For example, *E. coli* contains five polymerases. DNA polymerase I plays important roles in repair and the processing of Okazaki fragments, enabling their ligation into high molecular weight chromosomal DNA. DNA polymerases IV and V are class Y error-prone polymerases and function in translesion synthesis at unrepaired sites of DNA damage. DNA polymerase II has roles that contribute to error-free replication restart and also appears to contribute to replicative fidelity (Fijalkowska et al. 2012; Goodman 2002). DNA polymerase III functions as the chromosomal replicase within bacteria.

Chromosomal replicases from all branches of life are tripartite (Kornberg and Baker 1992). They contain a polymerase, a sliding clamp processivity factor, and an ATP-driven clamp loader. 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 (Fay et al. 1981).

Early functional studies in bacteria revealed β_2 as the key processivity factor (LaDuca et al. 1986), and an ensuing crystal structure elegantly showed the structural basis for its function (Kong et al. 1992). β_2 forms a ring that surrounds the DNA template and tethers the polymerase to it, enabling processive replication. The β_2 ring is loaded onto DNA by an ATP-powered clamp loader, the DnaX complex ($DnaX_{cx}$). The $DnaX_{cx}$ contains three copies of the DnaX protein and one copy each of δ, δ', ψ, and χ. δ binds β_2 when both are free in solution (Stewart et al. 2001), but when δ is part of the $DnaX_{cx}$, ATP is required for the interaction(Indiani and O'Donnell 2003). Upon loading

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β_2 onto DNA, ATP is hydrolyzed within the $DnaX_{cx}$. Concomitant with β_2 loading, the $DnaX_{cx}$ chaperones Pol III onto the newly loaded β_2 (Downey and McHenry 2010). The basic principles of a tripartite polymerase (special replicative polymerase(s), clamp, clamp loader) are conserved throughout biology (► Eukaryotic DNA Replicases, ► Mechanism of PCNA loading by RFC, ► PCNA Structure and Interactions with Partner Proteins).

Molecular Interactions at the Replication Fork

Other features of bacterial replication (Fig. 1) are similarly conserved among all life forms. A hexameric helicase initiates replication by separating two DNA strands at the replication fork. The helicase ($DnaB_6$ in *E. coli*) translocates $5' \rightarrow 3'$ along the lagging-strand template (Lebowitz and McMacken 1986). The exposed single-stranded DNA (ssDNA) is coated on the lagging strand by ssDNA-binding protein (SSB). Priming is catalyzed by a dedicated primase, generating short RNA primers that are elongated by a replicase, DNA polymerase III holoenzyme (Pol III HE) (Fig. 1). Upon completion of an Okazaki fragment, the lagging-strand replicase must release and recycle to the next primer synthesized at the replication fork (Fig. 1a). In contrast, the leading-strand polymerase elongates continuously and processively for at least 150 kb or perhaps the entire chromosome, before dissociating (Mok and Marians 1987a, b).

$DnaX_{cx}$, in addition to its clamp-loading/polymerase-chaperoning role, serves as the central organizer at the replication fork. The DnaX protein comes in two forms – the full length translation product τ and a shorter product γ, which results from programmed ribosomal frame shifting at a specific site on the *dna*X mRNA (Blinkowa and Walker 1990; Flower and McHenry 1990; Tsuchihashi and Kornberg 1990). τ contains five domains, the first three of which are required for

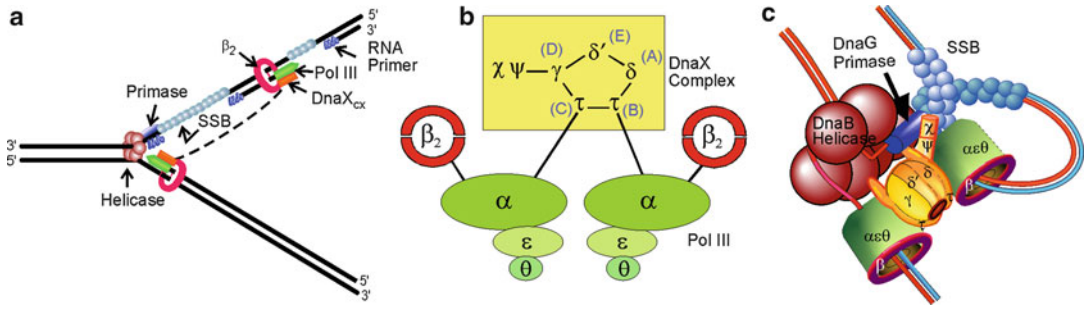

Fig. 1 DNA polymerase III holoenzyme contacts at the replication fork. (**a**) The hexameric helicase (DnaB) uses the energy of ATP hydrolysis to translocate down the lagging strand template, splitting two strands apart in advance of the leading strand replicase, Pol III HE. Single-stranded regions of the lagging strand template are coated by SSB. Primase interacts with the helicase and synthesizes short RNA primers for Okazaki fragment synthesis that are extended by the Pol III HE until a signal is received to recycle to the next primer synthesized at the replication fork. Gaps between Okazaki fragments are filled and RNA primers are excised by DNA polymerase I and the resulting nicks are sealed by 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 (*dotted line*). (**b**) Details of known subunit interactions within the Pol III HE. ψ threads through all three DnaX protomers (Simonetta et al. 2009) and is shown here to signify the unique cross-link observed with γ (Glover and McHenry 2000). In addition, there is a transient interaction between δ and, perhaps, additional $DnaX_{cx}$ subunits with β_2 during the clamp-loading reaction. (**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 (Gao and McHenry 2001c). 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 (Gao and McHenry 2001b; Kim et al. 1996a)

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ATP-dependent clamp loading. Domain III also serves as the oligomerization site, required for $DnaX_{cx}$ assembly (Gao and McHenry 2001a; Glover et al. 2001). Domains IV and V bind the DnaB replicative helicase and the Pol III α subunit, respectively (Gao and McHenry 2001b, c). γ contains only the first three domains. Two τ subunits contained within the DnaX complex dimerize the leading and lagging strand polymerases and bind two protomers within DnaB (Gao and McHenry 2001b; Kim et al. 1996a; McHenry 1982). This serves to condense all mechanical and enzymatic activities of the replication fork into one large assembly, potentially enabling communication between and coordination of all activities at the replication fork. Association of the DnaB with τ-containing Pol III HE accelerates its helicase activity tenfold (Kim et al. 1996a).

Replication forks are established at unique bacterial replication origins in a reaction initiated by the DnaA protein (▶ Replication Origin of *E. coli* and the Mechanism of Initiation). This protein binds origins and, in concert with other factors including the helicase loader, assembles the replicative helicase onto the lagging strand template. The replicative helicase associates, reversibly, with DnaG primase and primers for Okazaki fragment synthesis are made (Wu et al. 1992a). Because the lagging strand in one direction from the origin is the leading strand in the other, the first primer synthesized on each strand provides a unique primer for leading strand synthesis.

Once initiated, not all replication forks survive the 2 Mb journey to the replication terminus approximately halfway around the circular *E. coli* chromosome. A mechanism exists to reactivate dissociated replication forks (Heller and Marians 2005). This reaction is led by the PriA protein that binds to stalled forks and recruits additional proteins that lead to reassembly of the helicase. PriA also serves as a checkpoint protein, blocking a weak strand displacement activity within the Pol III HE, enforcing dependence upon DnaB (Manhart and McHenry 2013; Xu and Marians 2003; Yuan and McHenry 2009).

Stages of the Replicase Reaction

Initiation Complex Formation

Two alternative pathways exist for initiation complex formation in purified biochemical systems (Fig. 2). Incubation of all components of Pol III HE with ATP and primed DNA leads to rapid and nearly concerted assembly of initiation complexes. Alternatively, the β_2 loading and Pol III binding stages can be separated. In the first stage, $DnaX_{cx}$ assembles β_2 onto DNA in an ATP-dependent reaction. When assembly reactions are conducted this way, $DnaX_{cx}$s containing only γ appear to work as well as those that contain τ. In a second stage, Pol III binds to β_2 and associates with primer termini. Most work to date has focused primarily on the first stage of the two-stage initiation complex formation reaction, and significant differences have been found relative to the more physiological single-stage reaction (▶ Mechanism of Initiation Complex Formation).

The first structure of a sliding clamp was determined for *E. coli* β_2 (Kong et al. 1992). β_2 forms a head-to-tail dimeric ring that contains a 35 Å pore through the middle, large enough to accommodate hydrated duplex DNA. A structure for a sliding clamp surrounding DNA was initially predicted by molecular simulations with a PCNA homolog (Ivanov et al. 2006) and later verified by a primed DNA co-crystal structure (Georgescu et al. 2008). However, both the simulation and structure show the DNA positioned asymmetrically within the pore, making contacts with basic side chains in loops lining the pore. Maximizing interactions between these loops and the phosphodiester backbone leads to a conformation where the plane of the β_2 ring is tilted 22° relative to the axis of duplex DNA. It has been proposed that β_2 slides rapidly along DNA during replication by competition between charged residues on the inner surface during the sliding process. An alternative

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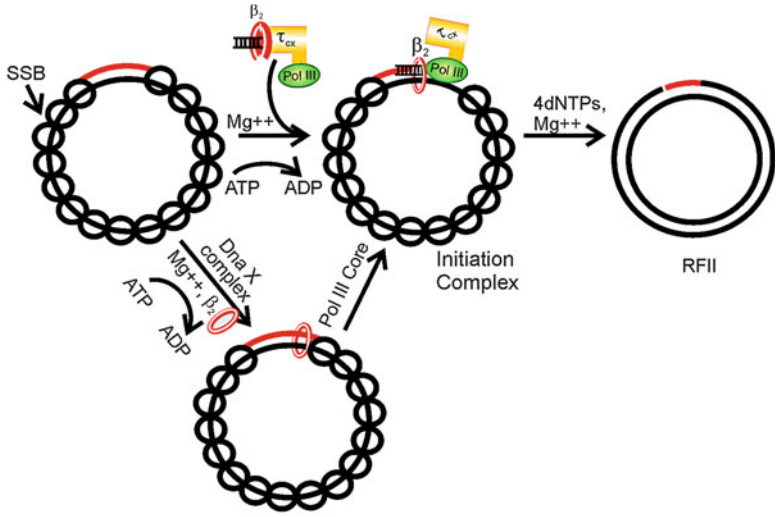

Fig. 2 Two pathways for initiation complex formation. In the two-stage reaction, the $DnaX_{cx}$ (composed of either the γ or τ forms of DnaX or any combination) can load a β_2 ring around DNA. After $DnaX_{cx}$ dissociates, Pol III can associate with the assembled β_2, forming a processive replication complex. Alternatively, Pol III HE that contains at least one τ DnaX subunit can assemble in a single-stage reaction where β_2 is loaded in an ATP-dependent manner and then bound Pol III is chaperoned onto the newly loaded β_2. During this process, the $DnaX_{cx}$ must dissociate from the primer terminus but remains part of the complex (not shown) because of the tight τ-α interaction (Downey and McHenry 2010; Downey et al. 2011)

conformation for the sliding clamp on DNA is observed in a structure from bacteriophage T4, where it is in a closed conformation with its clamp loader (Kelch et al. 2011). In this structure, the DNA is more centrally located and oriented roughly perpendicular to the axis of duplex DNA. Thus, the conformation might vary, depending on the protein partner of the sliding clamp. β_2 binds Pol III through an internal β binding domain within the Pol III α subunit that is positioned where double-stranded DNA exits a central channel that runs through the polymerase (Dohrmann and McHenry 2005; Wing et al. 2008) (► DNA Polymerase III Structure).

The $DnaX_{cx}$ opens the β_2 ring and closes it onto a primed DNA template. δ is a key $DnaX_{cx}$ subunit that enables this reaction. A structure has been solved of the complex between δ and a monomeric mutant of β that cannot form dimers because of replacement of key hydrophobic residues at the dimer interface (Jeruzalmi et al. 2001). An interaction occurs between three hydrophobic residues within domain I of δ and a cleft between domains two and three of β. Other interactions occur between δ domain I and a loop in β. Binding to this loop in β stabilizes a conformation that would disrupt the dimer interface. Whether this disruption is a consequence of contact with δ or whether this secondary interaction results from trapping a transiently open conformer of β_2 remains uncertain. FRET-based studies that monitor the open state of β_2 do not detect open β_2 in solution up to 6 μM δ (Paschall et al. 2011). Thus, δ by itself is not the sole clamp opener – it works in concert with the other subunits of the $DnaX_{cx}$.

The apoenzyme form of the $DnaX_{cx}$ binds β_2 weakly (K_d = 135 nM). Upon binding ATP, the $DnaX_{cx}$ undergoes a conformational change so that β_2 can be bound more effectively (K_d = 5 nM) (Thompson et al. 2009). Thus, ATP binding is required for extensive β_2 opening and is a necessary precursor to its loading onto DNA.

Many structures of the $DnaX_{cx}$ have been obtained, but most represent what appear to be inactive conformations. However, in all, five subunits appear in a pentameric ring of three truncated γ subunits, δ, and δ′ (Fig. 1b). To provide a convenient way to compare the pentameric clamp loaders

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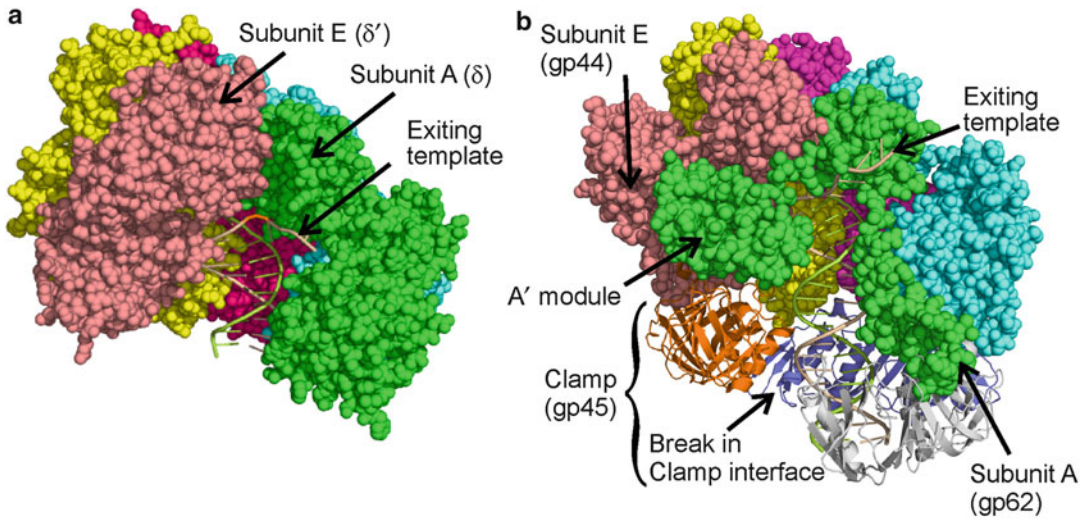

Fig. 3 Structures of the *E. coli* and bacteriophage T4 clamp loaders. (**a**) Structure of the *E. coli* $DnaX_{cx}$ (with three truncated γ subunits) and the N-terminal peptide of ψ in a complex with a primed template (pdb 3GLI) (Simonetta et al. 2009). (**b**) Structure of the T4 clamp loader ($gp44_4$-gp62) in a complex with a primed template and the T4 sliding clamp ($gp45_3$) in the open conformation (pdb 3U60) (Kelch et al. 2011). Both complexes are placed in approximately the same orientation, with the gap between subunits A and E facing the viewer (see naming convention in Fig. 1). The duplex template exits through the bottom of the clamp loaders as viewed and the ssDNA template exits across subunit A near the upper end of the gap between subunits A and E. The A' domain in the T4 clamp loader does not exist in the *E. coli* clamp loader

from different organisms, analogous subunits are labeled A-E as designated in Fig. 1b, following a convention established by O'Donnell and Kuriyan. Thus, δ and δ′ occupy the A and E positions, respectively, with DnaX occupying positions B-D. Early structures were obtained without the presence of the χψ subunits. A recent structure (Simonetta et al. 2009) in the presence of the N-terminal tail of ψ, which binds three DnaX subunits in the collar formed by interacting domain IIIs, placed all three DnaX subunits in a conformation that was competent for binding the ATP analog, ADP-BeF_3 (Fig. 3a). A short primed template was also present in the complex. Interactions between the $DnaX_{cx}$ and DNA occur primarily with the template strand. The three DnaX subunits and δ′ form a right-handed spiral around DNA in a slightly distorted B conformation. δ is not engaged, but it is argued (by analogy to a structure of the eukaryotic homolog RFC in complex with PCNA) that if β_2 were present, δ would participate in DNA template interactions as well. The only primer interaction is between Tyr316 of δ and a base at the primer terminus. It has been suggested that lack of extensive primer contacts allows the $DnaX_{cx}$ to bind both RNA- and DNA-primed templates. The ssDNA template exits the complex in an opening created by a gap between the δ and δ′ subunits. β_2 would bind (if present) the lower face of the complex as represented in Fig. 3a.

A critical deficit in our understanding pertains to a lack of knowledge of the contacts made by open β_2 and the *E. coli* $DnaX_{cx}$. A recent structure obtained for the bacteriophage T4 clamp loader ($gp44_4$-gp62) and an open T4 gp45 sliding clamp in complex with a primed template and an ATP analog (ADP-BeF_3) provide the best insight to date (Kelch et al. 2011). The gp62 protein is a homolog of the *E. coli* δ subunit (position A) but lacks a AAA^+ fold in domain 1 and has an extension off of the C-terminus (A' domain) that bridges to the adjacent subunit that occupies position E. A flexible tether extends from the N-terminus of gp62 that binds the gp45 clamp by a hydrophobic plug, analogous to the binding of β_2 by δ. Within the structure, there are multiple contacts with the open clamp that involve each of the subunits in the pentameric clamp loader, which

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holds it in an open "lock washer" conformation with both horizontal and vertical displacements of the clamp interface.

The 20-nucleotide duplex region of the primed template in the T4 structure is held in an A conformation. Thus, either DNA primers (present in the published structure) or RNA primers could be accommodated in the same conformation. The distorted B conformation in the *E. coli* structure might assume an A conformation if the clamp was present, necessitating tighter contacts by the δ subunit (A position) with its partners. The five subunits of the T4 clamp loader assume a spiral arrangement that matches the primed template, creating a 23 Å vertical displacement and a 9 Å horizontal displacement of the open clamp interface. Loops that line the interior of the clamp loader track and contact the minor groove, widening it. However, the 9 Å opening is too small to accommodate the entry of duplex DNA. Thus, the pathway to formation of this structure within cells remains unclear.

ATP (or ADP-BeF_3, as in the T4 structure) is required for the clamp loader to adopt a spiral structure. The ATP binding sites are bipartite, with an arginine finger that contacts the γ phosphate of ATP coming from an adjacent subunit. Once the clamp is loaded onto DNA, ATP hydrolysis likely destabilizes the spiral structure, leading to decreased affinity for DNA and breaking of contacts with the clamp, leading to the formation of a closed ring around the primed template.

The mechanism used for clamp opening remains unclear in most systems. In T4, the clamp has been shown to be open at one interface in solution (Millar et al. 2004), in spite of it being closed in a crystal structure (Moarefi et al. 2000; Shamoo and Steitz 1999). Thus, the T4 clamp loader probably just traps an already open clamp. Presumably, electrostatic interactions of the clamp with DNA drive its closing, concomitant with ATP hydrolysis, during the clamp-loading process. The Stokes' radius of β_2 is highly dependent upon solution conditions, suggesting that it too might be open in solution under some conditions (McHenry 2011). H-D exchange studies also show clamp structures are dynamic in solution (Fang et al. 2014). However, a recent kinetic study suggested opening of β_2 occurs after binding by the $DnaX_{cx}$ (Paschall et al. 2011). Whether that process is active (wrenching β_2 open) or passive (trapping open β_2 that forms when β_2 is bound to $DnaX_{cx}$) remains an "open" question. Molecular dynamics simulations performed with the eukaryotic homologs RFC and PCNA (Tainer et al. 2010) predict that initial contacts occur with closed PCNA and that, as PCNA spontaneously opens, the open conformation is trapped by more extensive interactions with remaining subunits in the pentameric ring of the clamp loader. By this model, the clamp loader does not open the clamp directly but merely positions itself to trap the open conformation once it forms. The energetics of opening for free PCNA and RFC-bound PCNA remain the same. This suggests the clamp opener does not actively pry the sliding clamp ring open. The major difference in the energy landscape is stabilizing the open complex, preventing it from closing. The dynamics of clamps in solution by themselves vary from organism to organism (Binder et al. 2014; Fang et al. 2014). Thus, the loading mechanism may vary between organisms.

The mechanism of *E. coli* initiation complex formation has been characterized kinetically using $DnaX_{cx}$s with three γ subunits (termed the γ_{cx}). Consistent with structural studies, ATP serves as an allosteric effector, increasing the affinity of the γ_{cx} for β_2 and DNA, bringing all reaction participants together (Ason et al. 2000; Thompson et al. 2009). The γ_{cx}, upon binding primed template, rapidly hydrolyzes bound ATP (Ason et al. 2003). Formation of the initial γ_{cx} – primed template – ATP complex is limited by the slow ATP-induced conformational change within the γ_{cx} of 3.3 s^{-1}(Thompson et al. 2009). β_2 binding is limited only by diffusion. β_2 is loaded before dissociation of the clamp loader (Cho et al. 2014; Hayner and Bloom 2013). Hydrolysis of three equivalents of ATP preceded β_2 closing. In these studies, using γ_{cx}, it was observed that β release might be the rate-limiting step in the initial β loading cycle.

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 (Downey and McHenry 2010). 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 that can support the rate of Okazaki synthesis required for chromosomal replication. Pre-steady state kinetic experiments show that the rate of functional initiation complex formation occurs 100-fold faster than in an unchaperoned reaction catalyzed by the γ complex (Downey et al. 2011). τ complexes bound to Pol III show rate constants for initiation complex formation of 25–50 s^{-1} (at 20 nM $DnaX_{cx}$), more in line with the rates required to support the physiological rate of initiation complex formation (Downey et al. 2011).

Elongation

Upon addition of dNTPs to initiation complexes, processive and rapid elongation ensues. Pol III alone exhibits a very low processivity (*ca*. 10 nt) (Fay et al. 1981), but complete Pol III HE is able to replicate an entire 5 kb circle without dissociating (Fay et al. 1981) and, judging from the static stability of initiation complexes, could have the processivity to replicate an entire chromosome (McHenry 1988).

The primary determinant of processivity of the *E. coli* replicase is the interaction of Pol III with β_2 (Kong et al. 1992; LaDuca et al. 1986) and ε and the other protein binding site within dimeric β_2 (Jergic et al. 2013; Ozawa et al. 2013; Toste-Rego et al. 2013). The presence of the ε subunit increases the apparent affinity of α for the primed template and the k_{cat} for the polymerization reaction (Kim and McHenry 1996b) and increases its processivity (Studwell and O'Donnell 1990). The presence of τ also increases the processivity of Pol III and permits stimulation by an important physiological polycation, spermidine, which decreases the processivity of Pol III in isolation (Fay et al. 1982). The presence of the remaining $DnaX_{cx}$ subunits 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 (Fay et al. 1982). 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 (Glover and McHenry 1998). 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 (Glover and McHenry 1998). Two τ protomers bind the DnaB helicase, further stabilizing the replicase at the fork (Gao and McHenry 2001b; Kim et al. 1996a; Fig. 1c). Pol III HE acting in concert with DnaB exhibits a processivity of >150 kb (Mok and Marians 1987a, b). These multiple interactions that contribute to processivity appear redundant (Marians et al. 1998).

During processive replication of long single-stranded templates, Pol III HE typically stops synthesis upon encountering a duplex (O'Donnell and Kornberg 1985) or after displacing a small number of nucleotides (Dohrmann et al. 2011). However, a strand displacement activity of the DNA Pol III HE has been observed under a variety of conditions (Canceill and Ehrlich 1996; Stephens and McMacken 1997; Xu and Marians 2003; Yao et al. 2000). 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 (Yuan and McHenry 2009). A recently discovered ε-β interaction is also required for strand displacement (Jergic et al. 2013). Extrapolating these findings to natural replication forks suggested the leading strand polymerase might be stabilized by interactions with the lagging strand coated with SSB, mediated through a τ-ψ-χ link. Firmly establishing this notion is the recent observation that χ mutants that are defective in interaction with SSB exhibit a defect in the

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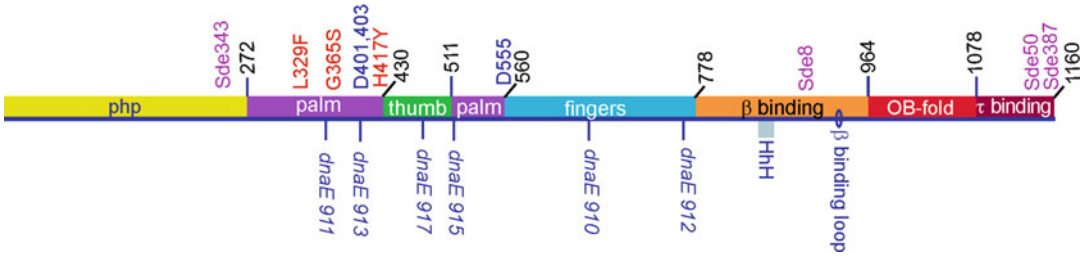

Fig. 4 Modular organization of Pol III α. The names and colors of the domains shown are from Bailey et al. (2006) except that their C-terminal domain was further divided into the OB fold and τ-binding domains. The residue numbers that define domain borders in *E. coli* α are shown above the bar in *black*. The position of antimutator mutations (marked below the *dnaE* gene in *blue*) and mutations selected to discriminate dideoxynucleotides (*red* above the bar) are indicated (Fijalkowska and Schaaper 1993; Hiratsuka and Reha-Krantz 2000; Oller and Schaaper 1994; Vandewiele et al. 2002). It is likely that these influence either the rate of polymerization or base selection and reside within the polymerase active site. Sde mutations (McHenry 2011) that likely interfere with initiation complex formation are shown in magenta above the bar. Mutator mutations (not shown) in *dnaE* (Maki et al. 1991; Strauss et al. 2000; Vandewiele et al. 2002) 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 (Vandewiele et al. 2002). *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 (Bierne et al. 1997)

processivity of leading strand synthesis (Marceau et al. 2011), even though SSB is thought to be exclusively associated with the lagging strand template.

τ serves an additional role of protecting β within elongating complexes from removal catalyzed by exogenous γ complex (Kim et al. 1996b). The δδ′ subunits of the $DnaX_{cx}$, best known for their roles in β_2 loading, are also required for optimal processivity (Song et al. 2001). It is not understood whether their role in processivity is in protecting β_2 from removal, in concert with τ, or in some other function.

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 (Fig. 4). Three acidic side chains (*E. coli* (Eco) D401, D403, and D555) in the internal polymerase domain coordinate two Mg^{++} ions, facilitating catalysis of nucleotide insertion (Pritchard and McHenry 1999). Antimutator and nucleotide selection mutants, presumably associated with polymerase function, helped to further define the limits of the polymerase domain (Fig. 4).

Like all polymerases, Pol III α contains palm, thumb, and finger domains, in the shape of a cupped right hand. Superposition of the α palm with that of mammalian Pol β aligns the three identified catalytic residues of α (Bailey et al. 2006) with those of Pol β (Sawaya et al. 1997). An apoenzyme structure of the full-length *Thermus aquaticus* (Taq) α subunit showed that the palm domain has the basic fold of the X family of DNA polymerases that includes the slow, non-processive Pol βs, placing bacterial replicases as a special class within that family (Bailey et al. 2006). A structure of Eco α truncated within the β-binding domain also exhibited a Pol β-like fold with perturbations in the active site which are presumably corrected upon substrate binding (Lamers et al. 2006).

A ternary complex of a dideoxy-terminated primed template, incoming dNTP, and full-length Taq α provided significant insight into the function of Class C polymerases (Wing et al. 2008). Among the primed template-induced conformational changes is the 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

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to make contacts with the sugar-phosphate backbone in the minor groove. The fingers of α also move, and a portion that rotates *ca*. 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 polymerase contacts the template from its terminus to a position 12 nucleotides behind the primer terminus, in excellent agreement with photo-cross-linking experiments (Reems et al. 1995). The finger domain creates a wall at the end of the primer terminus that forces a sharp kink in the emerging template strand (Wing et al. 2008).

The terminal domains of Pol III α confer special properties upon it, including the ability to bind to and communicate with other replication proteins. Analysis of α deletion mutants revealed that C-terminal domains are responsible for interactions with both τ and β (Kim and McHenry 1996a, b). An essential β_2 interaction site (Eco 920–924) (Dalrymple et al. 2001) was verified by mutagenesis, coupled with functional, genetic, and biophysical experiments (Dohrmann and McHenry 2005). 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 (Kim and McHenry 1996a). More detailed mutagenesis studies (Dohrmann and McHenry 2005) 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 similarity to elements found in other DNA-binding proteins. These include a helix-hairpin-helix motif (HhH) (Eco 836–854) (Bailey et al. 2006; Doherty et al. 1996) and an OB fold (Eco 964–1,078) (Bailey et al. 2006; Theobald et al. 2003; Fig. 4).

A structure of Taq α revealed a well-organized β_2-binding domain with dsDNA-binding capability. DNA binding occurs through an HhH motif and its flanking loops (Wing et al. 2008). 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^{o} and swings down into position as the enzyme binds DNA (Wing et al. 2008), 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.

The structure of the ternary complex of Taq α with a primed template and incoming dNTP also revealed a striking conformational change that includes the OB fold moving to a position near the single-strand template distal to the primer (Wing et al. 2008). 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 (Theobald et al. 2003). However, the β1-β2-β3 face that commonly interacts with ssDNA (Theobald et al. 2003) appears to "face away" from the emerging template and to face the τ binding domain. So, binding of the OB fold, if it takes place, occurs either in a nonstandard way or there are further rearrangements as the template strand becomes longer or when additional protein subunits are present.

The second of the two C-terminal domains in the Taq 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 (Bailey et al. 2006). Mutational studies support the importance of this subdomain in binding τ (Dohrmann and McHenry 2005). Further information regarding possible sites of interaction of this extreme C-terminal domain with τ was derived from a genetic screen for *s*uppression of a dominant lethal phenotype of an extra chromosomally expressed *dnaE* that formed initiation complexes but was unable to elongate (McHenry 2011). Two *sde* mutations in the C-terminal domain (W1134C and L1157Q) appeared to severely diminish the interaction with τ.

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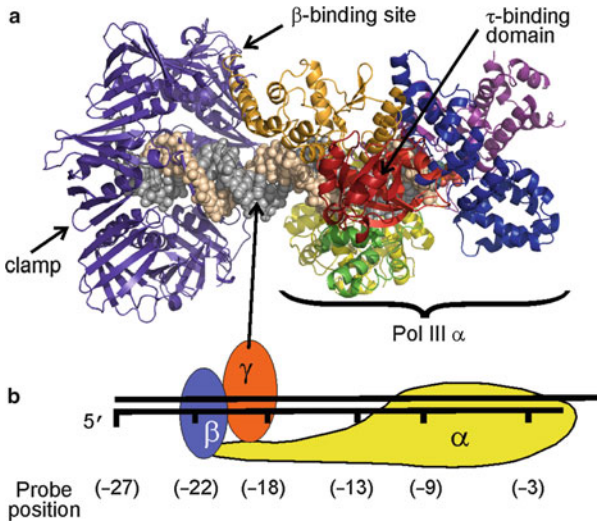

Fig. 5 Models of β_2 binding to Pol III α from reference (Wing et al. 2008) with a proposed position for binding of the γ subunit of the $DnaX_{cx}$. (**a**) The β_2 binding site in Pol III α is indicated, docked to one of two polymerase binding sites within the β_2 clamp (indicated by *arrow* to *blue-purple* β_2 structure). The remaining protein components represent Pol III α, colored as in Fig. 4. The structure in (**a**) was prepared from pymol files provided by R. Wing and rotated so that the PHP domain is facing away from the plane of the paper and the τ-binding domain is projecting toward the viewer. (**b**) Positions of primer contacts with Pol III HE subunits determined by photo-cross-linking (Reems et al. 1995). These results suggest the γ subunit of the $DnaX_{cx}$ fits into the open gap as indicated, contacting the primer strand (*grey*). Because domain V of the τ subunit is believed to contact the upper portion of the C-terminal domain of α (shown in *dark red* in (**a**)), 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, and to facilitate polymerase release and recycling during Okazaki fragment synthesis. This model would need to accommodate the newly discovered interaction between ε and β (Jergic et al. 2013; Ozawa et al. 2013; Toste-Rego et al. 2013)

The availability of a structure of β_2 on dsDNA (Georgescu et al. 2008), and a knowledge of the β_2-binding site for polymerases (Bunting et al. 2003; Burnouf et al. 2004), permitted construction of a model of these proteins interacting on DNA (Wing et al. 2008). The model places β_2 approximately 20 nucleotides behind the primer terminus (Fig. 5), in agreement with foot printing, FRET, and photo-cross-linking studies (Griep and McHenry 1992; Reems and McHenry 1994; Reems et al. 1995). 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 (Burnouf et al. 2004). The model accommodates such an interaction. The same photo-cross-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 photo-reactive probes were placed on nucleotide −18 of the primer (Reems et al. 1995). We note that the open cleft might accommodate γ, which could be sequestered in mixed $\tau/\gamma DnaX_{cx}$s by interaction of τ with the τ-binding C-terminal domain.

Termination Upon Completion of an Okazaki Fragment and Cycling to the Next Primer on the Lagging Strand

Simultaneous with the exceedingly highly processive leading strand Pol III HE, the lagging strand polymerase must, upon completion of an Okazaki fragment, rapidly dissociate and reassociate with a new primer for the next Okazaki fragment in under 0.1 s. Two primary models have been proposed for how this occurs. In the collision model, it was proposed that polymerase dissociation was triggered by collision of the polymerase with the preceding Okazaki fragment (Leu et al. 2003;

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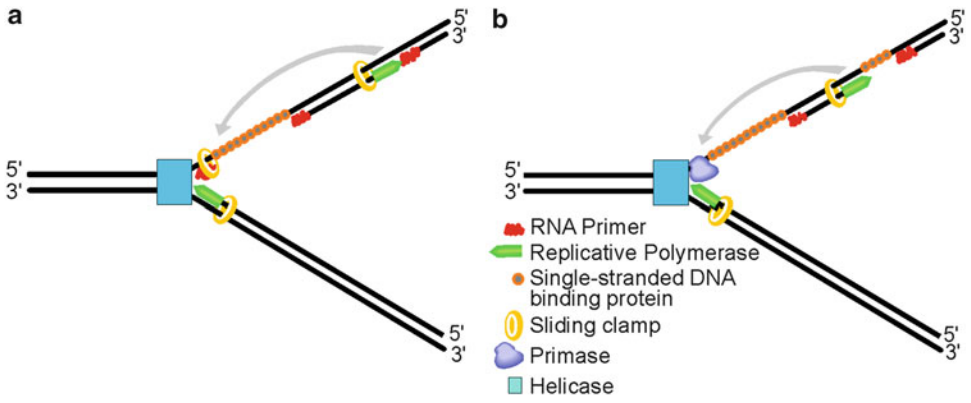

Fig. 6 Two models for lagging strand polymerase cycling. (**a**) In the collision model, it was proposed that all Okazaki fragments are synthesized to completion and collision of the elongating polymerase with the 5′ end of the preceding Okazaki fragment triggers release and recycling to the next primer at the replication fork (Georgescu et al. 2009). (**b**) In the signaling model, it was proposed that a signal that accompanies the synthesis of a new primer is transmitted to the elongating lagging strand polymerase and it dissociates, even if the gap between Okazaki fragments has not been filled (Wu et al. 1992b)

Fig. 6a). In the signaling model, synthesis of a new primer for the next Okazaki fragment drives cycling, even if the preceding Okazaki fragment is not finished (Wu et al. 1992b; Fig. 6b). In both models, polymerase leaves β_2 behind, and a new β_2 is loaded on the next primer (Stukenberg et al. 1994). Kinetic tests of the collision model suggest it is inadequate, by itself, to support a physiologically relevant rate of polymerase release (Dohrmann et al. 2011). A study that exploited selective modulation of lagging strand synthesis on rolling circle templates with highly asymmetric nucleotide composition supported the signaling model and refuted the collision model for *E. coli* (Yuan and McHenry 2014). A modification was required in the signaling model specifying the availability of a new primer as the signal rather than the action of primase, per se. A proposal was made that the clamp loader was the sensor in *E. coli* (Yuan and McHenry 2014), in agreement with a similar proposal made for T4 cycling (Chen et al. 2013). In model systems provided by bacteriophages T4 and T7, which encode their own replication proteins, the signaling model appears dominant (Hamdan et al. 2009; Yang et al. 2004).

Proofreading Within the Bacterial Replicase

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 are homologous and contain acidic residues that chelate two Mg^{++} ions that participate directly in catalysis (Beese and Steitz 1991). In *E. coli* and other bacteria that use only one Pol III replicase, the proofreading exonuclease exists as a separate polypeptide chain, ε (Scheuermann et al. 1983), which binds to the Pol III α subunit (Fig. 1) through α's N-terminal PHP domain (Jergic et al. 2013; Ozawa et al. 2013; Toste-Rego et al. 2013; Wieczorek and McHenry 2006).

The structure of the catalytic domain of ε has been determined and is consistent with two Mg^{++} catalysis, though the protein coordinates two Mn^{++} ions derived from the crystallization buffer in the structure (Cisneros et al. 2009; Derose et al. 2002; Hamdan et al. 2002). Based on this structure, a

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mechanism for nucleotide removal by hydrolysis has been proposed (Hamdan et al. 2002). The two metal ions are held in place by interaction with three essential acidic active site residues. Asp 12, Glu 14, and Asp 167 all coordinate metal ion A. Ion B is bound solely by Asp 12, suggesting it may be bound more weakly and thus could dissociate with each catalytic turnover. The proposed mechanism begins with metal ion A interacting with the reactive phosphate and coordinating the attacking hydroxide ion with the assistance of general base catalysis from an active site His 162. Metal ion B coordinates a phosphate oxygen and presumably, with A, withdraws electrons and makes the reactive phosphate more susceptible to nucleophilic attack. Both ions may serve to shield the charge on the phosphate, reducing charge repulsion of the attacking hydroxide anion. Metal ion B is proposed to coordinate the departing 3′-OH of the DNA chain, stabilizing the developing negative charge of the transition state.

ε binds a nonessential (Slater et al. 1994) auxiliary subunit, θ, whose only apparent function is to stabilize ε (Taft-Benz and Schaaper 2004), and ε also binds α through its C-terminal domain (Ozawa et al. 2008). 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 (Steitz 1999). 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.

Kinetic studies indicate that ε has a high catalytic capacity (280 nt removed/s) and, by itself, acts distributively (Miller and Perrino 1996). 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 (Reems et al. 1991). That exonuclease action within the Pol III HE is processive indicating 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 (Griep et al. 1990).

The PHP Domain of Pol 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 pyrophosphatase activity (Aravind and Koonin 1998). However, such an activity is not present (Lamers et al. 2006). The structure of YcdX, a protein more closely related to the Pol III PHP domain and whose function is unknown, revealed a Zn^{++} trinuclear center with characteristics similar to several phosphoesterases (Teplyakov et al. 2003).

This information prompted a search for intrinsic hydrolytic activity in α in the absence of ε, which led to the discovery of a second proofreading activity within Pol III (Stano et al. 2006). The second activity (the PHP exonuclease) follows the classical criteria for proofreading initially established for *E. coli* DNA polymerase I (Brutlag and Kornberg 1972). The PHP exonuclease exhibits higher activity on mispaired termini, and removal of a mispair precedes elongation by the associated polymerase (Stano et al. 2006). The activity was distinguished from the prototypical proofreading exonuclease by being dependent on an endogenous metal ion that is not Mg^{++}, likely Zn^{++}. Addition of a Zn^{++} chelator in the presence of excess Mg^{++} destroys activity (Stano et al. 2006). The PHP exonuclease activity in pure recombinant *Thermus thermophilus* (Tth) α was distinguished from mesophilic exonucleases by high thermal stability that decayed in parallel with polymerase activity (Stano et al. 2006).

The structure of Taq α revealed that a cluster of nine residues in the PHP domain, including eight of the ligands predicted from informatics approaches (Wieczorek and McHenry 2006), chelates three metal ions (Bailey et al. 2006), as shown directly for the *E. coli* YcdX homolog (Teplyakov

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et al. 2003). Kuriyan and colleagues, from the structure of the Eco α, pointed out a channel between the polymerase active site and the proposed PHP active site (Lamers et al. 2006). The PHP domain contains a long loop (Eco 107–116) that interacts extensively with the thumb. There may also be contacts between the PHP domain and DNA (Wing et al. 2008). This would explain the dependence of polymerase activity on the integrity of the PHP domain. Deletion of 60 N-terminal PHP residues or a D43A point mutation within the proposed active site abolishes polymerase activity (Kim et al. 1997), and mutation of single acidic residues within PHP decreases polymerase activity (Pritchard and McHenry 1999). Cooperative unfolding of the PHP and polymerase domains has been observed, consistent with an overall structural role for PHP (Barros et al. 2013). 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 (Banos et al. 2008; Blasius et al. 2006). In the case of Pol X from *B. subtilis*, the activity has been linked to PHP by point mutation and deletion analysis (Banos et al. 2008). Such domains may function as independent proofreaders or may bind a separate ε proofreading subunit. Possible functions of the PHP domain of Pol III α have been speculated upon (McHenry 2011).

Organisms that Contain Multiple DNA Polymerase IIIs: Functions and Interactions

Both eukaryotic and prokaryotic replicases use a nearly structurally identical sliding clamp processivity factor and a five-protein ring-shaped AAA^+ ATPase clamp loader (▶ Eukaryotic DNA Replicases). However, while in *E. coli*, the Pol III HE is the sole replicase, in eukaryotes, three replicases exist. Pol ε is the leading strand replicase, Pol δ is the lagging strand replicase, and Pol α is part of the priming apparatus and elongates nascent primers with dNTPs for a short distance before handing them off to Pol δ (Dua et al. 1999; Nethanel and Kaufmann 1990; Nick McElhinny et al. 2008; Pursell et al. 2007). Interestingly, Gram-positive bacteria also contain multiple Pol IIIs and, along with them, other features reminiscent of eukaryotic replication systems. The *E. coli* $DnaX_{cx}$, through the C-terminal domain of its τ subunit, binds to Pol III very tightly, with a K_d of 70 pM (Kim and McHenry 1996a), and because τ is oligomeric, the leading and lagging strand polymerases are maintained in one coupled complex. In contrast, Gram-positive DnaX complexes bind their cognate polymerases extremely weakly (Bruck and O'Donnell 2000; Bruck et al. 2005), suggesting a transient interaction and/or the presence of additional factors that make the interaction more stable.

In low-GC Gram-positive bacteria, two Pol IIIs exist, termed PolC and DnaE (Koonin and Bork 1996). They are homologous, but PolC has some of its domains rearranged and it contains an endogenous standard Mg^{++}-dependent proofreading activity. DnaE is more closely related to the *E. coli* Pol III. A suggestion was made, based on genetic/cell physiology studies, that PolC is the leading strand polymerase and DnaE is the lagging strand polymerase in *B. subtilis* (Dervyn et al. 2001). However, DnaE has low fidelity, at least in vitro (Bruck et al. 2003; Le Chatelier et al. 2004). Yet, its overproduction in vivo does not increase mutation rates (Le Chatelier et al. 2004). These observations argue against a major replicative role.

A *B. subtilis* rolling circle replication system has been reconstituted using 13 purified *B. subtilis* replication proteins (Sanders et al. 2010) predicted to be required by previous genetic or biochemical investigations (Bruand et al. 1995, 2001; Bruck and O'Donnell 2000; Dervyn et al. 2001; Polard et al. 2002; Velten et al. 2003). This system appears to accurately mimic the reaction at the replication fork of a Gram-positive bacterium, in terms of both its correspondence with genetic

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requirements and the replication fork rate in vivo (500 nt/s at 30 °C) (Wang et al. 2007). Leading strand replication requires 11 proteins, including the Pol III encoded by *polC*. The second Pol III encoded by *dnaE* will not substitute. In addition to these 11 proteins, lagging strand replication requires DnaE and primase (Sanders et al. 2010). This is consistent with proposals for a lagging strand role for DnaE (Dervyn et al. 2001). However, the elongation rate of DnaE is too slow (~25 nt/s) to keep up with the replication fork. In contrast, PolC supports a physiologically relevant elongation rate (~500 nt/s). PolC discriminates against RNA primers; DnaE uses RNA primers efficiently (Sanders et al. 2010). These characteristics suggest a role for *B. subtilis* DnaE, analogous to eukaryotic Pol α, in which it extends RNA primers initially and then hands them off to a replicase.

Consistent with the eukaryotic Pol α role, model systems using RNA-primed ssDNA show inefficient use by PolC, with a marked stimulation by low levels of DnaE to a level of synthesis greater than DnaE alone (Sanders et al. 2010). However, in the absence of PolC, DnaE can catalyze extensive synthesis. This makes it difficult to assess the exact position of the hand-off of an extended primer from DnaE to PolC, because DnaE appears to continue synthesis in the absence of another polymerase. This issue was addressed by using a specific PolC inhibitor (HB-EMAU) (Tarantino et al. 1999). This class of inhibitors likely acts as a dGTP analog, forming a ternary complex with the primed template and PolC and trapping the enzyme in a dead-end complex (Low et al. 1974). When HB-EMAU was included in cooperative RNA primer extension reactions containing DnaE and PolC, synthesis was drastically inhibited, indicating the hand-off to PolC occurs early in the reaction (Sanders et al. 2010). The precise window for the hand-off awaits further experimentation.

Cross-References

- ▶ DNA Polymerase III Structure
- ▶ Eukaryotic DNA Replicases
- ▶ Mechanism of Initiation Complex Formation
- ▶ Replication Origin of *E. coli* and the Mechanism of Initiation

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