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MicroReview

Chromosomal replicases as asymmetric dimers: studies of subunit arrangement and functional consequences

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Summary

Studies of the DNA polymerase III holoenzyme of *Escherichia coli* support a model in which both the leading and lagging strand polymerases are held together in a complex with the replicative helicase and priming activities, allowing two identical α catalytic subunits to assume different functions on the two strands of the replication fork. Creation of distinct functions for each of the two polymerases within the holoenzyme depends on the asymmetric character of the entire complex. The asymmetry of the holoenzyme is created by the DnaX complex, a heptamer that includes τ and γ products of the *dnaX* gene. τ and γ perform unique functions in the DnaX complex, and the interaction between α and τ appears to dictate the catalytic subunit's role in the replicative reaction. This review considers the properties of the DnaX complex including both τ and γ, with the goal of understanding the properties of the replicase and its function *in vivo*. Recent studies in eukaryotic and other prokaryotic systems suggest that an asymmetric dimeric replicase may be universal. The leading and lagging strand polymerases may be distinct in some systems. For example, Pol ε and Pol δ may function as distinct leading and lagging strand polymerases in eukaryotes, and PolC and DnaE may function as distinct leading and lagging strand polymerases in low GC content Gram-positive bacteria.

General properties of replicases

Cellular replicases from bacteria, archaea and eukaryotes are complex macromolecular assemblies that have a highly conserved structure. The complexes are tripartite assemblies that include a replicative polymerase, a 'sliding clamp' processivity factor (β in bacteria and PCNA in archaea and eukaryotes), and a complex multisubunit ATPase (DnaX complex in bacteria and RFC in archaea and eukaryotes) (see Fig. 1 for diagram of bacterial replicase). The ATPase complex performs multiple essential functions, one of which is to assemble the processivity factor onto DNA, giving it the alternative name of 'clamp loader.' β_2 and $PCNA_3$ are oligomeric ring-shaped molecules that encircle the DNA template and bind the polymerase, forming a tether or clamp that holds the polymerase on the DNA (Kuriyan and O'Donnell, 1993). Because a closed ring cannot assemble readily onto DNA by itself, it requires an ATP-powered machine to allow it to open and lock into place on the DNA. The 'clamp loaders' form a pentameric ring composed of structurally similar proteins. Clamp loader subunits are members of the extended AAA^+ family of motor-like ATPases, that perform a variety of cellular activities (Jeruzalmi *et al.*, 2001a). In eukaryotes each of the five subunits are encoded by distinct genes.

In bacteria, the five subunits are encoded by three genes: *dnaX*, *holA* and *holB*. The DnaX subunit appears to function as the sole ATPase in most bacterial DnaX complexes (Bullard *et al.*, 2002). In *Escherichia coli* and some other bacteria, the *dnaX* gene encodes two products, a shorter γ subunit and a longer τ protein. The γ subunit is encoded by an alternative reading frame that can be created by several mechanisms including programmed translational frameshifting in *E. coli* and transcriptional slippage in *Thermus thermophilus* (Blinkowa and Walker, 1990; Flower and McHenry, 1990; Tsuchihashi and Kornberg, 1990; Larsen *et al.*, 2000). Both γ and τ are ATPases and act as 'clamp loading' proteins. The longer protein τ includes two domains not present in γ; these domains facilitate interaction between τ and the DnaB helicase at the replication fork and between τ and the α subunit of Pol III (Dallmann *et al.*, 2000; Gao and McHenry 2001a, b). The *E. coli* DnaX complex also includes one copy each of the χ and ψ subunits. ψ binds to DnaX and χ, tethering the latter protein to the complex.

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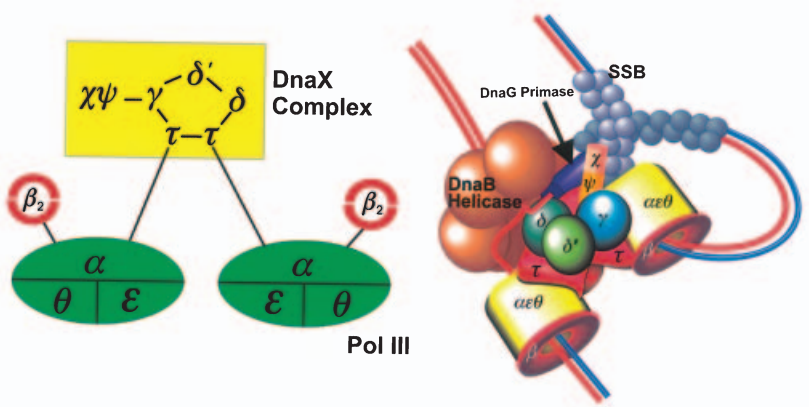

Fig. 1. Structural models of Pol III holoenzyme.
Left. Diagram showing Pol III holoenzyme subunit stoichiometry and major protein–protein contacts. The subunits in the yellow rectangle are the components of the DnaX complex, including the ATPase subunits encoded by the *dnaX* gene: τ and γ. The green ovals represent Pol III. The red ring represents the β_2 sliding clamp processivity factor. It is shown bound to the α subunit, as when assembled on DNA. β_2 is bound to δ during the 'loading' reaction prior to placement on DNA (Jeruzalmi *et al*., 2001b).
Right. A model for holoenzyme subunit arrangements at the replication fork showing the τ–DnaB interaction, placement of χ-ψ on the lagging strand side of the replication fork, and the χ–SSB interaction on the lagging strand. Parental DNA is in red; newly synthesized DNA is blue.

χ interacts with single-stranded DNA binding protein (SSB) with a much higher affinity when SSB is bound to DNA than when it is free in solution (Glover and McHenry, 1998). Because DNA replication models include single-stranded DNA only on the lagging strand of the replication fork, it has been suggested that χψ is associated with lagging strand replication (Kelman *et al*., 1998) (Fig. 1, right). The χ-ψ-SSB contact is essential for robust replicase activity in solutions at physiological ionic strength (Glover and McHenry, 1998).

Assembly of the replicative complex is often described as involving two stages, although this distinction is somewhat artificial. In the absence of polymerase, the DnaX complex can transfer β onto DNA in a reaction that requires ATP hydrolysis. Pol III can associate with the loaded β_2 and form a highly processive complex. However, *in vivo*, the DnaX complex is likely to contain τ and γ, so that it also can bind to α. Under these conditions, the functional replicase would assemble onto DNA in tightly coupled, nearly concerted steps. The distinction between the 'concerted' reaction and the two-stage reaction is revealed when assembly is compared in the presence of ATP or the non-hydrolysable analogue of ATP, ATPγS. In the presence of ATPγS, the initiation complex forms only if τ is present in the DnaX complex, revealing an advantage of α being held in a complex with the 'clamp loader' during the β 'loading' reaction (Dallmann *et al*., 1995). In addition, in the presence of ATPγS, the amount of complex formed is reduced by half. Further, when ATPγS is added to purified initiation complexes formed in the presence of ATP, half of the initiation complexes dissociate (Johanson and McHenry, 1984; McHenry and Johanson, 1984; Glover and McHenry, 2001).

Previous studies, including the aforementioned experiments employing ATPγS, led to the proposal that the DNA polymerase III holoenzyme (Pol III holoenzyme) is an asymmetric dimer, with distinguishable leading and lagging strand polymerases (McHenry and Johanson, 1984). This suggestion was substantiated in later studies that provided insight into the replicative mechanism of the prototypical *E. coli* asymmetric replicase (Glover and McHenry, 2001). This review will explore the unique role of τ in the *E. coli* replicase and the complex mechanism required for τ and γ to be assembled into the DnaX complex. This subject is critical because this assembly pathway and the interactions of τ with the polymerase are essential to the structure and function of the asymmetric replicase. Recent insights into the replicative mechanism of the prototypical *E. coli* asymmetric replicase will be discussed, and finally, the less well characterized putative asymmetric dimeric replicases of low GC content Gram-positive organisms and eukaryotes will be described. [Because of a necessarily limited focus for a *MicroReview*, several important topics regarding the function of DNA polymerase III holoenzymes have not been reviewed. Interested readers should refer to Kelman and O'Donnell (1995) for a more general review and Ason *et al*. (2003) and Jeruzalmi *et al*. (2001a,b) (and references therein) for a description of the kinetic mechanism of holoenzyme and structural studies respectively.]

Special contributions of the τ form of DnaX

The *E. coli* DnaX complex has the composition $DnaX_3\delta\delta'\chi\psi$ where the three DnaX subunits can be any mixture of τ and γ. Any of these complexes can load β onto DNA with nearly equal efficiency during the first stage of the assembly reaction in the absence of polymerase. Early replication models proposed that γ is the active subunit in 'clamp loading' and that the τ subunit merely provided an inert platform to organize the replicative complex. However, recent studies more closely approximating *in vivo* conditions suggest that τ, with its two unique domains, performs functions that cannot be performed by γ.

τ contacts the DnaB helicase and accelerates the rate of fork progression

The important and unique role played by τ was clearly demonstrated in studies of rolling circle DNA replication using an assay developed by Marians and colleagues (Kim *et al*., 1996a). These studies used the PriA replication restart pathway to load DnaB efficiently onto tailed-form II molecules. In this system, the rate limiting step is DNA unwinding by DnaB helicase in advance of the polymerase (Kim *et al*., 1996b). In the absence of polymerase, DnaB unwinds DNA at 40 nt/s, which is the rate of rolling circle replication in reactions reconstituted with γ but without τ. When τ is included in rolling circle DNA replication, the rate of fork progression increases to 500–700 nt/s at 30°C, approximating the rate of fork movement *in vivo* at that temperature. The most straightforward explanation for this observation is that τ interacts directly with DnaB, altering its conformation and stimulating catalytic efficiency. This hypothesis was verified by gel filtration and BIAcore surface plasmon resonance binding experiments, which demonstrate direct interaction between the two proteins (Kim *et al*., 1996b; Yuzhakov *et al*., 1996). BIAcore binding data indicate that at least two τ subunits must interact with $DnaB_6$ to form a complex with a physiologically relevant affinity (Gao and McHenry, 2001a). This stoichiometry reflects the fact that the energies for two linked τ subunits binding to $DnaB_6$ are additive and the microscopic K_ds are multiplicative.

τ couples the leading and lagging strand polymerase

When our laboratory purified a new form of Pol III in the early 1980s, Pol III was found to co-purify with a previously unknown subunit called τ that forms a complex in which two molecules of the Pol III were held together by τ (McHenry, 1982). Previous studies in the laboratory of Bruce Alberts suggested that replication of the leading and lagging strands of bacteriophage T4 were coupled (Sinha *et al*., 1980). Thus, it seemed possible that τ might similarly coordinate leading and lagging strand DNA synthesis in *E. coli*. This model was tested by examining whether coordinated DNA replication of leading and lagging strand DNA depends on the concentration of γ or τ during complex formation in the PriA-dependent rolling replication reaction (Kim *et al*., 1996a). When the concentration of DnaX (γ or τ form) is sufficiently high, leading and lagging strand replication proceeds. However, if complexes are formed at a higher concentration and then diluted to a lower concentration, only τ containing complexes replicate the lagging strand normally after dilution. This supports the notion that τ is required to hold the lagging strand polymerase at the replication fork, targeting it to the next primer synthesized at the fork. The γ complex can load β onto DNA, but cannot bind polymerase in a stable manner. Replicase assembled with the γ form of DnaX falls off after synthesizing an Okazaki fragment. In the absence of τ, an active replicase can not reassemble at the replication fork in a dilute reaction. Presumably, the role of τ is to act as a tether, holding the lagging strand polymerase at the fork through a stable interaction with the leading strand polymerase.

τ prevents removal of β by exogenous γ complex

When PriA-dependent rolling circle DNA replication is reconstituted with τ-containing Pol III holoenzyme, the reaction products reach greater than 100 000 bases in length. In contrast, in reactions reconstituted with γ in the absence of τ, reaction products are 9–23 kb in length and the amount of product is reduced. Product length decreases when the concentration of γ complex increases (Kim *et al*., 1996c). The γ complex loads β onto DNA, but it also can bind an elongating replication complex and displace β (Turner *et al*., 1999), thereby terminating processive DNA synthesis. The presence of τ inhibits this reaction and, in contrast, stimulates processive DNA synthesis (Kim *et al*., 1996c). These results suggest that τ is required for Pol III holoenzyme to achieve a high level of processivity, which is an essential property of an efficient chromosomal replicase.

τ enables lagging strand polymerase to cycle upon encountering a blocking oligonucleotide

Pol III holoenzyme and the replication apparatus encoded by bacteriophage T4 dissociate from a completely replicated template when they encounter the 5′ end of a blocking oligonucleotide (Hacker and Alberts, 1994; Stukenberg *et al*., 1994). This process may be similar to the process of polymerase recycling that occurs at the end of an Okazaki fragment. τ is required; γ cannot substitute. C-τ, the region unique to τ and missing from γ, can also trigger polymerase recycling (Leu *et al*., 2003). The mechanism used by τ to trigger breaking the Pol III–β_2 interaction and recycling is not yet clear. C-τ may sense the blocking oligonucleotide directly, or may be required so that α subunit can sense the block. β binds to at least two sequences in the C-terminal end of α. The affinity of α for β_2 is negatively modulated by the polymerase domain when it is not bound to DNA (Kim and McHenry, 1996). τ also binds to the C-terminal end of α. Binding of τ to α is abolished by deletion of 48 C-terminal residues – a deletion that decreased the affinity of α for β at least 10-fold. Thus, the interplay of α and β in binding to closely related C-terminal sequences of α and the modulation of β affinity by the conformational state of the polymerase domain of α may modulate or sensitize the β–α interaction.

τ is required for efficient replication at physiological salt concentration by binding χ and ψ

Native or fully reconstituted Pol III holoenzyme carries out processive elongation in a solution containing ≤800 mM potassium glutamate. However, SSB, χ-ψ and the τ form of DnaX are required for a stable interaction with the template at this ionic strength, and the γ-form of DnaX can not substitute (Glover and McHenry, 1998). This observation suggests a χ–ψ–SSB interaction (Glover and McHenry, 1998; Kelman *et al*., 1998). Evidence for such an interaction with rapid association and dissociation rates was obtained using SSB immobilized on a BIAcore chip and χ-ψ as the analyte in the flow cell. The K_d measured was 2.7 μM, which is physiologically irrelevant. The *in vivo* concentration of replicase is 10–20 nM, so an equivalent or lower K_d is required for the interaction to take place *in vivo*. However, when χ-ψ was studied in the context of the DnaX complex, and when SSB was associated with DNA to form a protein-coated fibre, a physiologically relevant K_d of 9.5 nM was observed (Glover and McHenry, 1998).

SSB inhibits replication by Pol III in the absence of auxiliary subunits. A primed SSB-coated oligonucleotide was used as a replication substrate, in order to determine which replicase components are required to support DNA synthesis in the presence of SSB. This assay showed that α, χ-ψ and τ are required, and that γ can not substitute for τ (Glover and McHenry, 1998). The simplest explanation for this observation is that χ-ψ must be tethered to α by τ so that the interaction between χ-ψ and SSB suppresses the ability of SSB to inhibit DNA synthesis. Because γ binds to χ-ψ but not to α, it cannot perform the tethering function provided by τ.

These results suggest that τ has both an active and a passive role as part of the replicase complex in *E. coli*. τ provides unique critical active functions that enable Pol III holoenzyme to carry out DNA synthesis at the replication fork, and τ passively tethers Pol III and χ-ψ together, allowing DNA synthesis by the α catalytic subunit in the presence of SSB. SSB binds to the lagging strand but not the leading strand at the replication fork, and there is only one χ-ψ per dimeric replicase, suggesting that χ-ψ participates specifically in lagging strand DNA replication (Glover and McHenry, 1998; Kelman *et al*., 1998).

Assembly of the DnaX complex

DnaX (indicating either γ or τ) is a remarkable 'transformer', because it exists in three distinct stable oligomeric forms, transforming from one form to the other in response to association with different protein partners. Each form of DnaX has well-defined subunit composition with a unique stoichiometry, and depending upon the presence of other accessory proteins, either γ or τ of a particular combination of DnaX proteins is preferentially present (Fig. 2). In a solution including only τ and/or γ, DnaX forms a stable tetramer with different stoichiometries of the two subunits (Dallmann and McHenry, 1995). Rigorous solution studies using equilibrium sedimentation detect only tetramer and monomer. This is typical for proteins that form a homotetramer, because the trimeric or dimeric structure tends to be much less stable than the tetramer. However, when δ-δ′ interacts with $DnaX_4$, the DnaX tetramer transforms to a trimer, and a stable $DnaX_3\delta\delta'$ complex is formed (Pritchard *et al*., 2000); this structure has been unequivocally confirmed, because it exists in the crystal structure of the γ complex (Jeruzalmi *et al*., 2001a). When the DnaX tetramer interacts with Pol III, τ_4 transforms to a dimer (Pol III-τ-τ-Pol III). It is important to understand these structural transformations, because they are critical for the mechanism by which Pol III holoenzyme is assembled.

The Pol III holoenzyme complex can be readily reconstituted from individual components in solution. Because reconstitution is so easy, it can be used as an assay to monitor purification of the components. Although the holoenzyme complex reconstitutes easily, it is difficult to assemble γ and τ into the same complex (Onrust *et al*., 1995; Pritchard *et al*., 2000). When all holoenzyme subunits are co-incubated in solution, the holoenzyme complex that forms has a $DnaX_3$ component with three τ subunits and the γ subunit is excluded from the complex. In contrast, γ and τ do co-assemble into the DnaX complex *in vivo*, when overproduced from an artificial operon expressing all five genes on a single transcript (Pritchard *et al*., 2000). Under these conditions, assembly favours

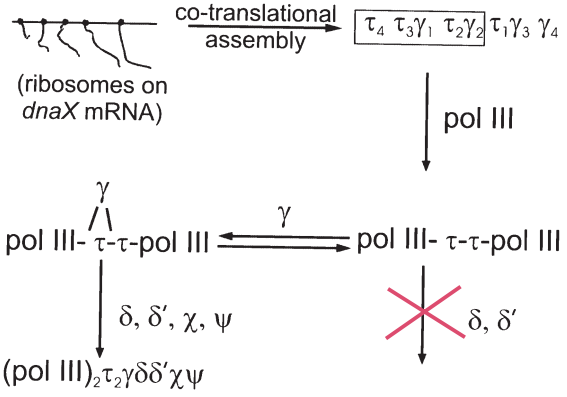

Fig. 2. A model for assembly of DNA Pol III holoenzyme. The two DnaX proteins, τ and γ, are presumably assembled into tetramers co-translationally. Pol III can bind DnaX assemblies containing two or more τ subunits and convert them to dimers within Pol III-τ-τ-Pol III. Kinetic experiments indicate that multimer composition changes over time [shown here as $(Pol\ III)_2$-$\tau_2\gamma$, but the stoichiometry is uncertain], with different species trapped by δ-δ′ and χ-ψ to form Pol III holoenzyme. (β_2 not shown.)

complexes rich in γ. In addition, the species likely to represent the most physiologically important form of the DnaX complex, $\tau_2\gamma\delta\delta'\chi\psi$, is the least abundant species that forms. We explored, *in vitro*, how the $DnaX_3$ balance might be tipped to favour Pol III holoenzyme complexes with an abundance of τ relative to γ.

A biotin-tagged form of τ was generated and used in an assay to measure how much γ is assembled into complexes (Pritchard and McHenry, 2001). Assembly reactions were carried out with different groups of components and products containing biotinylated τ were trapped on streptavidin beads. The remaining DnaX components were added to quench γ-τ co-assembly and to stabilize the complexes (i.e. δ′ and χ-ψ were added to an assembly reaction that included biotinylated τ, γ and δ) and the amount of γ bound to τ-containing complexes was quantified after washing. γ was found to be assembled into complexes with τ if δ′ and δ and/or χ-ψ are omitted from the co-assembly assay. Because δ and χ-ψ synergistically assist binding of δ′ to DnaX (Olson *et al*., 1995), the results suggest that γ and τ can exchange only if τ is partitioned from δ′ and δ/χ-ψ. Apparently, a very novel mechanism has evolved to permit the partitioning that depends on the stoichiometric 'transformer' properties of DnaX.

The first insight into the mechanism that partitions τ from δ-δ′, prior to co-assembly with g, came from the observation that Pol III stimulates the exchange of γ and τ in DnaX assemblies (Pritchard and McHenry, 2001). In simple mixing experiments, the stimulation is modest, reducing the half-life for exchange of γ into complexes with τ several fold. However, this stimulation may be centrally important in light of two facts: (i) Pol III, upon binding τ_4, converts it to τ_2. (ii) δ-δ′ does not appear to bind to τ_2 when in a complex with Pol III. These results suggest that the conversion of τ_4 to τ_2 is exploited because the τ_2 intermediate cannot bind δ-δ′. However, τ_2 can bind free γ in solution through its equilibrium with γ_4. The $\tau_2\gamma$ intermediate can be trapped by δδ′ and χ-ψ (Fig. 2). The $\tau_2\gamma$ intermediate has not been isolated, but it has been detected in kinetic assembly experiments. Complete experimental proof for this mechanism requires study of the assembly reaction starting with purified Pol III-τ-τ-Pol III. The model predicts that the reaction would nearly exclusively lead to Pol III holoenzyme with a $\tau_2\gamma$ DnaX core and that the assembly reaction would favour this species *in vivo*.

Asymmetric distribution of subunits within the DnaX complex

The δ-δ′ and χ-ψ subunits associate with τ_4 or γ_4 *in vitro*. *In vivo*, Pol III holoenzyme contains both τ and γ, which raises the question of how the subunits are physically arranged in the holoenzyme structure: δ-δ′ and χ-ψ subunits could be randomly arranged with respect to τ and γ, or the arrangement could be specific and defined. This question was addressed by treating authentic holoenzyme with a chemical cross-linking agent. This approach revealed that γ cross-links to ψ and δ′ (Glover and McHenry, 2000). A cross-link was also detected between δ and τ (Bullard *et al*., 2002). Using as a reference the $\gamma_3\delta\delta'$ pentameric ring revealed from the X-ray crystal structure (Jeruzalmi *et al*., 2001a) that has three γ subunits numbered sequentially counter-clockwise from γ#1 that is adjacent to δ′ in Fig. 1 (left), our cross-linking results suggest that τ occupies the positions of γ#2 and γ#3 (Bullard *et al*., 2002). This arrangement is consistent with the possibility that δ′ makes a unique contact with γ, and δ makes a contact with τ. Because χ-ψ uniquely cross-links with γ, it is assigned to the γ#1 position (Fig. 1).

Distinct leading and lagging strand halves of an asymmetric dimeric holoenzyme

The above discussion describes our current understanding of the structural asymmetry of *E. coli* Pol III holoenzyme. This structural asymmetry is linked to the functional asymmetry of the enzyme complex, which is also beginning to be understood. As mentioned earlier in this review, researchers noticed in early studies that holoenzyme forms half as many initiation complexes in the presence of ATPγS as in the presence of ATP (Johanson and McHenry, 1984; McHenry and Johanson, 1984). Furthermore, when ATPγS is added to complexes formed in the presence of ATP, half of the complexes dissociate (Fig. 3). Because of this, ATPγS can be used to form a dimeric complex that utilizes two distinct primer templates. These molecules are extremely useful for examining the biochemical requirements for complex formation, the placement of the β 'clamp-loading' (and unloading) activity relative to each template (Glover and McHenry, 2001) and the asymmetric function of the complex.

Because only one mole of initiation complex is formed per mole of Pol III holoenzyme in the presence of ATPγS, ATPγS can be used to assemble monomeric initiation complexes. If a different primer template is then added in the presence of ATP, a dimeric complex forms with distinguishable first and second templates. This experimental design was used to determine the components required for formation of each enzyme/template initiation complex. SSB, in addition to β and a nucleotide cofactor is required for formation of the second complex but not the first (Glover and McHenry, 2001). The requirement for SSB during formation of the second initiation complex suggests that this half of the complex may provide a model for the lagging strand half of the replicase, which is coated with SSB *in vivo*.

Loading and unloading of β_2 was also studied using

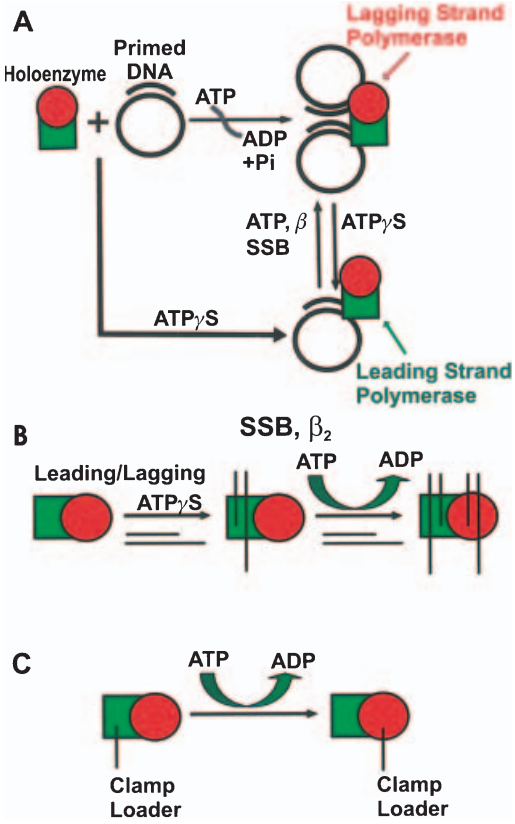

Fig. 3. Asymmetric function of DNA polymerase III holoenzyme as revealed by two stage initiation complex formation in the presence of ATPγS, then ATP. Green squares represent the leading strand polymerase half of an asymmetric dimeric Pol III holoenzyme; red circles represent the lagging strand half.
A. Dimeric Pol III holoenzyme can form a dimeric initiation complex in the presence of ATP, but only a monomeric initiation complex in the presence of ATPγS. Monomeric complexes (which are thought to provide a model for the leading strand complex) can be converted to dimeric complexes in the presence of ATP, β_2 and SSB. The lagging strand half of dimeric complexes specifically dissociates when ATPγS is added.
B. ATPγS can support association of the leading strand template-primer without ATP hydrolysis. ATP hydrolysis is required for assembly of the second (lagging strand) primer template.
C. One model to explain this observation is that ATP hydrolysis is absolutely required for an isomerization reaction that moves the orientation of the 'clamp loading' activity of the DnaX complex from the leading strand to the lagging strand. Primer template is omitted from the figure. The mechanism of 'isomerization' at the subunit conformational or quaternary arrangement level is not understood. Even though ATP hydrolysis is not required to form an initiation complex on the leading strand *in vitro*, it is possible that this reaction might be driven by ATP hydrolysis *in vivo*, because it may confer faster rates, greater specificity and more favourable equilibrium to the reaction.

dimeric holoenzyme complexes with two distinct primer templates. As mentioned above, DnaX loads and unloads β_2 onto and off of DNA (Turner *et al*., 1999). The β_2 loading and unloading reactions were used as an indication of the orientation of the 'clamp loading' activity of the DnaX complex to the 'first' and 'second' initiation complexes (Glover and McHenry, 2001). By definition, the 'clamp loader' must be oriented towards the first template primer assembled. This assembly reaction can take place without ATP hydrolysis in the presence of ATPγS, but ATP is required for assembly of the second primer-template complex. It is possible that ATP hydrolysis is required for an isomerization of the 'clamp loader' and re-orientation towards the second primer template (Fig. 3). When ATPγS was added to dimeric complexes, the 'clamp loader' was disclosed to have remained associated with the second primer template upon re-orientation. This observation is consistent with selective dissociation of the second primer template. These observations, taken together with our understanding of replication fork dynamics, suggest that an asymmetric dimeric Pol III holoenzyme first forms an initiation complex with the leading strand template and then, in a reaction requiring ATP hydrolysis, assembles on the second lagging strand template. Once the dimeric complex is associated with two DNA templates, the 'clamp loader' remains permanently oriented towards the lagging strand. With this orientation, the 'clamp loader' is in an appropriate position to form initiation complexes during Okazaki fragment synthesis, a reaction not required on the leading strand.

Do low GC Gram-positive organisms and eukaryotes have distinct leading and lagging strand polymerases?

Recent studies show that eukaryotic Pol δ and ε both interact with RFC and PCNA during chromosomal DNA replication and that both genes are required for cell viability. These results suggest that one polymerase might have a leading strand role and the other a lagging strand role. *In vivo* studies also determined the mutation frequency in proofreading exonuclease mutants of Pol ε and Pol δ. The results were consistent with the possibility that Pol ε is the enzyme that carries out leading strand DNA synthesis (Shcherbakova and Pavlov, 1996; Pavlov *et al*., 2002). The hypothesis that Pol δ is required for lagging strand synthesis is reinforced by its requirement during DNA synthesis of the C-rich strand of the telomere, which is a lagging strand reaction (Diede and Gottschling, 1999). However, the pol2-16 mutant of Pol ε is inviable due to a mutation in the C-terminal region, despite the fact that it has intact polymerase activity (Kesti *et al*., 1999). This result detracts from the idea that polymerase activity is the essential function provided by Pol ε. However, these mutants are quite sick (Ohya *et al*., 2002) suggesting that there may be some plasticity in the enzymatic requirements at the eukaryotic replication fork, but that a multi-polymerase ensemble that includes Pol ε is preferred.

Genome sequencing projects revealed that low GC content Gram-positive bacteria have two polymerases (Koonin and Bork, 1996). One polymerase is the prototyp-

ical Gram-positive bacterial PolC, which is characterized by a catalytic subunit with proofreading exonuclease activity; the second polymerase is homologous to the α subunit of *E. coli* Pol III (DnaE). Genetic analysis indicated that both polymerases are essential for viability (Dervyn *et al.*, 2001). It was proposed that both polymerases might be involved in elongation at the fork and that they might have distinct roles in leading and lagging strand DNA synthesis. This hypothesis was tested by measuring replication of a well-defined replicon in PolC and DnaE temperature-sensitive mutants at the non-permissive temperature. DnaE mutants produced a low level of incompletely replicated single-stranded intermediates. Strand-specific probes showed that the incompletely replicated regions were on the lagging strand, indicating a lagging strand replication defect. In PolC mutants, a severe drop in replicon copy number was observed, but no single-stranded intermediates were detected, which is consistent with a leading strand replication defect. It will be useful to study the relative function of these two proteins in reconstituted replication systems, which will provide an opportunity to define and understand the distinct function of each protein in the complete replication reaction.

Future studies

Significant progress has been made in understanding the mechanism of natural replicases at the replication fork. However, current findings have raised other important questions. The groundbreaking X-ray crystallography work of J. Kuriyan, M. O'Donnell and colleagues, especially with the DnaX complex, provides insight into how the β_2 assembly reaction on primed DNA might work. Full understanding will require knowledge of all of the protein–protein contacts, kinetic steps and structures of the relevant intermediates. Structures also remain unsolved for the unique C-terminus of τ, which is not found in γ, and for a representative of the DNA polymerase III class of polymerases. The structure of this enzyme will be critical for understanding how Pol III binds its auxiliary proteins, and will set the stage for further exploration of the communication channels between components of the replicative machinery.

In addition to understanding the structure of the unique C-terminus of τ and how it relates to the contact points on its protein partners, we need to understand how the τ-DnaB contact relates to the mechanism by which Pol III recycles on the lagging strand of the replication fork and maintains processive DNA synthesis. Current models focus on the idea that the lagging strand polymerase is in effect tethered to the leading strand polymerase as a mechanism for holding the lagging strand polymerase at the fork after it dissociates from a nascent Okazaki fragment and retargeting it to the next primer. A lagging strand Pol III–τ–DnaB tether could accomplish the same goal, perhaps even more effectively, given that the DnaG primase binds to DnaB. Whether this tether can facilitate recycling of the lagging strand polymerase and whether this mechanism is redundant or synergistic with the links proposed as part of the dimeric enzyme model remains uninvestigated.

Future studies are needed to confirm the assembly mechanism of the DnaX complex. To test the model proposed in Fig. 2, the assembly reaction should be started with Pol III-τ-τ-Pol III; as mentioned above, the prediction is that the product will be predominantly Pol III holoenzyme with a $\tau_2\gamma$ composition. Rigorous determination of the stoichiometry of the proposed intermediate in Fig. 2 [whether $(\text{Pol III})_2\tau_2\gamma$ or $(\text{Pol III})_2\tau_2\gamma_2$] will be daunting, given that the intermediate cannot be isolated by methods attempted to date. Cross-linking and other structural data will be needed to determine if the complex assembled *in vitro* has the same subunit arrangement as native holoenzyme.

Further studies are also needed on the function of the asymmetric dimeric polymerase at the replication fork. We need to determine how ATPγS facilitates assembly of β onto DNA, especially relative to current models that suggest hydrolysis is mandatory. In addition, the putative isomerization of the 'clamp loader' described above needs to be studied; however, this may not be possible until appropriate structural methods are available and until the loading of β onto DNA is more completely characterized.

It will be intriguing to consider the relationship of the two polymerases at the replication fork and how their interaction ensures efficient recycling of the lagging strand replication apparatus during Okazaki fragment synthesis. Further studies are needed to test the idea that low GC content Gram-positive organisms utilize two distinct polymerases at the replication fork, and it will be interesting to compare the structure of the replicase in these organisms to the replicase in *E. coli*. This is an especially important line of investigation, given that the most important class of bacterial pathogens derives from this class of organisms and because it has been proposed that eukaryotic replication forks may also include two distinct polymerases.

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