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The DNA Polymerase III Holoenzyme: An Asymmetric Dimeric Replicative Complex with Leading and Lagging Strand Polymerases

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Summary

The DNA Polymerase III holoenzyme forms initiation complexes on primed DNA in an ATP-dependent reaction. We demonstrate that the nonhydrolyzable ATP analog, ATPγS, supports the formation of an isolable leading strand complex that loads and replicates the lagging strand only in the presence of ATP, β, and the single-stranded DNA binding protein. The single endogenous DnaX complex within DNA polymerase III holoenzyme assembles β onto both the leading and lagging strand polymerases by an ordered mechanism. The dimeric replication complex disassembles in the opposite order from which it assembled. Upon ATPγS-induced dissociation, the leading strand polymerase is refractory to disassembly allowing cycling to occur exclusively on the lagging strand. These results establish holoenzyme as an intrinsic asymmetric dimer with distinguishable leading and lagging strand polymerases.

Introduction

The *E. coli* chromosome is replicated by nearly continuous synthesis of the leading strand and discontinuous synthesis of discrete Okazaki fragments on the lagging strand. The Okazaki fragments are subsequently processed and ligated into high molecular weight DNA (Ogawa and Okazaki, 1980; Kornberg and Baker, 1992). The DNA polymerase III holoenzyme (holoenzyme) contains two pol III cores (Pol III) that are held together by a τ dimer (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). This coupling by τ tethers the lagging strand polymerase to the fork, enabling its recycling through the successive rounds of Okazaki fragment synthesis (Kim et al., 1996a).

The DNA polymerase III holoenzyme is composed of ten distinct proteins (Onrust et al., 1995) that are organized into three functional modules: (1) Pol III, which contains the holoenzyme's polymerase (α subunit) and exonuclease (ε subunit) activities; (2) the toroidal-shaped processivity "clamp" β, which tethers Pol III to DNA; and (3) the ATP-powered DnaX complex "clamp-loader," which sets the β clamp around DNA. The DnaX complex has the composition $\tau_2\gamma_1\delta_1\delta'_1\chi_1\psi_1$ (Pritchard et al., 2000). Both τ and γ possess ATPase activity and are products of the DnaX gene, with γ being a truncated version of τ (Kodaira et al., 1983; Mullin et al., 1983). The τ protein serves as an organizing center by binding the DnaB helicase (Kim et al., 1996b; Yuzhakov et al., 1996), polymerase/exonuclease, clamp loading, and SSB binding activities into one complex. The auxiliary subunits, $\delta\delta'\chi\psi$, can bind to either DnaX protein in vitro, but within authentic holoenzyme, reside on γ and not τ (Glover and McHenry, 2000). The presence of only one copy each of the auxiliary subunits imparts a structural asymmetry to the holoenzyme. The $\delta\delta'$ subunits are primarily involved in β loading (Onrust and O'Donnell, 1993), while the $\chi\psi$ subunits bind SSB (Glover and McHenry, 1998; Kelman et al., 1998).

We previously demonstrated that substitution of ATP by ATPγS results in formation of half as much functional initiation complex. Furthermore, addition of ATPγS to isolated complexes formed in the presence of ATP results in the loss of activity by one-half of the initiation complexes (Johanson and McHenry, 1984; Dallmann et al., 1995). The 2-fold differential nucleotide phenomenon is not an equilibrium artifact since final activity is independent of ATPγS concentration once the ATP binding site of DnaX is saturated. Reactions did not progress beyond the 50% level over time. This differential nucleotide effect has been traditionally attributed to the functional asymmetry of holoenzyme (McHenry, 1988a). It was proposed that the observed functional heterogeneity arose not from two distinct enzymes in solution, but from distinguishable leading and lagging strand halves within the holoenzyme (Johanson and McHenry, 1984; McHenry, 1988a; Maki et al., 1988; Hawker and McHenry, 1987; McHenry, 1988b).

In spite of the original ATPγS observation being made 16 years ago, it was not known if the effect observed reflected a true difference between two halves of the holoenzyme. Furthermore, it was not known if the effect was at the DNA binding, initiation complex formation, or on a downstream step that influenced the ability of complexes to elongate. We resolve these issues in this report and extend our understanding of the holoenzyme as an intrinsically asymmetric dimer with distinguishable leading and lagging strand polymerases.

Results

ATPγS-Initiation Complexes Contain One Molecule of DNA per Dimeric Holoenzyme

ATPγS supports the formation of one-half as much initiation complex as ATP (Johanson and McHenry, 1984; Dallmann et al., 1995). We sought to ascertain whether the 2-fold difference in activities of initiation complexes formed in the presence of ATP and ATPγS could be explained by a 2-fold difference in the amount of DNA sequestered within these complexes. We developed an endonuclease protection assay that allowed us to quantify primed templates bound by initiation complexes. A BamHI restriction endonuclease site was placed 10 nt behind the 3′-hydroxyl of the ^{32}P-50-mer primer. The endonuclease site was protected within initiation complexes; free DNA was cleaved producing a 37-mer fragment. When ATP was left out of the initiation complex formation reaction, all of the ^{32}P-labeled 50/87-mer appeared to be cleaved (Figure 1A, lane 1). This indicates

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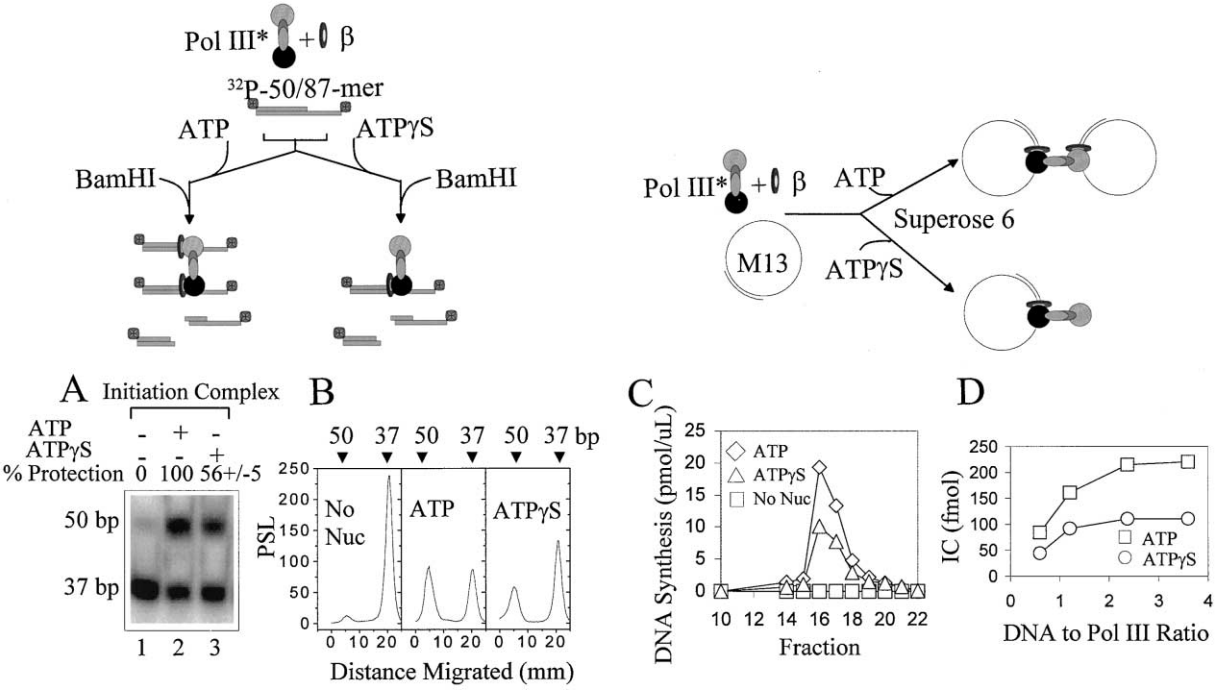

Figure 1. ATPγS-Initiation Complexes Contain One Molecule of DNA per Dimeric Holoenzyme

(A) Comparison of the amount of DNA protected from cleavage by initiation complexes formed in the presence of ATP and ATPγS. Initiation complexes were formed on a ^{32}P-labeled 50/87-mer DNA primed template (^{32}P-label is on the 5′-end of the primer) containing a BamHI restriction site −10 from the 3′-primer terminus in the absence of nucleotide or presence of ATP or ATPγS. After initiation complex formation, 50 U BamHI was added to cleave any unprotected primed template DNA. Samples were subjected to 12% urea PAGE and visualized by phosphorimagery. We have demonstrated previously that holoenzyme subunits in initiation complexes protect from DNase I digestion the first 30 nucleotides of the primer (Reems and McHenry, 1994). A Neutravidin molecule was bound to the biotinylated 5′-end of both the primer and template serving as a "molecular bumper" preventing the ring-shaped β subunit from sliding off the end of the DNA. The amount of 50-mer remaining for the ATP experiment was used as the 100% value. Residual amounts of uncleaved 50-mer in the experiment performed in the absence of nucleotide were subtracted from both the ATP and ATPγS experiments as background.

(B) Phosphorimager traces of lanes of the gel shown in (A). PSL, photostimulated luminescence.

(C) Isolated initiation complexes formed in the presence of ATPγS possess half the activity of those formed in the presence of ATP. Initiation complexes were formed on primed M13Gori in the presence of either ATP (diamond, 1 mM final), ATPγS (triangle, 1 mM final), or buffer lacking nucleotide (square). Complexes were isolated by gel filtration on a Superose 6 FPLC column and identified in collected fractions by replication assay.

(D) ATPγS-initiation complexes are saturated in DNA. Initiation complexes (IC) were formed in the presence of either ATP (square) or ATPγS (circle) with DNA to Pol III ratios of 0.6:1, 1.2:1, 2.4:1, and 3.6:1. Complexes were isolated by gel filtration and assayed for activity.

that protection is afforded by bona fide initiation complex formation, but not by holoenzyme binding in itself. Utilizing this endonuclease protection assay, initiation complexes were formed with holoenzyme in the presence of ATP or ATPγS and subjected to denaturing electrophoresis (Figure 1A, lanes 2 and 3). Quantification indicated that initiation complexes formed in the presence of ATPγS protected only 56 ± 5% of the ^{32}P-50/87-mer primed template protected by initiation complexes formed in the presence of ATP (Figures 1A and 1B). This suggests that in the presence of ATPγS, the dimeric holoenzyme only bound and protected one equivalent of DNA and complexes formed in the presence of ATP protected two equivalents, consistent with our previous observation of a 2-fold difference in active initiation complexes formed.

When we formed initiation complexes with holoenzyme in the presence of ATP or ATPγS and isolated them by gel filtration, we found that complexes formed with ATPγS possessed half the activity as those formed using ATP (Figure 1C). We were concerned that the lowered activity of ATPγS-initiation complexes could be due to a diminished affinity for DNA resulting in complexes that were subsaturated in DNA. However, the activity levels of ATPγS-initiation complexes were not increased to that supported by ATP when we increased the DNA to enzyme ratio (Figure 1D). The ratio of DNA to enzyme required to saturate initiation complexes formed in the presence of ATP was 2-fold higher than the ratio required to saturate complexes formed in the presence of ATPγS. These results are consistent with the notion that ATPγS-initiation complexes have an occupancy of one DNA molecule per dimeric enzyme leaving one Pol III assembled within holoenzyme unoccupied.

ATPγS-Initiation Complexes Can Bind and Replicate a Second Molecule of DNA Only in the Presence of ATP, β, and SSB

We next determined whether the unoccupied Pol III within the ATPγS-initiation complex could bind, incorporate, and replicate a second molecule of DNA. We

formed and isolated an ATPγS-initiation complex on primed-M13Gori DNA and incubated it with a ^{32}P-50/87-mer in the presence of ATP, β, and SSB. Elongation of the ^{32}P-labeled 50-mer following addition of dGTP resulted in formation of an 87-mer. The ^{32}P-50/87-mer was replicated by an ATPγS-initiation complex only in the presence of β, ATP, and SSB (Figure 2A, compare lanes 1–4). These results demonstrate that ATPγS-initiation complexes with an M13 molecule bound in one Pol III active site can form a second, distinct, replication-competent initiation complex in the dimeric holoenzyme's other Pol III site. Unlike the initial ATPγS-initiation complex formation step, which requires ATP and β, complex formation in the second Pol III site also requires the presence of SSB.

Both Pol III molecules within an isolated ATP-initiation complex are occupied by an M13 DNA molecule and therefore should be unable to replicate additional 50/87-mer DNA. As a control, we formed ATP-initiation complexes at a limiting ratio of M13 DNA to Pol III (1.2:1). After isolation, the DNA-deficient ATP-initiation complexes were able to replicate a second molecule of 50/87-mer DNA with their vacant Pol III site, provided SSB was present (Figure 2B, compare lanes 1 and 2). When ATP-initiation complexes were assembled at excess saturating ratios of M13 DNA to Pol III (3.6:1), isolated complexes no longer had an available site to load and replicate the added oligonucleotide primed template (Figure 2B, lanes 3 and 4). In contrast, isolated ATPγS-initiation complexes could still load and elongate a second primed template even with complexes formed with excess M13 DNA to Pol III ratios prior to isolation (Figure 2C, compare lanes 1 and 2 with 3 and 4). This result demonstrates that the endogenous DnaX complex which loaded β onto the first strand can also load β onto the second strand in the presence of ATP and SSB.

One potential concern with our conclusion that endogenous DnaX can load β for both halves of holoenzyme is that trace-free DnaX complex eluting with initiation complexes upon gel filtration could lead to the same result. We addressed this issue in a separate control experiment in which we omitted nucleotide in the initial initiation complex formation reaction and subjected the "mock initiation complex" to gel filtration. We tested an aliquot from the fraction where initiation complex would elute and found that it was unable to sustain replication of exogenously added ^{32}P-50/87-mer in the presence of added Pol III, SSB, β, and ATP (data not shown).

The experiments reported so far address the ability of holoenzyme to bind primed DNA in functionally active initiation complexes. We next determined the minimum requirements allowing the ^{32}P-50/87-mer to bind to and gel filter with a preformed isolated ATPγS-initiation complex. ^{32}P-50/87- mer was added to isolated ATPγS-initiation complexes and incubated with either ATP and β, SSB alone, or ATP, β, and SSB and gel filtered on a Superose 6 column. Radioactivity associated with the ^{32}P-50/87-mer would be found in the early excluded initiation complex fraction only if it is bound to the ATPγS-initiation complex. We separately quantified the amount of M13 present in initiation complexes by replication. The amount of M13 in initiation complex-containing fractions appeared to be constant throughout the various experiments (Figure 2D). We found that ATP and β alone

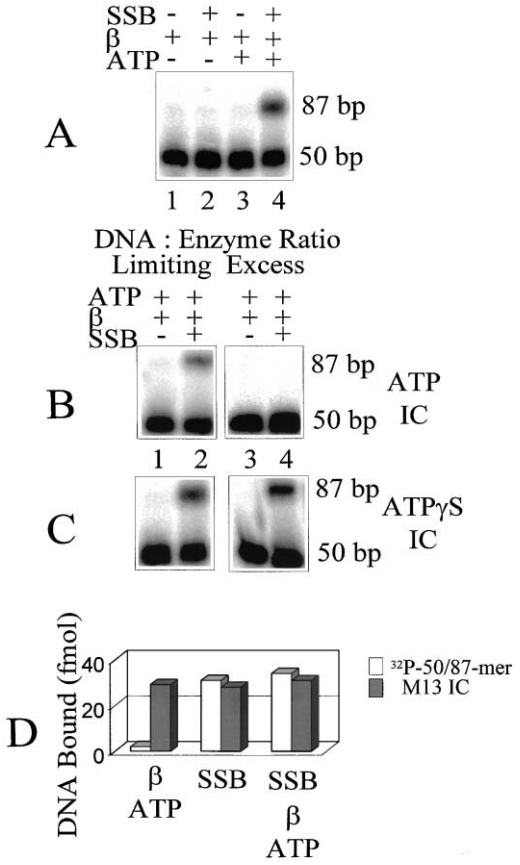

Figure 2. ATPγS-Initiation Complexes Can Bind and Replicate a Second Distinct Molecule of DNA Only in the Presence of ATP, β, and SSB

(A) Initiation complex was formed on primed M13Gori in the presence of ATPγS and isolated by gel filtration. Replication of a ^{32}P-50/87-mer by the isolated ATPγS-initiation complex was assayed in the presence of ATP, β, and SSB. Elongation of the ^{32}P-50-mer appears as a ^{32}P-87-mer product. Reaction products were subjected to 12% urea PAGE and visualized by phosphorimagery.

Initiation complexes were formed in the presence of either ATP (B) or ATPγS (C) with DNA to Pol III ratios 1.2:1 and 3.6:1 and isolated by gel filtration. Replication of the ^{32}P-50/87-mer by the initiation complex was assessed in the presence of added ATP, β, and SSB.

(D) SSB facilitates binding of a second DNA molecule to an isolated ATPγS-initiation complex. Isolated ATPγS-initiation complex was incubated with the ^{32}P-50/87-mer in the presence of either ATP and β, SSB only, or ATP, β, and SSB and gel filtered on a Superose 6 column. The amount of M13 present in column fractions containing isolated initiation complex was determined by replication assay and the amount of ^{32}P-50/87-mer was assessed by scintillation counting. Quantification of the amount of M13 and 50/87-mer present in the initiation complex is shown.

were not sufficient to incorporate the second DNA molecule into the ATPγS-initiation complex. The presence of SSB alone, in the absence of β and ATP, was sufficient to allow the ATPγS-initiation complex to bind a second molecule of DNA (Figure 2D). However, this second bound DNA molecule is not replication competent.

One possible explanation for our observation is presented as a model depicting the requirements for second DNA strand binding and replication (Figure 3). In the absence of SSB, β and ATP alone are unable to

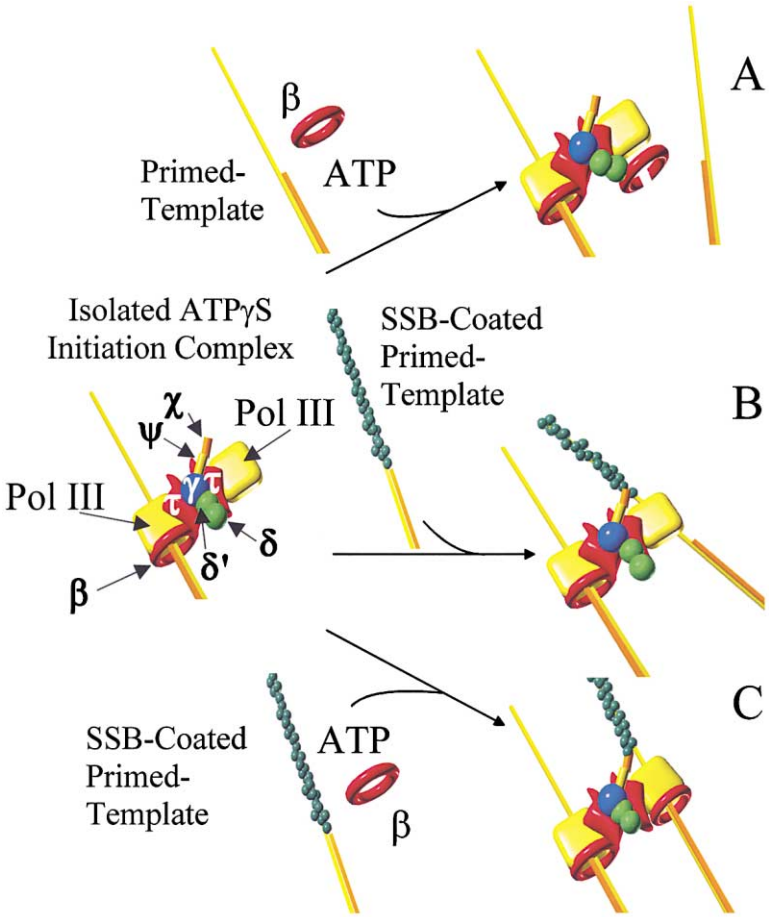

Figure 3. Model for Second Strand Binding and Replication by Isolated ATPγS-Initiation Complexes

β and ATP alone are not sufficient for initiation complex formation on the second strand (A). Although SSB leads to binding of the second strand (B), β and ATP are required for initiation complex formation (C).

support initiation complex formation in the second half of the enzyme (Figure 3A). The presence of SSB on the second DNA molecule could serve to localize the clamp loader to the primed template through the χ-SSB interaction (Figure 3B) (Glover and McHenry, 1998; Kelman et al., 1998). The mere localization of the second DNA molecule to the polymerase is not sufficient to allow its replication. All three components, SSB, β, and ATP are required for second strand initiation complex formation and replication (Figure 3C).

Initiation Complexes Formed Stepwise Exhibit Asymmetric Function

It has been demonstrated that upon addition of ATPγS to a preformed isolated ATP-initiation complex, half of the complex dissociates (Johanson and McHenry, 1984). This begs the question, does ATPγS induce disassembly of a specific "side" of a dimeric initiation complex? We developed an assay allowing for the controlled stepwise ordered assembly of a dimeric initiation complex, which allowed us to determine whether the disassembly was ordered.

We formed an ATPγS-initiation complex on an avidin bead-DNA matrix using a biotin-tagged 50/87G-mer as the first primed template incorporated into an initiation complex (Figure 4). The 50/87G-mer is replicated upon the addition of only dGTP. We designated initiation complex formation in the first side of the dimeric replicase as the first initiation complex (IC-1). Initiation complex formation in the second side of the dimeric replicase (IC-2) was accomplished by adding a 50/87T-mer (either unlabeled [Figure 4A] or ^{32}P-labeled [Figure 4B]), β, SSB, and ATP to the immobilized IC-1. The second DNA molecule, the 50/87T-mer, is a nonbiotinylated primed template replicated by the sole addition of dTTP. After IC-2 formation in the vacant site in the enzyme, the column was washed to remove all unattached DNA and protein.

To determine whether IC-1 or IC-2 would disassemble upon addition of ATPγS and thus be unable to replicate, the bead-initiation complex was incubated with either ATPγS to induce initiation complex disassembly or a buffer control. dGTP or dTTP was added to enable replication of either the 50/87G-mer or the 50/87T-mer, respectively. Addition of ATPγS had no effect on the ability of IC-1 formed with the 50/87G-mer to replicate (Figure 4A, compare lanes 1 and 2, arrows). Addition of ATPγS disassembled IC-2 and converted it to a replication-incompetent state (Figure 4B, compare lanes 1 and 2, arrows). Although not replicated, the ^{32}P-50/87T-mer remained bound to the initiation complex as demonstrated by the absence of radioactivity in the elution profile at the ATPγS addition step (data not shown), consistent with the expected remaining χ-SSB-template association. Therefore, ATPγS had no effect on the replication of the initiation complex formed first (IC-1), but dissociated and inhibited the replication of the initiation complex

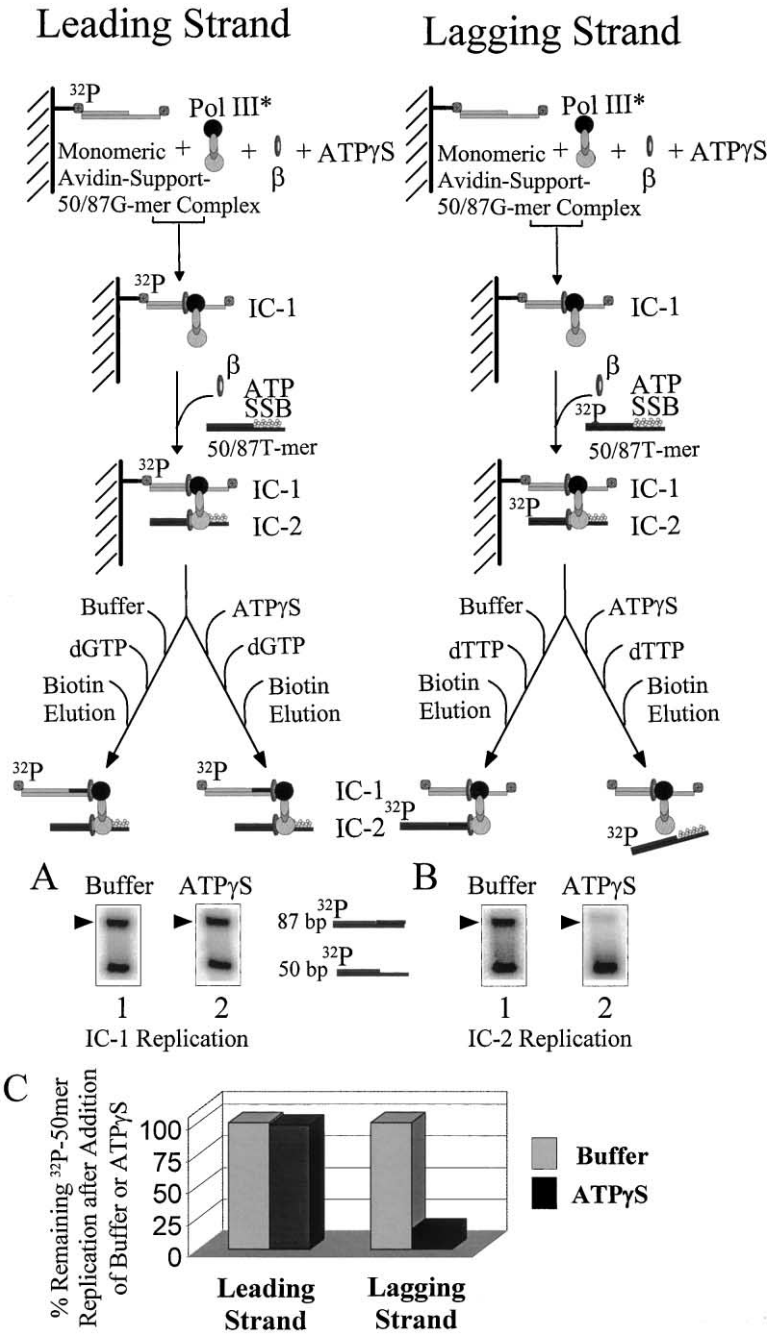

Figure 4. Initiation Complexes Formed Stepwise Exhibit Asymmetric Function

ATPγS-initiation complexes were formed on the bead-50/87G-mer (either ^{32}P-labeled [A] or unlabeled [B]). Incorporation of the second DNA molecule into the immobilized ATPγS-initiation complex was done by adding 50/87T-mer (either ^{32}P-labeled [B] or unlabeled [A]), β, SSB, and ATP to the complex. After excess unbound protein, DNA and nucleotide were washed away, the bead-initiation complex was then incubated with buffer or ATPγS to allow initiation complex dissociation. Subsequently, either dGTP (A) or dTTP (B) was added to facilitate primer elongation on the bead-DNA matrix. dGTP allows for the specific replication of the 50/87G-mer while dTTP allows for the replication of the 50/87T-mer. The elongated DNA was eluted from the beads, analyzed by 12% urea PAGE, and visualized by phosphorimagery. Quantification (C) of the % remaining ^{32}P-50-mer replication after addition of buffer or ATPγS was accomplished by phosphorimagery using ImageQuant software.

formed last (IC-2), in the stepwise reaction (Figure 4C). These results indicate that holoenzyme "remembers" the order in which a dimeric initiation complex is formed and allows DNA dissociation, and, presumably, cycling in only one side. The ATPγS-sensitive side is likely the lagging strand side of the asymmetric holoenzyme, consistent with the requirement for SSB to form replication complexes in this side only and the established lagging strand role of SSB and its association with χψ in lagging strand replication (Kelman et al., 1998; Yuzhakov et al., 1999). To test this hypothesis, we needed to determine whether a bona fide lagging strand replication complex was quantitatively destroyed by ATPγS.

Rolling circle replication systems have been developed to examine the characteristics of replication forks in vitro (Alberts et al., 1983; Minden and Marians, 1985). These systems mimic their in vivo counterparts by replicating both the leading and lagging strands of DNA by a coupled mechanism. Using the *E. coli* rolling circle system, we tested our hypothesis that the lagging strand complex should be ATPγS sensitive. We were unable to examine the effect of ATPγS in a fully reconstituted coupled system because the helicase action of DnaB is blocked by ATPγS and the dynamics of primer synthesis would be expected to be affected (LeBowitz and McMacken, 1986). Thus, we exploited a cleverly de-

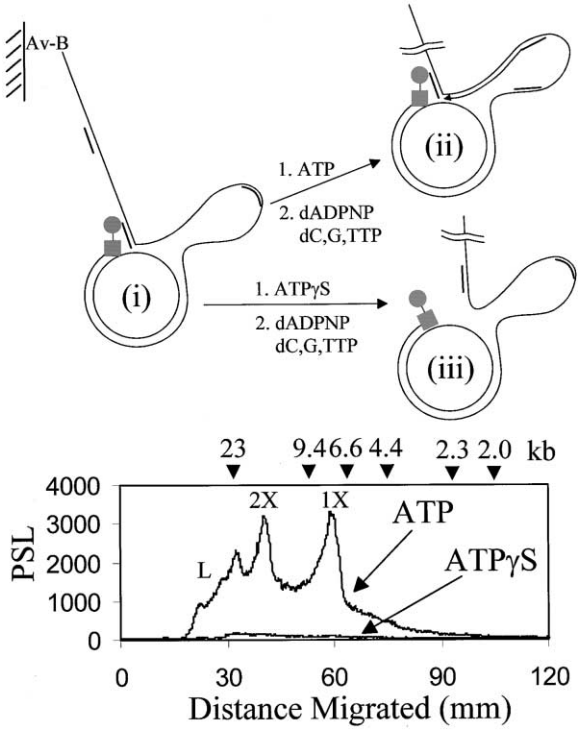

Figure 5. ATPγS Disassembles the Lagging Strand Polymerase in an Isolated Replisome Complex Containing a Preformed Leading and Lagging Strand Initiation Complex

Replisome complexes containing long leading strands and lagging strands primed with an exogenously added DNA primer (Li and Marians, 2000) were captured and prepared on monomeric avidin beads (i). Isolated complexes containing a *preformed* leading and lagging strand initiation complex were incubated with either ATP (ii) or ATPγS (iii) followed by an addition of [α^{32}P]-dNTPs. Nonradioactive dADPNP was used in place of dATP to prevent reinitiation during replication elongation. Replication products were analyzed by alkaline-agarose gel electrophoresis. An overlay plot of phosphorimager traces of gel lanes containing the ATP-mediated (ii) and ATPγS-mediated (iii) dissociation experiments is shown. 1X and 2X, migration positions of oligo-primed lagging strand products one and 2 times the M13 genome length, respectively. L, migration position of leading strand replication products. PSL, photostimulated luminescence.

signed system whereby lagging strand synthesis catalyzed by the unoccupied half of a dimeric polymerase at the replication fork can be observed independently by addition of exogenous primers to a long lagging strand generated by rolling-circle synthesis in the absence of primase (Li and Marians, 2000).

Rolling circle complexes containing long leading strands and DNA oligonucleotide-primed lagging strands were prepared and immobilized on avidin-coated agarose beads (Figure 5 (i)). These replisome complexes bound to the avidin beads contained an initiation complex in both sides of the polymerase (leading and lagging strand). Immobilization on beads was possible because the M13 template used to prepare tailed form II DNA was primed with a 5′-biotinylated DNA primer. In separate parallel experiments, either ATP (Figure 5 (ii)) or ATPγS (Figure 5 (iii)) was added to an avidin bead-replisome matrix possessing leading and lagging strand complexes. We assessed whether ATPγS versus ATP had disassembled the lagging strand half of the replisome by measuring residual lagging strand replication upon addition of dADPNP and [α^{32}P]-dGTP, dCTP, and dTTP. dADPNP was used instead of dATP because, unlike dATP, it cannot facilitate initiation complex formation (Burgers and Kornberg, 1982). Although incorporated slower than dATP, dADPNP does function as an acceptable substrate for the polymerase in replication. ^{32}P-labeled replication products were subjected to alkaline-agarose gel electrophoresis and phosphorimager traces were made of each gel lane. The ATP control gave the expected replication pattern: long leading strand products and nascent lagging strand DNA synthesized from the oligonucleotide primer. The long ca. 100 kb leading strand product (Figure 5, marked with L), which serves as the lagging strand template, contains multiple copies of the M13 genome. The exogenously added DNA oligonucleotide, which anneals once per M13 genome and functions as a lagging strand primer, was extended by the polymerase producing an M13 genome-length 8 kb product (Figure 5, marked as 1X). Because oligonucleotide annealing to the lagging strand template was not 100% efficient, leaving 16 kb gaps between primers, some DNA primers were extended to replication products two times M13 genome length (Figure 5, marked as 2X) (Li and Marians, 2000). In the ATPγS experiment, no leading or lagging strand replication products were observed (Figure 5) as anticipated because of the inhibitory action of ATPγS on DnaB helicase function (Shrimankar et al., 1992). Lagging strand replication is not contingent upon leading strand replication in a system in which primed-lagging strand template is available to the lagging strand half of the holoenzyme (Li and Marians, 2000). Therefore, the absence of lagging strand replication products in the ATPγS experiment indicates that the lagging strand initiation complex was disassembled by ATPγS and converted to a replication-incompetent state. This observation is consistent with our assignment of the ATPγS-sensitive half of the dimeric DNA polymerase III holoenzyme as the lagging strand half.

Discussion

Holoenzyme Asymmetry Revealed by ATPγS

Previously, suggestions have been made regarding the role of a dimeric polymerase in the replication process (Sinha et al., 1980; McHenry, 1982). We extended this hypothesis based on the activity of ATPγS to include a dimeric polymerase that is asymmetric (Johanson and McHenry, 1984). In recent years, data have emerged which support the notion that one molecule of holoenzyme can replicate both the leading and lagging strand in the presence of the primosome (Kim et al., 1996a). In this report, we present studies which reveal that the intrinsic properties required of holoenzyme to function asymmetrically reside solely within the replicase and do not require additional proteins such as DnaB.

ATPγS supported the formation of a leading strand complex which bound and replicated lagging strand DNA in the presence of β, ATP, and SSB (Figure 6A). SSB coats the single-stranded portion of the lagging strand in vivo, and reported biochemical observations

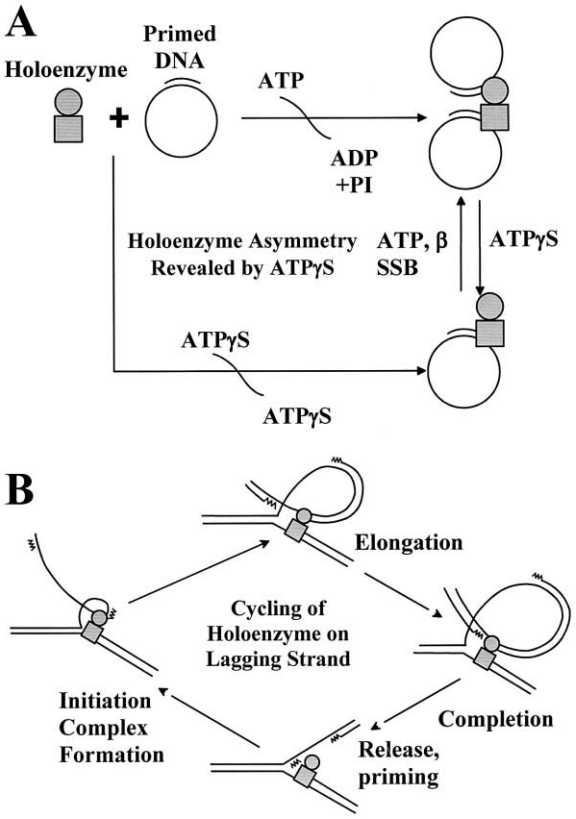

Figure 6. ATPγS Reveals Holoenzyme Asymmetry

(A) ATPγS supports the formation of a leading strand complex which binds and replicates lagging strand DNA in the presence of β, ATP, and SSB.

(B) The functional asymmetry observed with holoenzyme is consistent with replication in vivo where the leading strand of DNA remains bound to the same side of the polymerase throughout semi-discontinuous replication. The lagging strand of DNA, on the other hand, is replicated by a cycle of binding, elongation, and dissociation events.

are consistent with an exclusive role for the holoenzyme χ-ψ subunits and SSB in the replication of the lagging strand (Kelman et al., 1998; Yuzhakov et al., 1999). Consistent with this, we found that the χ-SSB interaction aided in localizing the second DNA molecule to the second vacant Pol III site during initiation complex formation. Once a dimeric complex had formed, the replication complex disassembled in the opposite order by which it assembled. Upon ATPγS-mediated dissociation, the leading strand polymerase was refractory to disassembly allowing cycling to occur exclusively on the lagging strand. This functional asymmetry (Figure 6B) is consistent with replication in vivo where the leading strand of DNA remains bound to the same side of the polymerase throughout semi-discontinuous replication. The lagging strand of DNA on the other hand is replicated by a cycle of binding, elongation, and dissociation events.

Potential Underlying Mechanisms Explaining the Effect of ATPγS in Initiation Complex Formation and Dissociation

Initiation complex formation has been defined by a requirement for ATP (Wickner, 1976; Burgers and Kornberg, 1982). ATPγS, a nonhydrolyzable ATP analog, has been shown to support initiation complex formation (Johanson and McHenry, 1984; Dallmann et al., 1995). Consistent with this, the first initiation complex formed in the dimeric holoenzyme is β and nucleotide dependent. We do not mean to imply that leading strand initiation complex formation is not coupled to ATP hydrolysis in the natural reaction. Such coupling could accelerate the rate or specificity of the natural reaction. However, unlike the second initiation complex formed by the lagging strand half of holoenzyme, the leading strand half can occur in the absence of ATP hydrolysis. The second initiation complex formation step requires ATP hydrolysis. Nucleotide hydrolysis may be required for isomerization of the DnaX complex directing clamp loading activity from the leading to the lagging strand. ATP-dependent isomerization or rotation is an established mechanism used by several enzymatic systems, for example, the F_1F_0-ATP synthases (Schulenberg et al., 1999; Stock et al., 2000). Recent work of the O'Donnell laboratory has demonstrated that the binding of ATPγS facilitates formation of a γ complex-open β-DNA "composite" (Hingorani and O'Donnell, 1998). This composite is not stable when ATP is used, as nucleotide hydrolysis allows the complex to quickly turn over yielding free γ complex and a β-DNA complex.

Initiation complex formation studies which exclusively utilize the γ complex are limited in the number of issues which can be addressed. Unlike authentic DnaX complexes containing τ, the γ complex is unable to bind to the polymerase and form holoenzyme-based initiation complex. γ complex supports little or no ATPγS-mediated replication (Dallmann et al., 1995). The presence of τ in holoenzyme unites the clamp loader and polymerase. In the presence of τ, ATPγS likely supports the formation of a Pol III-DnaX complex-open β-DNA composite. Nonhydrolytic turnover of this composite resulting from nucleotide diffusion out of the ATPase active site would release the β clamp. If the open β clamp was positioned such that the DNA could "breathe" in and out of the open processivity factor, closure would result in productive initiation complex formation. The positively charged inner diameter of the β ring could interact with the negatively charged phosphates on the DNA backbone imparting additional stability, pulling the β-DNA catenation step forward.

What mechanism might be at work when ATPγS dissociates preformed ATP-initiation complexes? We have demonstrated here that ATPγS disassembles the initiation complex formed last in a stepwise reaction. The addition of ATPγS to the dimeric initiation complex could induce a conformational change in the DnaX complex which allows the δ subunit to bind to the β subunit on the second DNA strand, facilitate β_2 opening, and its dissociation from DNA (Hingorani and O'Donnell, 1998). This dissociation might be driven by charge repulsion upon the lagging strand template by the leading strand DNA. Why does the addition of ATPγS not dissociate the initiation complex in the other side of the dimeric polymerase? During leading strand replication within the cell, there must exist a mechanism preventing the endogenous clamp-loader from removing β from the leading strand polymerase. One likely explanation is that the DnaX complex has undergone a nucleotide-depen-

dent isomerization which limits its clamp loading (or unloading) activity exclusively to the lagging strand. τ is required for protection of replication complexes by exogenous γ complex (Kim et al., 1996c), perhaps because of its role in organizing and maintaining the asymmetry of the replicating complex.

Is There a Connection between Holoenzyme's Structural and Functional Asymmetry?

Our model (Figure 6) demonstrates that once the first initiation complex forms, the holoenzyme dedicates that half to the task of continuous leading strand replication. Whether the holoenzyme prededicates a specific endogenous Pol III molecule to the task of leading or lagging strand synthesis before initiation complex formation remains to be determined. Our data do demonstrate that once the first complex formation event occurs, a holoenzyme-directed functional asymmetry is observed in the subsequent complex formation step. In this report, we have demonstrated that the DnaX complex can load a β processivity factor onto both strands of a dimeric initiation complex. We have shown that once a dimeric initiation complex forms, the clamp loading activity is directed solely to one side of the complex. Whether this arises from steric-based prevention of the removal of β on the leading strand side, or whether the clamp loader has undergone an isomerization which limits its activity to one side of the polymerase, remain to be determined. Whatever the mechanism is which focuses the clamp loading activity exclusively on the second side of the initiation complex, the asymmetry required for this process is intrinsic to the asymmetric dimeric DNA polymerase III holoenzyme. However, these properties may be further modified by association with DnaB and other primosomal components, altering the rate, fidelity, and recycling properties as the lagging strand polymerase cycles between primers on the lagging strand of the replication fork during Okazaki fragment synthesis (Kim et al., 1996b; Li and Marians, 2000).

Experimental Procedures

Proteins, Nucleic Acids, and Reagents

Recombinant exonuclease-deficient Pol III was expressed in *E. coli*, then purified using the procedure described for the wild-type protein (Kim and McHenry, 1996). β (Johanson et al., 1986), SSB (Griep and McHenry, 1989), and τ DnaX complex (Glover and McHenry, 2000) were purified as described. Pol III* refers to a τ-reconstituted exonuclease-deficient Pol III* prepared by incubating a 4-fold molar excess of exonuclease-deficient Pol III with τ complex at room temperature for 10 min. In all experiments within this report, we use a DNA-primed template in initiation complex assays; thus, it was necessary to use the exonuclease-deficient Pol III containing an active-site mutation in ε. This exo-deficient Pol III functions identically to wild-type Pol III in all assays tested with the exception of the absence of the 3′ to 5′ exonuclease activity.

ATPγS was from Roche Molecular Biochemicals. dADPNP was from TriLink BioTechnologies, Inc. Oligonucleotides were synthesized and PAGE-purified by Synthetic Genetics Inc. The sequence of the 50T-mer is: 5′ A C T T C T C C T A G G T A T T C T C A C T C C G C T G A A C T G T T G A A T T C T G T T A A C C 3′. The 87T-mer's sequence is: 5′ A $(A)_{35}$ A G G T T A A C A G A A T T C A A C A G T T T C A G C G G A G T G A G A A T A C C T A G G A G A A G T 3′. The underlined sequence corresponds to the EcoRI restriction site. The sequence of the 50G-mer is: 5′ T T G T* T C A G A T G A A G G C G C A T A G G C T G G C T G A C C T T C G G A T C C A G T A A T C C 3′. The 87G-mer's sequence is: 5′ T* C $(C)_{35}$ C G G A T T A C T G G A T C C G A A G G T C A G C C A G C C T A T G C G C C T T C A T C T G A A C A A 3′. The underlined sequence corresponds to the BamHI restriction site and the asterisk to a thymidine residue containing a biotin molecule. The sequence of the 30-mer, which anneals to M13Gori (position 979–1008), is: 5′ A G G C T G G C T G A C C T T C A T C A A G A G T A A T C T 3′. Primer-template annealing and 5′ [^{32}P]-end-labeling of primers were described previously (Glover and McHenry, 1998). In all experiments except those involving monomeric avidin beads (Pierce), the 50/87G-mer had a Neutravidin molecule (Pierce) bound to each 5′ end. This construct was prepared by incubating a 15-fold molar excess of Neutravidin over the 50/87G-mer for 5 min at room temperature. Protein concentration determination for individual protein subunits was determined by extinction coefficient while protein complex concentration was determined using the Pierce Coomassie Plus assay. Bovine serum albumin (fat-free, Sigma) was used as an assay standard.

Endonuclease Protection Assay

Initiation complexes were formed by incubating Pol III* (1.8 pmol), β (12 pmol dimer), and either ATP (570 μM) or ATPγS (570 μM) with Neutravidin bound [^{32}P]50/87G-mer (2.8 pmol) for 5 min at 30°C. Cleavage of unprotected DNA was effected by adding 50 U BamHI to initiation complexes and incubating the mixture for 10 min at 37°C (Figure 1). Samples were subjected to 12% urea PAGE. Quantification of the amount of 50G-mer remaining after endonuclease digestion was done by phosphorimagery using ImageQuant software. The value obtained for the amount of 50G-mer remaining in the ATP-containing sample was defined as 100%. Results are presented as the averages ± standard deviation from three separate experiments.

M13 Initiation Complex Formation and Gel Filtration

Initiation complexes were formed by incubating Pol III* (15 pmol), β (120 pmol dimer), 30-mer-primed M13Gori (18 pmol circles) in the presence of either ATP (1 mM final), ATPγS (1 mM final), or without nucleotide for 5 min at 30°C (Figure 1). Initiation complexes with varying DNA to Pol III ratios (Figure 1C) were formed as described above, except that the amounts of M13 (18–35 pmol) and Pol III* (2.5–15 pmol) were adjusted to yield the desired products.

Initiation complex (100 μl sample volume) was loaded onto a 24 ml Superose 6 FPLC gel filtration column equilibrated in buffer S (25 mM HEPES [pH 7.5], 100 mM sodium chloride, 5% glycerol, and 8 mM magnesium acetate). Gel filtration fractions (0.5 ml) were collected at 4°C with a flow rate of 0.2 ml/min. Aliquots (10 μl) of each fraction were assayed for processive replication after adding 48 μM unlabeled dNTPs (dATP, dCTP, and dGTP), and 18 μM [^{3}H]TTP (specific activity = 520 cpm/pmol TTP), then incubating the mix for 5 min at 30°C. Assays were quenched by trichloroacetic acid precipitation and quantified by scintillation counting. 9 μg SSB was added to each replication assay.

Characterization of Initiation Complexes Containing Two Distinct Primed Templates

Replication of the ^{32}P-50/87G-mer by isolated ATP- or ATPγS-initiation complexes (Figures 2A–2C) was accomplished by incubating initiation complex with ATP (0.6 mM), β (36 pmol dimer), SSB (120 pmol tetramer), ^{32}P-50/87G-mer (1 pmol), and dGTP (50 μM) for 2 min at 30°C in a 200 μl reaction. Reaction products were subjected to 12% urea PAGE and visualized by phosphorimagery.

Experiments determining conditions which facilitate binding of the ^{32}P-50/87G-mer to and gel filtration with preisolated ATPγS-initiation complex (Figure 2D) were performed as follows. One hundred femtomoles initiation complex were incubated with the ^{32}P-50/87G-mer (800 fmol) and either ATP (0.6 mM) and β (36 pmol dimer), SSB only (570 pmol tetramer, preincubated with the 50/87G-mer prior to addition to initiation complex), or ATP, β, and SSB for 2 min at 30°C. The mixture (100 μl) was loaded onto a 24 ml Superose 6 column equilibrated in buffer S. Gel filtration fractions (0.5 ml) were collected at 4°C in buffer S with a flow rate of 0.2 ml/min. Aliquots (20 μl) of each fraction were assayed for processive as described previously. The elution position of the ^{32}P-50/87G-mer (165 cpm/fmol) was determined by scintillation counting.

Avidin Bead Assay for Initiation Complex Formation

A 300 μl slurry of monomeric avidin beads (150 μl beads) was incubated with 1 ml biotin elution buffer (2 mM D-biotin, 100 mM sodium phosphate [pH 7.2] and 150 mM sodium chloride) for 5 min. The beads were microcentrifuged, the supernatant was removed, and 1 ml of Buffer S was added to equilibrate the beads in column buffer. The beads were incubated in Buffer S for 5 min, collected by microcentrifugation, and resuspended in 150 μl Buffer S.

Bead-DNA complexes were made by incubating 300 μl equilibrated bead slurry with 3 pmol ^{32}P-50/87G-mer for 10 min at room temperature. When a ^{32}P-50/87T-mer was utilized, a nonradioactively labeled 50/87G-mer was used. Initiation complexes were formed on the bead-DNA matrix by adding 10 pmol Pol III*, 120 pmol β, and 1 mM ATPγS (final) to 300 μl equilibrated bead-DNA slurry and incubated 5 min at 30°C. Bead-initiation complex was then loaded onto a Micro Bio-Spin Chromatography Column (Bio-Rad Laboratories). The column was then washed with four 300 μl aliquots while collecting the flow through as 300 μl fractions. Incorporation of the second DNA molecule into the immobilized ATPγS-initiation complex was done as follows. An aliquot (prewarmed to 30°C) containing 8 pmol 50/87T-mer (either ^{32}P-labeled or unlabeled), 120 pmol β, 570 pmol SSB (tetramer), and 1 mM ATP (final) was added to the column and incubated for 5 min at room temperature to facilitate the second initiation complex formation. A 300 μl aliquot was then collected and the column was washed with three 300 μl aliquots of column buffer. Next, a 300 μl aliquot (prewarmed to 30°C) of column buffer or one containing ATPγS (1 mM final) was added to the column and incubated for 5 min to allow for initiation complex dissociation. Subsequently, a 300 μl fraction was collected.

To facilitate differential primer elongation on the bead-DNA matrix, a 300 μl aliquot (prewarmed to 30°C) of column buffer containing 42 μM (final) either dTTP or dGTP and 1 mM ATPγS (final, when present in the dissociation step) was added to the column and incubated for 5 min at room temperature. dTTP allows for the specific replication of the 50/87T-mer while dGTP allows for the replication of the 50/87G-mer. The column was then washed with three 300 μl aliquots of column buffer (containing 1 mM ATPγS (final) in the dissociation experiment). In order to elute elongated DNA from the monomeric avidin beads, 300 μl biotin elution buffer was added to the column followed by a 10 min incubation at room temperature. A 300 μl fraction was collected and the column was washed with 300 μl aliquots of biotin elution buffer until >80% of the ^{32}P-labeled DNA was recovered. The elution position of the ^{32}P-labeled DNA was determined by scintillation counting. Fractions containing elongated primer were analyzed by 12% urea PAGE and visualized by phosphorimagery. Quantification of the % remaining ^{32}P-50mer replication after addition of buffer or ATPγS was done by phosphorimagery using ImageQuant software. The amount of 50-mer remaining for the buffer experiment was used as the 100% value.

Replication by Isolated Replisome Complexes

Replisome complexes containing long leading strands and DNA oligonucleotide-primed lagging strands were prepared according to a procedure described elsewhere (Li and Marians, 2000) with the following modifications. TFII was prepared from M13mp18 primed with a synthetic biotinylated (5′-end) DNA primer which supports primosome and DnaG-dependent leading and lagging strand replication (data not shown). Replisome complexes (Figure 5) containing long leading strands were added to 50 μl monomeric avidin beads equilibrated in buffer R1 (50 mM HEPES-KOH [pH 7.5], 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM magnesium acetate, 0.1 M potassium glutamate, 200 μg/ml bovine serum albumin, 0.02% (v/v) Tween 20, 50 μM dGTP, 50 μM dCTP), placed in a Micro Bio-Spin column, and washed with buffer R1 to remove free protein and nucleotide. An initiation complex was formed in the lagging strand half of the bead bound replisome complex by adding 30-mer primer (2.2 μM), ATP (1 mM), β (1.2 μM), SSB (11 μM) to the to the bead complex and incubating for 10 min at 30°C. The column was then washed with three 100 μl aliquots of either buffer R2 (buffer R1 plus 100 μM ATP) for the ATP dissociation experiment or buffer R1 for the ATPγS dissociation experiment. One hundred microliters buffer R2 or R3 (buffer R1 plus 1 mM ATPγS) was then added to the ATP and ATPγS experiments, respectively, and allowed to incubate for 15 min at 30°C. Next, a 100 μl aliquot of buffer R4 (buffer R2 plus 400 μM dTTP, dCTP, dGTP, dADPNP, and [α^{32}P]-dTTP, dCTP, and dGTP) or buffer R5 (buffer R3 plus 400 μM dTTP, dCTP, dGTP, dADPNP, and [α^{32}P]-dTTP, dCTP, and dGTP) was added to the ATP and ATPγS, respectively, and allowed to incubate for 10 min at 30°C to facilitate replication. The ATP and ATPγS experiments were washed with five separate aliquots of buffer R1. The bead-replisome complexes in the spin columns were spun to dryness, removed, and placed in a 1.5 ml eppindorf tube. Seventy-five microliters alkaline agarose sample buffer (150 mM NaOH, 10 mM EDTA, 5% glycerol, and 0.1% bromophenol blue) was added to the beads and incubated for 30 min at room temperature. The bead sample buffer suspension was briefly centrifuged and the supernatant removed. Twenty microliter aliquots of the supernatants were subjected to 0.5% alkaline agarose gel electrophoresis as previously described (Li and Marians, 2000). The gel was dried and visualized by phosphorimagery.

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