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A Dynamic Polymerase Exchange with *Escherichia coli* DNA Polymerase IV Replacing DNA Polymerase III on the Sliding Clamp*[S][◆]

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An assay that measures synchronized, processive DNA replication by *Escherichia coli* DNA polymerase III holoenzyme was used to reveal replacement of pol III by the specialized lesion bypass DNA polymerase IV when the replicative polymerase is stalled. When idled replication is restarted, a rapid burst of pol III-catalyzed synthesis accompanied by ~7-kb full-length products is strongly inhibited by the presence of pol IV. The production of slower-forming, shorter length DNA reflects a rapid takeover of DNA synthesis by pol IV. Here we demonstrate that pol IV rapidly (<15 s) obstructs the stable interaction between pol III* and the β clamp (the lifetime of the complex is >5 min), causing the removal of pol III* from template DNA. We propose that the rapid replacement of pol III* on the β clamp with pol IV is mediated by two processes, an interaction between pol IV and the β clamp and a separate interaction between pol IV and pol III*. This newly discovered property of pol IV facilitates a dynamic exchange between the two free polymerases at the primer terminus. Our study suggests a model in which the interaction between pol III* and the β clamp is mediated by pol IV to ensure that DNA replication proceeds with minimal interruption.

Successful DNA replication requires a high fidelity DNA polymerase to replicate the entire genome accurately. In *Escherichia coli*, DNA polymerase III holoenzyme (pol III HE)[2] is a replicative polymerase that catalyzes elongation of DNA chains at a rate of about 1 kb/s with high fidelity. pol III HE is a multisubunit complex that contains two pol III core catalytic subassemblies, each linked to a τ subunit of the DnaX complex. Polymerase activity resides in the α subunit, the largest subunit of pol III HE. pol III*, a subassembly composed of two pol III cores and one DnaX complex, binds to a dimer of the β subunit (the β clamp) loaded onto the primer/template by the DnaX complex to form a stable structure, pol III HE, which can synthesize DNA processively (over 50 kb per binding event) (1, 2).

When a replisome encounters a lesion, pol III is generally believed to stall on DNA at a lesion site on the leading strand, resulting in uncoupling of leading and lagging strand DNA synthesis and arrest of the replication fork (3–5). Two major pathways are thought to play a role in overcoming DNA damage at the replication fork; one is recombinational repair, and the other is the translesion synthesis (TLS). In the TLS pathway, a switch is likely to occur from the stalled replicative polymerase to a specialized polymerase, which replaces the former to bypass the lesion. It is not yet clear, however, how a specialized polymerase gains access to the primer terminus when a stalled replicative polymerase exists at the site or how it acts in coordination with the replicative polymerase (6).

In *E. coli*, three polymerases, pol II, pol IV, and pol V, have been identified as specialized polymerases. pol IV, encoded by the *dinB* gene, belongs to the Y family and is up-regulated by the SOS response (7). pol IV can replicate undamaged DNA together with the β clamp, but its fidelity is much lower than that of pol III HE (8). pol IV is able to bypass various lesions such as AP, BaP, AAF, and N^2-furfuryl-dG adducts *in vitro* (6, 9, 10). Although a Δ*dinB* strain shows no apparent defect in UV sensitivity and UV-induced mutagenesis, it displays increased sensitivity to agents that cause N^2-dG adducts, suggesting that pol IV can bypass such damaged DNA *in vivo* (9).

In a previous study (3), we examined the dynamics of the replication fork when it encounters an abasic lesion on a template DNA, using an *in vitro* assay based on semi-bidirectional DNA replication of an *oriC*-containing plasmid. We demonstrated that an abasic lesion on both strands blocks chain elongation by pol III HE. A lesion on the lagging strand did not impede the progression of the replication fork, whereas a lesion on the leading strand greatly reduced replication fork velocity, implying that a pol III core on the lagging strand easily detaches from the β clamp to bind to the next primer but that a pol III core on the leading strand does not (3). To investigate the mechanism of polymerase switching, we reconstituted a model TLS reaction at a replication fork and observed whether pol IV could bypass a non-coding abasic lesion. We supposed that pol

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[2] The abbreviations used are: pol, DNA polymerase; pol III HE, DNA polymerase III holoenzyme; TLS, translesion synthesis; ssDNA, single-stranded DNA; IC, initiation complex; SSB, *E. coli* single-stranded DNA-binding protein.

IV would bypass the lesion readily on the lagging strand because of rapid dissociation of pol III, but not on the leading strand, owing to a continued presence of pol III. We found instead that pol IV inserted a nucleotide opposite the lesion and yielded −1-frameshifted bypass products efficiently on both strands.[3] We speculated either that pol III was detached from the lesion site on both strands or that pol IV could access the primer terminus even if the stalled pol III remained bound. During these experiments, we also noted a striking inhibition of DNA synthesis on non-lesion-containing templates when we added pol IV at high concentration.[3] This last result implied that pol IV might have a direct inhibitory effect on DNA synthesis by pol III HE, raising the possibility that pol IV might replace pol III at a primer terminus.

In this study, we examine whether pol IV inhibits DNA synthesis of pol III HE and whether it can take over the primer from pol III HE. The assay measures burst DNA synthesis by pol III HE on a singly primed circular single-stranded DNA (ssDNA) template without a lesion. We find that pol IV inhibits DNA synthesis by pol III HE in several ways, one of which involves a polymerase switch reaction from pol III* to pol IV. When pol IV gains access to the 3′-primer end, pol III* rapidly dissociates from the β clamp. The data suggest that pol IV actively displaces pol III during the switching process by interfering with the interaction between pol III* and the β clamp.

EXPERIMENTAL PROCEDURES

Nucleic Acids—Purification of circular ssDNA from M13mp18 phage was carried out as described previously (11). A 25-mer oligonucleotide primer, uni25 (12), was labeled with ^{32}P at its 5′-end by T4 polynucleotide kinase (Toyobo). Labeled or unlabeled uni25 primer and M13mp18 ssDNA were mixed at a molar ratio of 3:1 in 265 mM NaCl, incubated at 96 °C for 3 min and at 59 °C for 15 min, and cooled slowly to room temperature. M13mp18 ssDNA primed with labeled uni25 was purified on a MicroSpin S-400 HR column (GE Healthcare) to remove unbound primer. The synthetic homopolymer poly(dA)300 and oligo(dT)10 were mixed at a weight ratio of 20:1 in 20 mM Tris-HCl (pH 8.0) containing 20 mM KCl and 1 mM EDTA, incubated at 65 °C for 10 min, and cooled slowly to room temperature.

Enzymes—Enzymes required for the burst DNA synthesis reaction were purified as described (12), except that the final Superose 6 column purification step for pol III* was omitted. The concentration of pol III* was estimated by determining the amount of initiation complex (IC) on primed M13mp18 ssDNA as described (13). Non-tagged wild-type pol IV was purified as described (8) and used in experiments shown in Figs. 1, *C*, *D*, and *F*, and 2. In other experiments, His-tagged wild-type pol IV or mutant pol IV was used. For purification, whole or partial *dinB* gene fragments were amplified by PCR from the *dinB* coding sequence in pET16b-*dinB* (8) and then cloned into the NdeI and BamHI sites of pET15b (Novagen) to generate expression constructs for His-tagged wild-type pol IV and mutant pol IV proteins. For polymerase-null mutant pol IV (pol IV D8A), we introduced an A to C base substitution at position 23 of *dinB* on the expression construct by PCR site-directed mutagenesis. For the β interaction-deficient mutant pol IV (pol IV $\Delta\beta$2C5), we introduced T to G base substitutions at positions 908 and 910 and C to G substitutions at position 913 of partial *dinB* (1–1038) on the expression construct by PCR site-directed mutagenesis. All constructs were checked by DNA sequencing. Each His-tagged pol IV protein was expressed in *E. coli* BL21 (DE3)/pLysS and purified as described (8, 14).

Burst DNA Synthesis on Primed M13mp18 ssDNA—Burst DNA synthesis reactions were carried out as described below. Primed M13mp18 ssDNA (0.03 pmol), 20 units of pol III* (1.2-fold molar excess over template DNA molecules), 0.6 μg of SSB, and 0.12 μg of β clamp were preincubated at 30 °C for 3 min in buffer 1 (7.5 μl) containing EDBG (20 mM Tris-HCl (pH 7.5), 4% glycerol, 8 mM dithiothreitol, 80 μg/ml bovine serum albumin, 1 mM ATP, 8 mM $MgCl_2$), and 100 μM each of dATP, dCTP, and dGTP to form the IC. DNA chain elongation was then started by adding prewarmed buffer 2 (22.5 μl) containing EDBG, 100 μM each of dATP, dCTP, and dGTP and 133 μM [α-^{32}P]dTTP at 30 °C. To stop the reaction, an equal volume of stop buffer (50 mM EDTA, 0.15% SDS, pH 8.0) was added at each time point. Replication products were extracted and analyzed by electrophoresis for 3.5 h at 2 V/cm on alkaline 0.9% agarose gels followed by autoradiography as described (3). In Figs. 1 and 2, the indicated amount of pol IV or mutant pol IV was added 15 s before the start of DNA synthesis. In Fig. 2, the indicated amount of pol III* was added at the same time as the start of DNA synthesis.

Inhibition of the Movement of pol III HE—Primed M13mp18 ssDNA (unlabeled, 0.06 pmol), 40 units of pol III* (1.2-fold molar excess over template DNA molecules), 1.2 μg of SSB, and 0.24 μg of β clamp were preincubated at 30 °C for 3 min in buffer 1 (8 μl) containing EDBG, 100 μM each of dATP, dCTP, and dGTP to form the IC. DNA chain elongation was then started by adding prewarmed buffer 2 (37.5 μl) containing EDBG, 100 μM each of dATP, dCTP, dGTP, and [α-^{32}P]dTTP. To stop the reaction, an equal volume of stop buffer was added at the appropriate time point. Replication products were extracted and analyzed by electrophoresis for 5.5 h at 2 V/cm on alkaline 0.8% agarose gels followed by autoradiography. pol IV was added (to a final concentration of 890 nM) 5 s after the start of DNA synthesis.

*Separation of IC and pol III**—A 2-ml glycerol gradient (20–41%) in EDBG was prepared as described (13). Primed M13mp18 ssDNA (unlabeled, 0.08 pmol), 26.7 units of pol III* (0.6-fold numbers of molecules relative to those of template DNA molecules), 1.6 μg of SSB, and 0.32 μg of β clamp were preincubated at 30 °C for 3 min in 80 μl of EDBG containing 100 μM each of dATP, dCTP, and dGTP to form the IC. The reaction mixture was loaded onto a glycerol gradient and centrifuged at 55,000 rpm for 75 min at 4 °C in a Beckman TLS-55 rotor in a TL-100 ultracentrifuge as described (13). Fractions (200 μl) were collected from the bottom of the tube. Polymerase activity in each fraction was measured as the amount of nucleotide incorporated in 5 min at 30 °C in 20-μl aliquots of each fraction, after the addition of 5 μl of a solution containing 800 pmol of poly(dA)/oligo(dT) and [α-^{32}P]dTTP (to a final concentration of 100 μM) in EDBG. After incubation, reactions

[3] A. Furukohri, M. F. Goodman, and H. Maki, unpublished data.

were stopped by adding an equal volume of 50 mM EDTA (pH 8.0). Aliquots were spotted onto DE81 filter paper (Whatman), which was then dried, washed three times with 0.5 M Na_2HPO_4 for 10 min, exposed for 30 min to an imaging plate, and analyzed using a BAS 2500 (Fuji Film). The total amount of DNA synthesis was calculated and plotted. To detect primed M13mp18, 0.2-μl aliquots of each fraction were used as a template for PCR amplification with the primer set (forward, 5′-GCAATTAATGTGAGTTAGCTCACTC-3′, reverse, 5′-GTGGGAACAAACGGCGGATTGACCG-3′). The products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. In the experiment shown in Fig. 3*A*, primed M13mp18 was omitted from the reaction; the reaction carried out with primed M13mp18 is shown in supplemental Fig. 1*A*. In Fig. 3, *B* and *C*, 1.5 μg of His-tagged polymerase-null mutant pol IV D8A or the same volume of control buffer was added (to a final pol IV concentration of 890 nM) after the formation of the IC. The reaction was incubated at 30 °C for 15 s, chilled, and subjected immediately to glycerol gradient centrifugation as described above.

IC Disruption Assay—Primed M13mp18 (unlabeled, 0.02 pmol), 6.7 units of pol III* (0.6-fold numbers of molecules relative to those of template DNA molecules), 0.4 μg of SSB, and 0.08 μg of β clamp were preincubated at 30 °C for 3 min in EDBG (20 μl) containing 100 μM each of dATP, dCTP, and [α-^{32}P]dTTP to form the IC. Poly(dA)/oligo(dT) (200 pmol) was then added to the reaction. His-tagged polymerase-null mutant pol IV D8A or the same volume of control buffer was added, to the final pol IV concentration indicated, 15 s before the addition of poly(dA)/oligo(dT). After incubation for the indicated time, aliquots were mixed with an equal volume of 50 mM EDTA (pH 8.0), and the amount of nucleotide incorporation was determined as described above. The final salt concentration, contributed by pol IV storage buffer, was 17 mM in Fig. 4, 7–12 mM in Figs. 5 and 6*B*, and 37 mM in Fig. 6*C*.

RESULTS

pol IV Inhibits DNA Synthesis by pol III HE on a Primed M13 ssDNA Template and Rapidly Takes Over the Primer Terminus from an Idled pol III HE—To analyze how the presence of pol IV might influence pol III HE-catalyzed replication, we used an assay for synchronized, processive DNA synthesis on a singly primed M13mp18 circular ssDNA template (Fig. 1*A*) (13). Preincubation of pol III*, β clamp, SSB, and the template DNA, in the presence of ATP and three dNTPs, leads to the formation of an IC in which an idling pol III HE binds to the primer terminus on the template DNA (Fig. 1*B*). The IC is stable for several minutes, mainly because the α subunit of pol III* binds tightly to the β clamp (15–17). On the addition of dTTP, every IC starts a synchronous burst of DNA synthesis during which pol III HE catalyzes processive DNA chain elongation at a rate of 800–900 nt/s As shown in Fig. 1*C*, the 5′-^{32}P-labeled primer was extended to about 4.5 kb within 5 s (*lane 2*) and to 7.25-kb full-length product within 10 s (*lane 3*) during the burst DNA synthesis. Using this assay, we investigated the effects of pol IV on the stability of the IC and on DNA chain elongation by pol III HE. When pol IV was added to the reaction mixture containing preformed IC 15 s prior to the start of burst DNA synthesis, almost no full-length product was formed even after 30 s (Fig. 1*C*, *lanes 5–8*), showing that the burst DNA synthesis of pol III HE was inhibited by the addition of pol IV. We fractionated purified pol IV by gel filtration to confirm that the inhibition observed was due solely to pol IV and found that the peak of inhibitory activity coincided with the peak of pol IV protein (data not shown).

A characteristic pattern of elongation products appeared when pol IV was added to the burst DNA synthesis reaction, with three discernible size classes: long smearing products (class I), strong paused products of ~1.2 kb (class II), and a group of shorter products (class III). Class I products were extended slowly, at a rate of ~100–200 nt/s, and remained shorter than the full-length product even after incubation for 30 s (Fig. 1*C*, *lane 8*). Thus, pol IV appeared to slow down DNA chain elongation by pol III HE. Class II products accumulated throughout the time course, and their sizes increased slowly. Since the class II products were already clearly visible at the 5-s time point, the pol III HE-elongated DNA chain was probably blocked by pol IV at a pause site, although we were unable to identify any particular structural features such as repetitive sequences or long hairpins in this region that could explain why pol III HE might have paused. It appears likely that both class I and class II products are derived from an inhibitory action of pol IV on a moving pol III HE. To explore this possibility, we measured DNA chain elongation by pol III HE when pol IV was added 5 s after the burst DNA synthesis started (Fig. 1*D*). At the 10-s time point, a major fraction of the 4.5-kb product DNA persists, accompanied by a diffuse series of longer products. The diffuse products continued to accumulate over a 15-s interval but did not reach the 7.25-kb full-length product characteristic of pol III replication. Thus, we concluded that pol IV has the capacity to inhibit a rapidly moving pol III HE.

The short products comprising class III were extended slowly (5–6 nt/s) during a 30-s incubation (Fig. 1*C*, *lanes 5–8*). Since this slow elongation rate was similar to that observed with pol IV together with the β clamp (18), we surmised that the pol IV added to the reaction might generate the short products. As shown in Fig. 1*E*, this appeared to be the case. A catalytically defective mutant pol IV (pol IV D8A) showed inhibitory effects on burst DNA synthesis similar to those found with wild-type pol IV, except that the class III products were not produced. This indicates clearly that the polymerase activity of pol IV is dispensable for the inhibition of burst DNA synthesis by pol III HE but is required for the synthesis of class III products. Since pol IV showed far weaker DNA polymerase activity with the same singly primed template in the absence of the β clamp (18), it appears to generate class III products with the help of the β clamp that was loaded onto the primer/template during IC formation. We therefore conclude that pol IV is able to take over a 3′-primer end from pol III*, which is an integral part of the preformed IC. pol IV performs the takeover with high efficiency and within 15 s of incubation prior to burst DNA synthesis. Considering the relatively long half-life of pol III* within the IC, pol IV seemed to promote polymerase switching from the idling pol III* to pol IV on the primer/template DNA.

The inhibitory effects of pol IV on the action of pol III HE were concentration-dependent (Fig. 1*F*). Increasing the con-

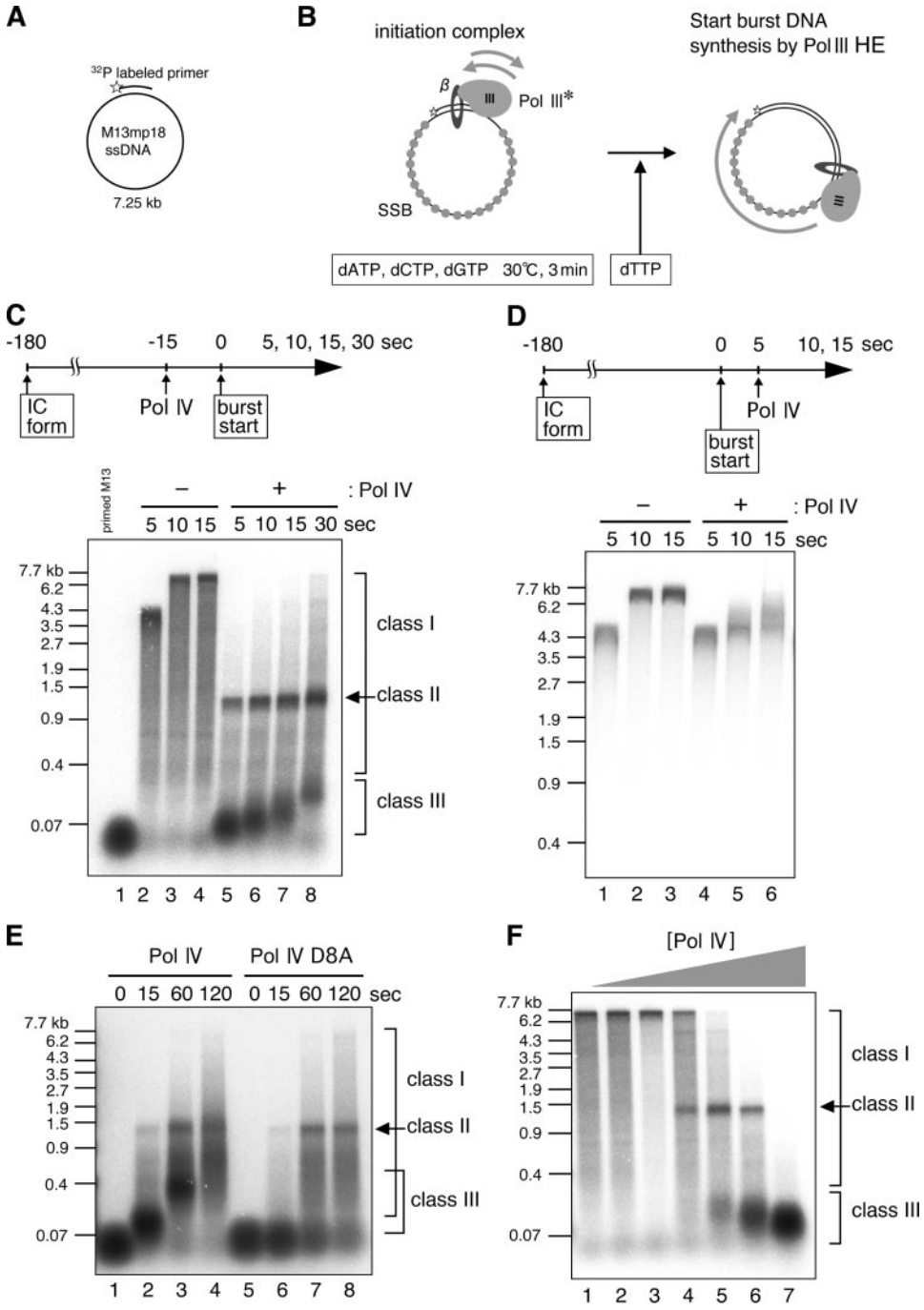

FIGURE 1. **Effects of pol IV on pol III burst DNA synthesis on a primed M13 ssDNA template.** *A*, M13 primer/template DNA. A 7.25-kb M13mp18 closed circular ssDNA template was annealed to a 5′-^{32}P-labeled 25-mer primer. *B*, a sketch showing the steps in the pol III burst DNA synthesis assay. A pol III IC is preassembled on the primer/template DNA and is incubated for 3 min in the presence of dATP, dCTP, and dGTP. On the addition of dTTP, pol III HE initiates burst DNA synthesis. *C*, *upper panel*, a sketch depicting the temporal series of events in the burst synthesis assay. Reactions were carried out as described under "Experimental Procedures." Briefly, pol III*, SSB, β clamp, and primed M13mp18 ssDNA were preincubated at 30 °C for 3 min to form the IC. pol IV (0.27 μg, final concentration of 220 nM at the start of DNA synthesis) was added 15 s before the addition of dTTP. Following the initiation of DNA synthesis, the reaction was incubated for the times shown above the *horizontal arrow*. *Lower panel*, replication products in the absence (*lanes 2–4*) or presence of pol IV (*lanes 5–8*) at the indicated times after the addition of dTTP are shown. The non-extended primer is shown as a control (*lane 1*). *D*, the experimental scheme is illustrated in the *upper panel*. Replication products at the indicated times (5, 10, and 15 s) after the addition of dTTP are shown. pol IV was added (to a final concentration of 890 nM) to the reaction 5 s after the start of DNA synthesis (*lanes 5* and *6*). The reaction was carried out with unlabeled primer/template DNA, as described under "Experimental Procedures." *E*, replication products at the indicated times after the addition of dTTP are shown. Assays were carried out as in *C* with 0.53 μg of wild-type pol IV (final concentration of 450 nM at the start of DNA synthesis) (*lanes 1–4*) or the same amount of pol IV D8A (*lanes 5–8*). *F*, replication products 15 s after the addition of dTTP are shown. Assays were carried out as in *C*, with 0, 0.03, 0.05, 0.11, 0.27, 0.53, or 1.1 μg of pol IV (final concentrations of 0, 22, 45, 89, 220, 450, or 890 nM, respectively, at the start of DNA synthesis) added to the reaction 15 s before the addition of dTTP.

centration of pol IV in the burst DNA synthesis reaction decreased the amount of full-length products, whereas the amount of class III products increased. A 50% decrease of full-length products was achieved with 89 nM pol IV (Fig. 1*F*, *lane 4*), representing a ~100-fold molar excess of pol IV over pol III HE in the IC. At 890 nM pol IV (Fig. 1*F*, *lane 7*), there were virtually no class I or class II products, indicating that almost all the primer DNA was extended into class III products by pol IV. It seems likely that at lower concentrations, pol IV rapidly releases the primer terminus during preincubation so that pol III* can return to it, and that much higher concentrations are needed for pol IV to occupy the primer exclusively.

Mass Action-mediated Exchange from pol IV to pol III during DNA Synthesis*—As shown in Fig. 1, *C* and *E*, after the start of DNA synthesis, class III products were extended by pol IV, but their total amount decreased with incubation time. These products were slowly converted to class I and II products, showing that pol III* can recapture the primer from the pol IV-β clamp complex after DNA synthesis has resumed. This is a slower reaction than the first switch from pol III* to pol IV. About 30% of the primers remained occupied by pol IV even after 60 s (Fig. 1*E*, *lane 7*, compare the amount of non-extended primer with that of class I and II products).

On the other hand, we observed a rapid polymerase switch from a moving pol IV to pol III* with increasing amounts of pol III* (Fig. 2). First, pol IV was added to the pol III IC to take over the primer from pol III*, and then increasing amounts of pol III* were added to the reaction coinciding with the start of DNA synthesis. Increasing the levels of pol III* did not significantly affect the pol IV-dependent reduction in the elongation speed of pol III HE, nor did it alter the pausing site in class II products. However, an increase in pol III* levels resulted in a reduction in the amount of class III products and a concomitant increase in the amount of class I and II products, indicating that the polymerase switch from moving pol IV to pol III* occurred immediately and depended on the concentration of pol III*. This result has two consequences. First, the inhibitory effects of pol IV on the elongation reaction of pol III and the polymerase switch by pol IV observed in Figs. 1 and 2 appear to be caused by different mechanisms; second, a rapid polymerase exchange from pol IV to free pol III*, and not to pol III* on the β clamp, occurs during DNA synthesis, with the relative amounts of each free polymerase determining the primer occupancy in accord with mass action.

pol IV Causes pol III to Dissociate from Primer/Template DNA*—There are two apparent explanations for the results described above. One is that the two polymerases dissociate both rapidly and "spontaneously" from the primer/template DNA and that a rapid exchange occurs between the free polymerases. However, this possibility would seem to be ruled out by numerous previous studies showing that pol III HE is highly processive and that a single pol III* remains bound to the β clamp for more than 5 min (15, 19). Although pol IV is far less processive than pol III HE, pol IV has been estimated to bind to the β clamp for ~1–2 min (18), which is much longer than the switching time observed here (<15 s). The second possibility is that a new polymerase might gain access to a primer terminus and β clamp, even if the first polymerase remains bound, and

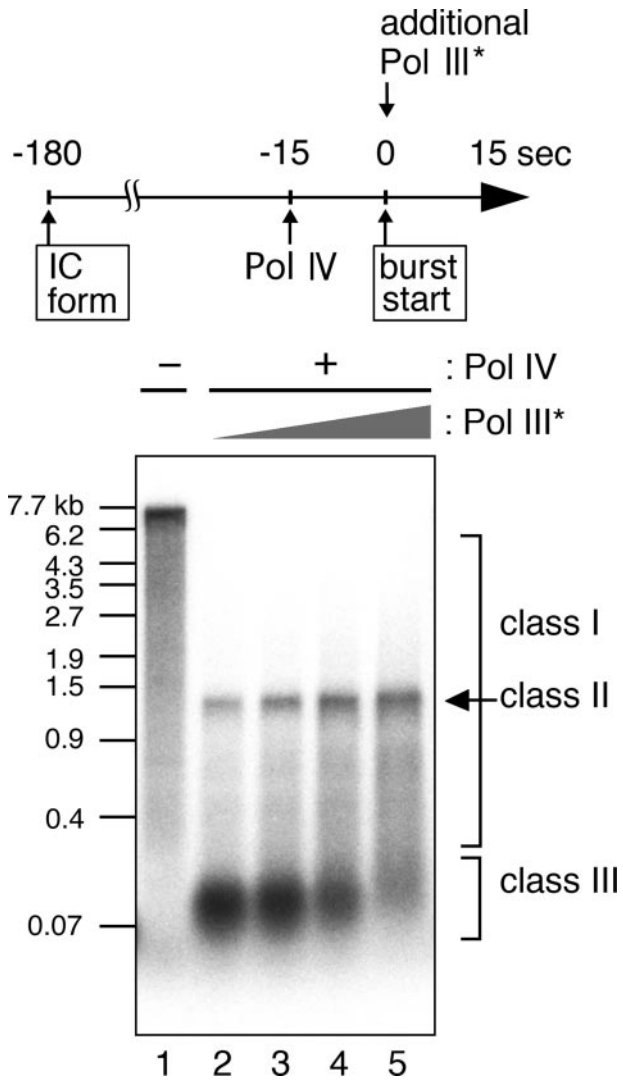

FIGURE 2. **Rapid polymerase switching from pol IV to pol III* during DNA synthesis is pol III* concentration-dependent.** The experimental scheme is illustrated in the *upper panel*. After the formation of the IC, 0.53 μg of pol IV (final concentration of 450 nM) was added to the reaction 15 s before the addition of dTTP. Increasing amounts of pol III* (0, 20, 60, or 180 units) were added at the start of DNA synthesis, and replication products formed after a 15-s incubation are shown in *lanes 2–5*. A reaction without pol IV or additional pol III* was carried out as a control (*lane 1*).

take over the primer from the first polymerase. If pol IV is already bound to the β clamp, it is possible that a second polymerase might bind to the β clamp and the primer terminus because, as shown in a previous structural study (20), pol IV can "angle off" the side of the β clamp, away from the primer terminus, perhaps to allow room for a second polymerase. If, conversely, pol III* binds first to the β clamp and holds the primer terminus, such a conformational change would presumably not be possible, although pol IV might be able to act proactively to promote the dissociation of pol III* from the β clamp and thereby take over the 3′-primer end from pol III HE.

To examine this "proactive removal" idea, we determined whether pol III* dissociates from primer/template DNA when pol IV is added. It has been reported that the pol III IC is stable enough to be isolated by sedimentation through a glycerol gradient (13). pol III*, the β clamp, SSB, and the template DNA

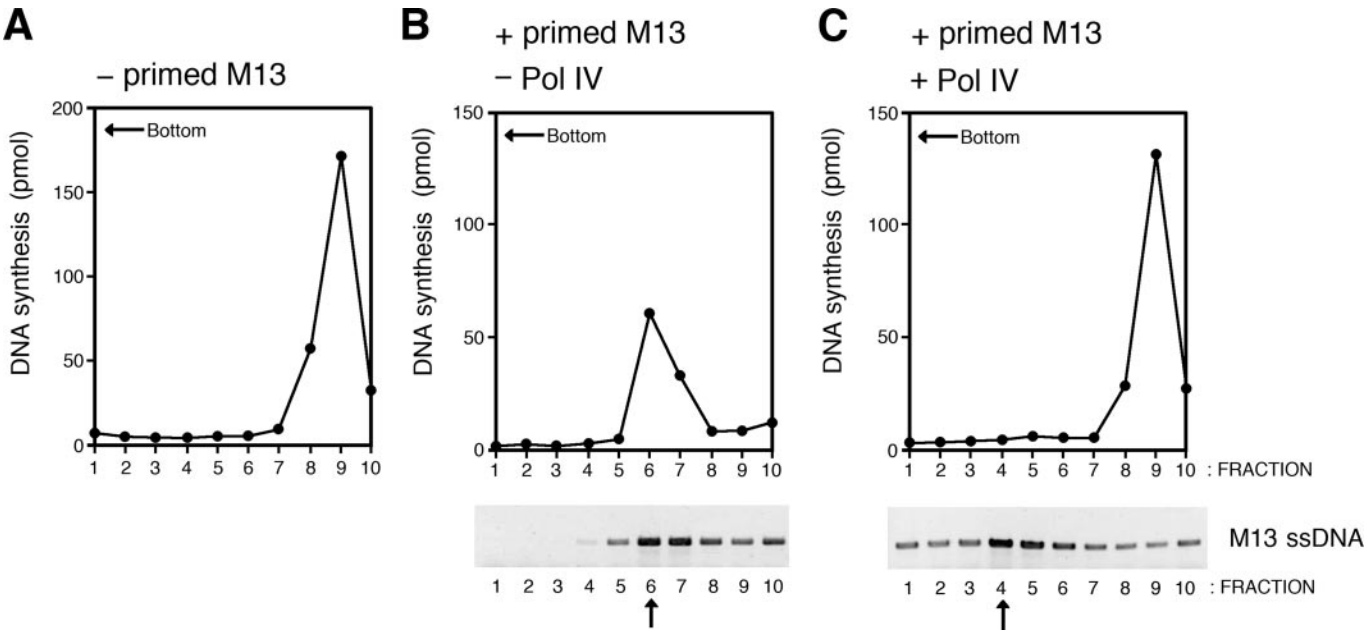

FIGURE 3. **pol IV promotes pol III* dissociation from primer/template DNA.** Isolation of initiation complexes by sedimentation through glycerol gradients. The IC was formed as described under "Experimental Procedures," except that the reaction was scaled up 2.7-fold (to 80 μl), and the products were sedimented in a glycerol gradient and then fractionated. Polymerase activity in each fraction was measured (*upper panels*). *Arrows* in the *lower panels* indicate the position of the primed template M13mp18 (indicated by *primed M13*) ssDNA peak. See "Experimental Procedures" for details. *A*, a reaction without primed M13mp18 ssDNA was carried out as a control to determine the position of free proteins. *B*, the reaction was carried out with primed M13mp18 ssDNA. *C*, pol IV D8A was added to the reaction to a final concentration of 890 nM just before sedimentation.

were preincubated to form an IC, and then the reaction mixture was loaded onto a glycerol gradient, centrifuged for 75 min, and fractionated. The peak of polymerase activity was detected in fraction 6, coincident with the template DNA peak (Fig. 3*B*), whereas free pol III* was detected in the top fractions (Fig. 3*A*, *peak* in *fraction 9*). In contrast, after the formation of a pol III IC, when we added a polymerase-null mutant pol IV D8A (890 nM) to the reaction, just before centrifugation, the peak of polymerase activity (*i.e.* the peak of pol III*) was detected in fraction 9, similar to free protein, whereas the template DNA peak was located in fraction 4 (Fig. 3*C*). This indicates that the pol III IC was disrupted and that pol III was actively displaced from the template DNA by pol IV. In Fig. 3*C*, the addition of pol IV D8A shifted the template DNA peak to a position nearer the bottom of the gradient (fraction 4) when compared with the control reaction (Fig. 3*B*, *fraction 6*), and a significant amount of pol IV D8A was detected in that fraction (see supplemental Fig. S1*B*). Therefore, it is possible that pol IV binds to the SSB-coated ssDNA.

Based on the burst DNA synthesis assay, we designed an experiment (Fig. 4*A*) to see whether pol III* dissociates from the primer/template DNA on the addition of pol IV, during preincubation, in the same reaction shown in Figs. 1 and 2, except that the IC was preformed on the primer/template (M13 ssDNA primed with unlabeled primer) in the presence of dATP, dCTP, and α-^{32}P-dTTP. After the formation of the pol III IC, an excess of the synthetic homopolymer poly(dA)/oligo(dT) was added to the reaction. If pol III* dissociates from primed M13 ssDNA, it should be captured by poly(dA)/oligo(dT) and detected as DNA synthesis because only dTTP is needed to synthesize DNA on poly(dA)/oligo(dT), whereas all four dNTPs are needed to replicate M13 ssDNA. We added a

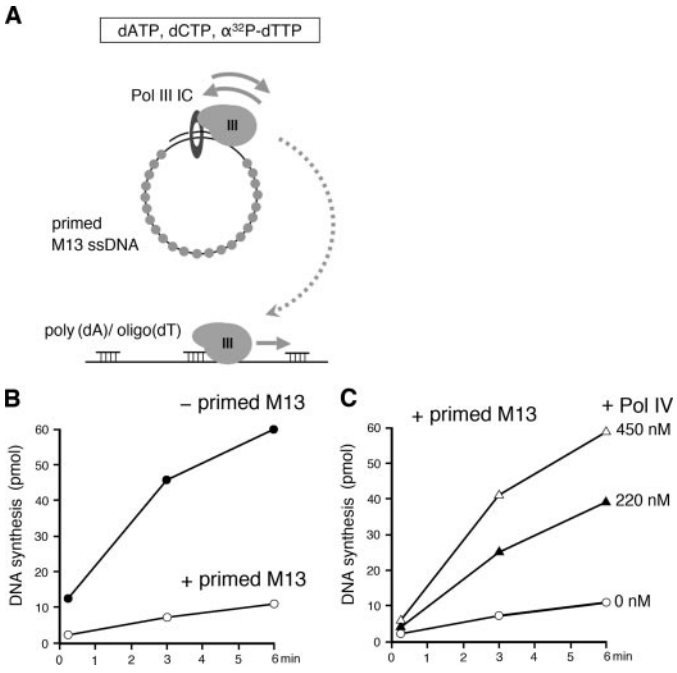

FIGURE 4. **pol IV disrupts a stable IC and promotes dissociation of pol III*.** *A*, scheme of the IC disruption assay. The IC was allowed to form, and then excess poly(dA)/oligo(dT) was added to the reaction. pol III* on primed M13mp18 ssDNA cannot start DNA synthesis with three nucleotides; however, if pol III* dissociates, it will be captured by poly(dA)/oligo(dT), on which it can synthesize DNA with only dTTP. *B*, pol III* and other proteins were preincubated with (*open circles*) or without (*filled circles*) primed M13mp18 (indicated by *primed M13*) ssDNA. After a 3-min preincubation, poly(dA)/oligo(dT) was added to the reaction. After 15-s, 3-min, and 6-min incubations, the amount of incorporated nucleotides in the reaction was quantified. See "Experimental Procedures" for details. *C*, IC disruption assays were carried out at final concentrations of 0 (*open circles*), 220 (*filled triangles*), and 450 nM (*open triangles*) pol IV D8A. The mutant pol IV was added to the IC 15 s before the addition of poly(dA)/oligo(dT). Time courses of nucleotide incorporation during the reactions are shown.

smaller amount of pol III* than primed M13 ssDNA, preincubated the mixture for 3 min to form the IC, and then added an excess amount of poly(dA)/oligo(dT) to the reaction. Incubations were performed for 15 s, 3 min, and 6 min, and dTTP incorporation into DNA was measured. When compared with the control reaction with free pol III* only, DNA synthesis was reduced substantially when the IC was preformed on the primed M13 ssDNA (Fig. 4*B*, compare *closed* and *open circles*). This shows that the pol III IC on primed M13 ssDNA cannot use poly(dA)/oligo(dT) as a template and that the pol III IC is stable for at least 6 min.

Since pol IV inhibited burst DNA synthesis on primed M13 ssDNA by pol III*, we then assessed whether pol III* can synthesize DNA on poly(dA)/oligo(dT) in the presence of pol IV. We used pol IV D8A to ensure that the DNA synthesis in the reaction was due to pol III*. When compared with its effect on burst DNA synthesis, pol IV D8A had little effect on DNA synthesis on poly(dA)/oligo(dT) (compare supplemental Fig. S2, *A* and *B*). pol IV D8A had no effect at 220 nM, whereas 450 nM pol IV D8A slightly reduced DNA synthesis on poly(dA)/oligo(dT). We speculate that the effect of pol IV is much weaker when pol III* performs distributive DNA synthesis on poly(dA)/oligo(dT) than when it performs processive DNA synthesis on primed M13 ssDNA because pol III* will bind easily to another primer on the homopolymer.

We examined whether pol IV promotes pol III* dissociation in this assay. After the formation of the IC, pol IV D8A was added to the reaction 15 s before the addition of poly(dA)/oligo(dT). The addition of the inactive pol IV mutant caused a marked increase in DNA synthesis, dependent on the pol IV D8A concentration (Fig. 4*C*), showing that the amount of free pol III* in the reaction increased on the addition of pol IV D8A and further confirming that pol IV promotes the dissociation of pol III* from primed M13 ssDNA. At 450 nM pol IV D8A, the amount of nucleotide incorporated within a 6-min incubation was approximately equal to that in the free pol III* control, indicating that the majority of pol III* was dissociated (compare Fig. 4*B*, *closed circles*, and 4*C*, *open triangles*).

pol IV Rapidly Obstructs the Interaction between pol III and the β Clamp*—The first polymerase switching reaction from idling pol III* to pol IV occurs rapidly (within ~15 s; Fig. 1). If pol IV promotes dissociation of pol III* when it takes over the primer, the dissociation should occur within the same time period. To test this prediction, we evaluated the effect of varying the preincubation time after the addition of pol IV D8A on the amount of dissociated pol III*. After the formation of the pol III IC, pol IV D8A (450 nM) was added to the reaction. The mixture was preincubated for varying times in the range of 0–120 s, and the amount of incorporated nucleotide within a fixed time of incubation (30 s) was measured (Fig. 5). When compared with the reaction without preincubation (0 s), the amount of product of dissociated pol III* increased when the reaction was preincubated for 15 s. The amount of product of dissociated pol III* remained almost constant between 15 and 120 s of incubation, indicating that pol III* was released from the template DNA within the first 15 s after the addition of pol IV. Because longer incubation does not cause an increase of free pol III*, we assumed that the amount of free pol III* was in a

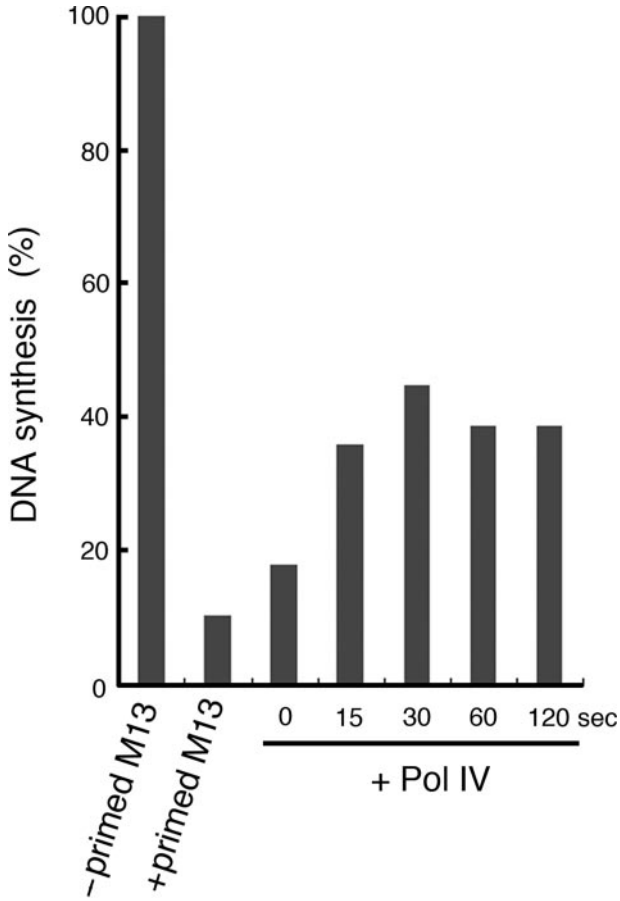

FIGURE 5. **pol III* dissociates immediately after the addition of pol IV.** IC disruption assays were carried out at a final concentration of 450 nM pol IV D8A; the preincubation times for the IC and mutant pol IV before the addition of poly(dA)/oligo(dT) were 0, 15, 30, 60, and 120 s. After the addition of poly(dA)/oligo(dT), the amount of nucleotide incorporation during a 30-s incubation was quantified. The percentages of DNA synthesis relative to the positive control (indicated by − *primed M13*; 100%) were calculated. The control assays indicated by − *primed M13* and + *primed M13* were carried out as described in the legend for Fig. 4*B*.

balance determined by the pol IV concentration; the dissociated pol III* might be able to quickly rebind to the β clamp on the primer/template DNA. This hypothesis appears consistent with the results in Figs. 1 and 2, which suggest that the β clamp remains bound to the template DNA when pol IV takes over the primer from pol III*. These results suggest that pol IV rapidly obstructs the interaction between pol III* and the β clamp on the primer/template DNA, causing the removal of pol III*, and that a rapid exchange between free and bound polymerases then ensues.

pol IV Directly Promotes pol III Release from the β Clamp*—It has been reported that pol III* and pol IV each have the ability to bind to the β clamp. We therefore tested whether pol IV acts on the β clamp to promote release of pol III* or on pol III* directly. pol IV binds to the β clamp via a C-terminal interaction domain, and a pol IV mutant lacking a conserved motif in the five C-terminal amino acids fails to interact with the β clamp (21). Thus, we made a series of polymerase-null pol IV mutants and evaluated their ability to promote dissociation of pol III* (Fig. 6). At 220 nM, as shown above, full-length pol IV D8A removed pol III*, whereas deletion of the five C-terminal

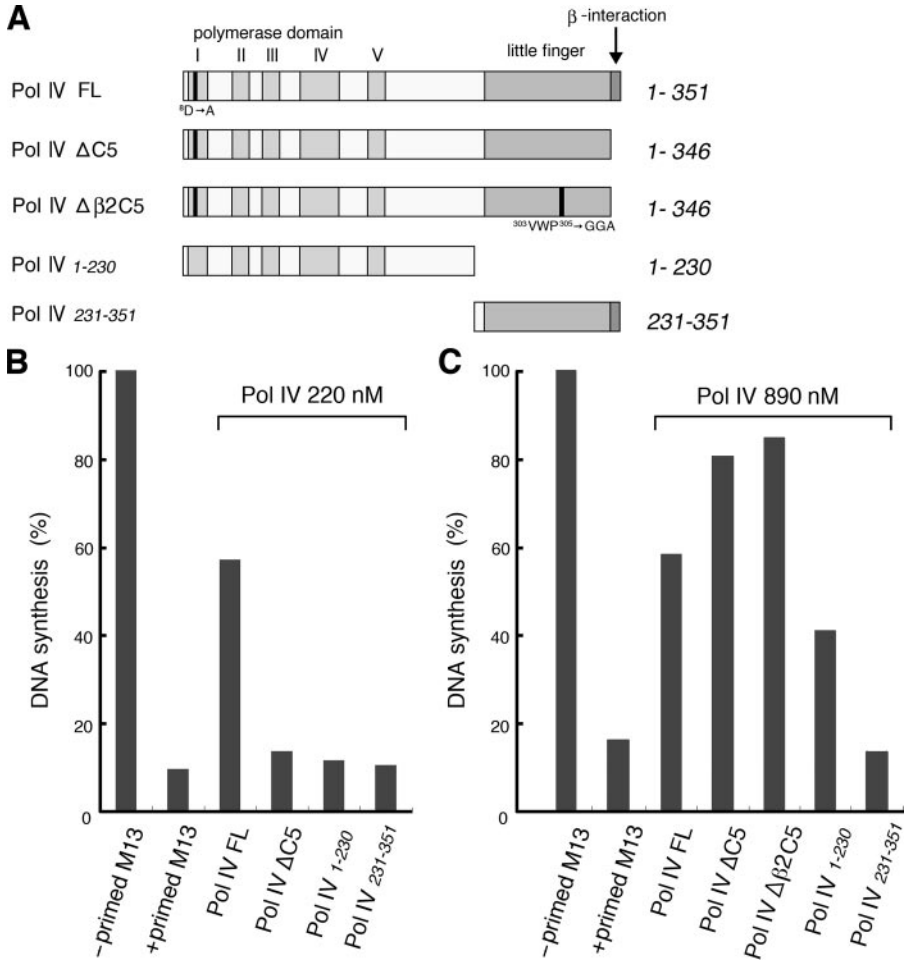

FIGURE 6. **Effects of deletion mutations in pol IV on pol III-catalyzed DNA synthesis in the IC disruption assay.** *A*, schematic representation of His-tagged pol IV mutants. *B*, IC disruption assays were carried out at a final concentration of 220 nM of full-length pol IV D8A (*pol IV FL*), pol IV D8A with the C-terminal five amino acids deleted (*pol IV ΔC5*), the N-terminal portion of pol IV (residues 1–230) (*pol IV 1–230*), and the C-terminal portion of pol IV (residues 231–351) (*pol IV 231–351*). After the addition of poly(dA)/oligo(dT), the amount of nucleotide incorporation during a 180-s incubation was quantified as in Fig. 5. *C*, the experiment was carried out as described in *B* with 890 nM pol IV FL, pol IV ΔC5, pol IV Δβ2C5 (pol IV ΔC5 residues ^{303}VWP^{305} changed to ^{303}GGA^{305}), pol IV 1–230, and pol IV 231–351, respectively. The amount of nucleotide incorporation during a 180-s incubation was quantified.

residues in pol IV D8A caused a marked decrease in the ability of pol IV to promote the dissociation of pol III* (Fig. 6*B*, compare *pol IV FL* and *pol IV ΔC5*). pol IV *1–230* (the N-terminal two-thirds of pol IV) and pol IV *231–351* (the C-terminal one-third of pol IV, containing the little finger domain with two β clamp-interacting regions) failed to cause pol III* dissociation at 220 nM. These results demonstrate that an interaction between pol IV and the β clamp is effective in displacing pol III* but that binding of the little finger domain to the β clamp is insufficient to promote pol III* dissociation.

On the other hand, we observed that pol IV D8A ΔC5 strongly promoted pol III*-catalyzed DNA synthesis at 890 nM (Fig. 6*C*), suggesting that pol IV can act directly on pol III*, causing it to dissociate without the aid of the β clamp at high pol IV concentration. Because there may be an additional binding site (^{303}VWP^{305}) in the little finger domain of pol IV that interacts with the side of the β clamp (20), it is possible that pol IV gains access to the β clamp only via the second site. However, another β interaction-deficient pol IV changed hydrophobic residues ^{303}VWP^{305} of pol IV D8A ΔC5 to ^{303}GGA^{305} (Fig. 6*C*, labeled as *Pol IV Δβ2C5*) showed a pol III*-releasing activity comparable with that observed with pol IV FL and pol IV ΔC5 at 890 nM. In addition, there was a considerable increase in the amount of nucleotide incorporation with pol IV *1–230*, whereas no measurable increase was observed with pol IV *231–351* at 890 nM. These results suggest that the pol III*-releasing activity of pol IV resides mainly in its N-terminal portion rather than in the C-terminal β-binding regions.

From the above results, we propose that the replacement of pol III* on the β clamp with pol IV is mediated by two processes, an interaction between pol IV and the β clamp (Fig. 6*B*) and a separate interaction between pol IV and pol III* (Fig. 6*C*). Both interactions are needed to drive a rapid release of pol III*, and it is possible, perhaps likely, that interactions involving the two polymerases are facilitated by an anchoring of the two enzymes in close proximity when bound simultaneously, albeit transiently, to the β clamp.

DISCUSSION

We examined the influence of pol IV on replication by pol III HE in a burst DNA synthesis assay to gain a clearer perspective on how pol IV replaces pol III* on the β clamp and takes over DNA synthesis. Our data provide clear evidence for a polymerase switch between pol III* and pol IV. We observed that pol IV rapidly takes over the primer terminus from a pol III HE that is stalled in an idled IC due to omission of a single dNTP, confirming a previous report (19). Our data suggest that pol III* dissociates rapidly from the β clamp on the primer/template DNA at roughly the same time that pol IV takes over the primer. A potentially important new aspect of the data is that the displacement of pol III* appears to be caused by the ability of pol IV to obstruct the interaction between pol III* and the β clamp. When the replication fork encounters a lesion in the leading strand template, pol III* is thought to remain bound to the β clamp and stalls at the lesion site. A "toolbelt" model has been proposed to account for how a TLS polymerase is able to access the primer terminus, whereas a stalled pol III* remains attached to the β clamp (19, 22). In this model, the TLS polymerase simultaneously associates with the β clamp bound to pol III*, and a polymerase switch then occurs within the double polymer-

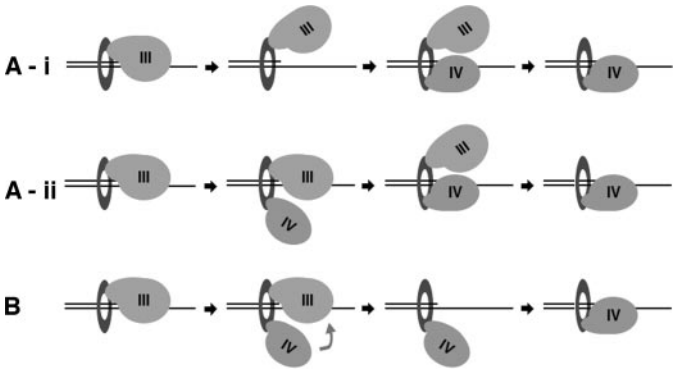

FIGURE 7. **Alternative molecular pathways regulating the replacement of pol III* by pol IV.** Our data show that when pol IV takes over a primer 3′-end from pol III IC, pol III* dissociates rapidly from the β clamp. *A*, the switching reaction occurs first resulting in the subsequent dissociation of pol III*. *Row i*, pol III* bound to the β clamp releases the primer terminus frequently, thereby allowing pol IV to bind to the β clamp and gain direct access to a 3′-primer end. *Row ii*, pol IV first binds to the vacant side of the β clamp, without access to the 3′-primer end. pol IV then changes its conformation to access the primer terminus and pol III* is displaced. *B*, pol IV acts directly on pol III* to cause its release. pol IV first binds to the vacant side of the β clamp and then removes pol III* to allow access to a 3′-primer end, and then binds to the primer terminus. Each of the pathways is expanded upon under "Discussion."

ase complex to bypass the lesion. After translesion synthesis is complete, the bound pol III* returns to the primer terminus to resume processive DNA synthesis. A biochemical mechanism to account for a relatively lengthy residence time for two polymerases bound to the β clamp, switching from one to the other to access and release the 3′-primer end, has not yet been established.

Our data suggest a possible alternative scheme in which a distinct polymerase switch occurs, resulting in a rapid dissociation of pol III* from the IC engendered by a direct interaction with pol IV. The β interaction-deficient pol IV mutant displayed a markedly reduced but residual ability to detach pol III* (Fig. 6). We posit that when pol IV binds to the β clamp, it obstructs the interaction between pol III* and the β clamp and actively facilitates polymerase exchange.

Additional work will be needed to substantiate and clarify the relationship between polymerase switching and pol III* dissociation. Although both events occur rapidly, within approximately the same time period after the addition of pol IV, we cannot say which happens first. In the model-based sketch (Fig. 7*A*), we have assumed that switching precedes "forced" pol III* dissociation. However, that raises the issue of how pol IV gains access to the 3′-primer end, whereas pol III* remains bound to the β clamp. One possibility is that pol III* bound to the β clamp releases the primer terminus frequently during preincubation, enabling pol IV to bind (Fig. 7*A*, *row i*). Because pol IV can interact with a unique site on the side of the β clamp, which is not used by pol III α subunit (20, 23), another possibility is that pol IV binds to one side of the β clamp, initially without access to the primer terminus to which pol III* remains bound. A subsequent conformational change in pol IV may facilitate pol III* displacement, enabling pol IV to gain access to the primer terminus (Fig. 7*A*, *row ii*). In this picture, binding of pol IV to the primer terminus and to one of the common binding sites of the β clamp might weaken the interaction between pol III core and the β clamp, resulting in pol III* dissociation. On the other hand, we found that a pol IV mutant lacking two interaction sites with the β clamp promotes dissociation of pol III* (Fig. 6*C*), although a high concentration of the pol IV mutant was required to cause pol III* dissociation. It is possible that pol IV acts directly on pol III* to force it to dissociate. If true, however, how does pol IV obstruct the interaction between pol III* and the β clamp so quickly? Since pol III core readily dissociates from the β clamp to allow rapid polymerase recycling in Okazaki fragment synthesis (24–26), one possibility is that pol IV can take advantage of the dissociation mechanism used by pol III* itself for recycling. In Fig. 7*B*, we proffer an alternative picture in which pol IV first binds to the side of the β clamp and then removes pol III*, thereby allowing pol IV to bind at the 3′-primer end.

Once pol III* has dissociated from the β clamp, pol III* and pol IV are able to exchange rapidly enough so that the primer occupancy is determined by random binding of each polymerase, *i.e.* by the relative concentration of free polymerases, in apparent accord with mass action. A rapid polymerase switching at the primer terminus occurs repeatedly in both directions, pol III* to pol IV and *vice versa*. pol III* rapidly regains a 3′-primer end undergoing elongation by pol IV (Fig. 2), with the rate of takeover by pol III* dependent on the concentration of pol IV (compare Fig. 1*E* with Fig. 2). Since it appears that pol IV dissociates rapidly from the primer and that continuous switching is needed for pol IV to saturate primer ends at high pol IV levels, it will be important to determine whether pol IV dissociation is predominantly spontaneous or whether it is influenced by direct interaction with free pol IV or pol III* molecules. This is a salient issue since our data imply an "active" removal of pol III* by pol IV, so one can ask whether the reverse might also be true. In addition, to what extent might pol III* exchange be rapid or slow, spontaneous or induced in the presence of pols I, II, and V?

A type of "dynamic" polymerase processivity, reported by Benkovic and colleagues (27), occurs when T4 gp43 undergoes rapid replacement (<1 min) on the gp45 clamp by an inactive gp43 mutant polymerase. They showed that an active polymerase exchange process takes place, without disrupting continued DNA synthesis, and that it requires a specific protein-protein interaction between the C terminus of gp43 polymerase and the gp45 clamp. Our data suggest that pol III* and pol IV might exchange in a similar way during preincubation. Consistent with this idea is that overexpression of pol IV has been reported to cause an increase in spontaneous mutagenesis, suggesting that pol IV can access a primer terminus during normal DNA synthesis, competing with pol III HE (14, 28). Using our system, we wanted to determine whether pol III* and pol IV exchange rapidly during DNA synthesis. Although we showed that pol IV removes "idling" pol III* and takes over the primer when one dNTP substrate is omitted from the reaction, we were uncertain as to whether pol IV could likewise act on moving pol III HE during DNA synthesis. We observed a reduction in the elongation speed of pol III HE in the presence of pol IV (Figs. 1 and 2, class I products), suggesting that pol IV indeed inhibits moving pol III HE; moreover, we observed a clear inhibition of the movement of pol III HE when pol IV was added after the start of DNA synthesis (Fig. 1*D*). However, it is not yet clear

whether the switch from pol III* to pol IV slows down elongation or whether binding of pol IV to the template ssDNA impedes the movement of pol III HE.

Do the effects of pol IV on pol III HE observed *in vitro* also occur *in vivo*? pol IV is relatively abundant in normal cells (250 molecules/cell) (29) when compared with pol III* (10–20 molecules/cell) and when compared with pol III HE at the replication fork (2–6 molecules/cell). pol IV is up-regulated in SOS-induced cells to 2,500 molecules/cell, whereas pol III* levels remain unchanged. Considering that both effects of pol IV (the polymerase switching and pol III* dissociation) could be clearly observed at a pol IV:pol III HE concentration ratio similar to that found *in vivo* (220 nM pol IV to 0.5–1 nM pol III IC in our system; Figs. 1*C* and 4*C*), it is possible that pol IV affects pol III HE at the replication fork *in vivo*. However, if pol IV frequently displaces the pol III core from the β clamp during DNA replication, this might cause a collapse of the replication fork or at least an increase of ssDNA gaps on both strands, resulting in SOS induction and a growth defect in the cell. We therefore hypothesize that there might be a regulatory mechanism to prevent pol IV from continually inhibiting DNA replication. If this is true, however, why does pol IV possess an activity that enables it to inhibit pol III HE?

As described earlier, a toolbelt model has been formulated to explain how a TLS polymerase accesses the primer terminus. In the model, the same pol III* on the β clamp returns to the primer terminus after TLS so that an intact replication fork can await resumption of DNA synthesis until TLS is completed. Although we do not know yet whether pol IV affects pol III HE *in vivo*, we suggest that if pol III* rapidly dissociates from the lesion site, leaving a damaged nucleotide-containing ssDNA gap with the β clamp behind it, then pol III* might be able to resume synthesis more rapidly. The ssDNA gap would then be filled by pol IV, by other polymerases such as pol V or pol II, or even by pol III*. Consistent with this speculation, it has been shown that a replication fork keeps moving even when a lesion exists on both strands *in vivo*, leaving an ssDNA gap behind it (30, 31). Heller and Marians showed (32) that the primase bound to DnaB helicase on the lagging strand can prime on the leading strand *in vitro*, implying that the replication fork restarts on the leading strand. We think it is possible that the interaction between the pol III core and the β clamp might be mediated by pol IV so as to ensure that fork movement proceeds with minimal interruption.

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