The β clamp targets DNA polymerase IV to DNA and strongly increases its processivity

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The recent discovery of a new family of ubiquitous DNA polymerases involved in translesion synthesis has shed new light onto the biochemical basis of mutagenesis. Among these polymerases, the *dinB* gene product (Pol IV) is involved in mutagenesis in *Escherichia coli*. We show here that the activity of native Pol IV is drastically modified upon interaction with the β subunit, the processivity factor of DNA Pol III. In the absence of the β subunit Pol IV is strictly distributive and no stable complex between Pol IV and DNA could be detected. In contrast, the β clamp allows Pol IV to form a stable initiation complex ($t_{1/2} \approx 2.3$ min), which leads to a dramatic increase in the processivity of Pol IV reaching an average of 300–400 nucleotides. *In vivo*, the β processivity subunit may target DNA Pol IV to its substrate, generating synthesis tracks much longer than previously thought.

INTRODUCTION

Point mutations, a source of genetic instability, may arise spontaneously or as a consequence of endogenous damage or xenobiotic exposure and imply generally inaccurate replication events. Significant advances in the field of mutagenesis have recently been achieved with the discovery of a vast family of novel DNA polymerases specialized in translesional DNA synthesis in both prokaryotic and eukaryotic organisms (reviewed in Friedberg *et al.*, 2000).

In *Escherichia coli*, the derepression of the SOS system, a coordinated cellular response to adverse conditions, leads to the elevated expression of three DNA polymerases, namely DNA Pol II (*polB*; Bonner *et al.*, 1988), DNA Pol IV (*dinB*; Wagner *et al.*, 1999) and DNA Pol V (*umuDC*; Bacher-Reuven *et al.*, 1999; Tang *et al.*, 1999). Together with the RecA protein, the *umuDC* gene products have been known for a long time to be essential

for mutagenesis triggered by UV light and abasic lesions (Friedberg *et al.*, 1995). On the other hand, the *dinB* gene product (Pol IV) is known to be involved in untargeted mutagenesis of phage λ in a RecA-independent pathway (Brotcorne-Lannoye and Maenhaut-Michel, 1986; Kim *et al.*, 1997) while the role of DNA Pol II in mutagenesis has remained undetermined. However, Napolitano *et al.* (2000) have recently shown that all three SOS-induced DNA polymerases are actually involved in lesion-induced (i.e. targeted) mutagenesis in *E. coli*.

Both DNA Pol IV and V belong to the aforementioned family of novel DNA polymerases (the so-called UmuC/DinB superfamily) and share with their homologues a low processivity as well as the lack of 3' to 5' exonuclease (proofreading) activity. Together with the *in vitro* demonstrations that these polymerases possess a clear propensity to elongate distorted primer/template (P/T) structures (reviewed in Friedberg *et al.*, 2000), these findings led to a new model for mutagenesis called the DNA polymerase switch model (Cordonnier and Fuchs, 1999). Briefly, translesional DNA polymerases are thought to be temporarily recruited at the site where the replisome has stalled (or dissociated), thus allowing bypass to occur before highly accurate and processive synthesis resumes by means of the replicative polymerase. Although this model appears attractive, many points remain to be established. Among them are the factors allowing the targeting of these specialized polymerases to their substrates, as well as the length of the polymerization track these enzymes will generate before the replicative machinery takes over and resumes processive synthesis. Importantly, as most of these polymerases lack proofreading activity it has been argued that the polymerization track generated by these enzymes *in vivo* should be as short as possible in order to avoid high rates of replication errors. This hypothesis is actually supported by the low processivity of all these novel DNA polymerases as

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exemplified by the strict distributivity of DNA Pol IV *in vitro* (Wagner *et al.*, 1999).

Once loaded onto DNA by the multi-subunit γ complex, the so-called β clamp is topologically linked to DNA and confers high processivity to the replicative DNA Pol III. In the present paper, we describe the effects of the β subunit on native Pol IV activity. In the absence of the β subunit, Pol IV is strictly distributive and no stable complex between Pol IV and DNA could be detected. In contrast, when loaded onto DNA the β clamp allows Pol IV to form a stable initiation complex ($t_{1/2} \approx 2.3$ min), which results in a dramatic increase in the processivity of Pol IV, reaching an average of 300–400 nucleotides (nt). The significance of these results is discussed within the DNA polymerase switch model for translesion synthesis.

RESULTS

We previously purified and analysed the activity of a histidine-tagged version of DNA Pol IV and demonstrated its strict distributive mode of replication (i.e. only one nucleotide is incorporated per substrate binding event) as well as its capacity to elongate mismatched base pairs through the generation of frameshift mutation intermediates (Wagner *et al.*, 1999). More recently, these properties were also found with the native form of DNA Pol IV, which has been used throughout the present study.

The β clamp targets DNA Pol IV to the P/T terminus

During attempts to determine the kinetic parameters of the native DNA Pol IV, we were unable to detect any stable Pol IV–DNA complex (see below). This finding indicates a very low affinity of Pol IV to P/T junctions and, consequently, suggests the need for this enzyme to be targeted to its DNA substrate by a co-factor. As the β clamp is associated with the replication complex (through direct interaction with the catalytic α subunit of Pol III; Kornberg and Baker, 1992) and is also known to interact with another DNA polymerase (DNA Pol II; Bonner *et al.*, 1992), we hypothesized that β is a good candidate for such a targeting factor. We tested this hypothesis by implementing the following trap assay (Figure 1A). The DNA substrate is obtained by annealing a 5'-radiolabelled 30mer primer (P*) to a 90mer template (T). This substrate (P*/T) contains 5' and 3' ssDNA overhangs 25 and 35 nt long, respectively. These overhangs allow stable loading of the β clamp in the presence of single-stranded binding protein (SSB) (Bloom *et al.*, 1997). Pol IV is pre-incubated with P*/T followed by simultaneous addition of a large excess of cold trap DNA and the dNTP required for a single addition event (dTTP). The reaction is quenched by EDTA after 7 s of incubation, and elongation of the radiolabelled primer is monitored by gel electrophoresis. With naked or SSB-coated P*/T, in the presence of trap DNA (Figure 1A, TA lanes), no primer elongation is seen, while in the absence of trap (Figure 1A, NT lanes) ~50% of the P* primer is elongated under the reaction conditions. The lack of primer elongation when trap DNA and dTTP are added simultaneously shows clearly the absence of a stable complex between Pol IV and the radiolabelled P*/T. In contrast, when the β clamp is loaded by the γ complex onto SSB-coated substrate, addition of trap DNA at the same time as dTTP does not prevent ~20% of the primer being elongated (same extent of elongation as in the

Fig. 1. The β subunit stabilizes Pol IV onto DNA substrate. Trap assays with the 30/90 DNA hybrid were used to demonstrate the stabilization of Pol IV onto its substrate. In all experiments, the trap is the unlabelled form of the substrate and is added in large molar excess as specified. (A) Trap assays were performed by mixing equal amounts (4 μ l) of solutions A (sA) and B (sB), and the reaction was allowed to proceed for 7 s before EDTA quenching. sA and sB compositions are as follows. Control lanes (C): sA = labelled substrate (*DNA) + Pol IV (Pol); sB = trap DNA. No trap lanes (NT): sA = *DNA + Pol; sB = dTTP. Trap efficiency lanes (TE): sA = *DNA + trap; sB = Pol + dTTP. Trap assay lanes (TA): sA = *DNA + Pol; sB = trap + dTTP. In assays involving naked or SSB-coated DNA, substrate *DNA was 100 nM, trap 30 μ M and Pol IV 30 nM. In assays involving SSB-coated DNA plus γ complex and β , substrate *DNA was 1 nM, trap 30 nM and Pol IV 0.3 nM. Stable Pol IV–DNA complex formation is visualized by the formation of products during the trap assay (TA) lanes. A stable complex is observed only when β subunit is initially loaded onto DNA (right panel; see text for details). (B) The decay of substrate-bound Pol IV as a function of time was fitted to a simple exponential curve to determine the dissociation rate constant k_{off} . All measurements were performed in duplicate. Substrate DNA, trap and Pol IV were 1.4, 35 and 1 nM, respectively.

absence of trap). These data show that in the presence of β , a stable complex between Pol IV and the substrate is formed. For all three substrates, trap effectiveness is demonstrated by mixing trap and labelled DNA before adding the polymerase and dTTP (Figure 1A, TE lanes).

In order to measure the dissociation rate of Pol IV from the β -loaded substrate, we pre-incubated Pol IV with the substrate, added the trap, and at various time delays dTTP was added for 7 s before the reaction was quenched with EDTA. The amount of labelled primer elongation was plotted as a function of the time delay between addition of trap and dTTP (Figure 1B) (Creighton *et al.*, 1995). The Pol IV dissociation rate (k_{off}) was determined by fitting the data to a single exponential curve. The value $k_{off} = 0.005 \text{ s}^{-1}$ corresponds to a half-life of the Pol IV– β -DNA complex of ~138 s (2.3 min). Together, these results show that the β clamp targets Pol IV onto the P/T substrate and allows the formation of a stable initiation complex.

Fig. 2. Only β loaded onto DNA significantly alters Pol IV activity. DNA Pol IV activity was tested on either primed closed circular pUC118 ssDNA (A) or 30/90 synthetic hybrid for 5 min (B) (see Methods). (A) Reaction mixture contains 1 nM Pol IV, 1 nM substrate DNA and the indicated proteins. (B) Reaction mixture contains 2 nM Pol IV, 10 nM substrate DNA and the indicated proteins as described in Results and Methods.

Effects of SSB, RecA, γ complex and β on the processivity of DNA Pol IV

When assayed on naked closed circular single-stranded (ss) or synthetic oligonucleotide DNA, Pol IV incorporates only one nucleotide per binding event (Wagner *et al.*, 1999). Here we tested whether the SSB, RecA, γ complex and/or the β subunit of Pol III holoenzyme modify the activity of Pol IV. As shown in Figure 2A, coating the closed circular ssDNA with SSB prior to addition of Pol IV results in a slight increase in the polymerase activity of the *dinB* gene product. We interpreted this result as a decrease in non-specific binding of Pol IV to naked ssDNA rather than to a targeting effect, as SSB does not stabilize Pol IV onto P*/T (Figure 1A). Addition of γ complex or β subunit alone to SSB-coated DNA does not modify the polymerization profile (Figure 2). However, when γ complex and β subunit are added together to SSB-coated or naked ssDNA, the elongation pattern is drastically modified (Figure 2). Through an ATP-driven reaction, the multi-subunit γ complex is responsible for the loading and targeting of the β ring onto P/T termini (Ason *et al.*, 2000). Thus, the results presented in Figure 2 suggest that the Pol IV processivity is increased upon interaction with the β subunit. The same conclusions can be reached when analysing the results obtained with the 30/90mer P*/T substrate (Figure 2B). The only observed difference is the strict requirement for SSB in the latter assay (Figure 2B). As discussed by Bloom *et al.* (1997), this type of short and linear DNA substrate supports processive DNA synthesis mediated by Pol III provided that SSB prevents β from sliding off the template before association with the polymerase.

Fig. 3. Processivity of Pol IV upon interaction with β subunit. Pol IV-mediated DNA synthesis on β -loaded M13 ssDNA (0.1 nM) primed with a labelled oligonucleotide in the presence of trap DNA (20-fold excess of unlabelled substrate) and all four dNTPs as a function of time (lanes 5–11). Reaction products were analysed by electrophoresis on alkaline agarose gel. Lanes 1–4, trap efficiency experiment (see text for details).

Finally, and at least at the ratio tested here (one RecA per 13 nt), RecA protein does not modify Pol IV activity (Figure 2A). This latter result is in agreement with the genetic data, indicating the independence of *dinB*-mediated mutagenesis with respect to *recA* (Brotcorne-Lannoye and Maenhaut-Michel, 1986).

In order to determine the processivity of Pol IV upon association with the β clamp, we measured the replication product's length generated by Pol IV in an assay involving circular M13mp18 ssDNA plus SSB, γ complex and β subunit in the presence of trap DNA (see Methods). When polymerase is added to a mixture of labelled P*/T (0.1 nM) and trap (2 nM), very low DNA synthesis is observed (Figure 3, lanes 1–4), demonstrating trap effectiveness. When trap DNA and dNTPs are added simultaneously to a pre-incubation mix containing the labelled P*/T and the enzyme, replication products resulting from a single polymerase binding event can be seen (Figure 3, lanes 5–11). After 1 min of reaction, the product length is quite homogenous with an average length of ~300 nt (Figure 3, lane 5). At longer times, a progressive dissociation of the polymerase is revealed by the appearance of a smear (Figure 3, lanes 6–11). From this experiment, we estimated the average and maximal processivity of β -associated Pol IV to be equal to 300 and 1300 nt, respectively. From this assay, the average nucleotide incorporation rate (k_{pol}) can be estimated to be between 3 and 5 nt/s, a number close to the k_{pol} value of 2 nt/s measured during a pre-steady-state single nucleotide incorporation experiment using a rapid quench flow apparatus (time range 10–100 ms; data not shown). Mathematically, the processivity of the enzyme is determined by the ratio k_{pol}/k_{off} , which is 400 nt (i.e. 2/0.005), a value close to the one observed experimentally.

Fig. 4. Pol IV affinity for β -associated DNA and dTTP. The K_m for β -loaded DNA (K_{mDNA}) and for dTTP (K_{mdTTP}) were assessed through the measurement of initial velocities of Pol IV-mediated dTTP incorporation onto 30/90 P/T using variable concentrations of substrates. For measurement of K_{mDNA} , 30/90 substrate was varied from 0.1 to 0.8 nM with a constant Pol IV concentration of 0.1 nM. For measurement of K_{mdTTP} , dTTP was varied from 0.05 to 15 μ M with a constant Pol IV concentration of 0.2 nM. **(A)** Examples of velocities measurements. **(B)** Double reciprocal plots of the Michaelis–Menten curves obtained from the velocities measurements.

Pol IV has high affinity for both β -associated DNA and dNTP

In order to get further insight into the affinity of Pol IV for β -associated DNA and dNTP, we measured the initial velocities of single dNTP incorporation as a function of substrate (β -associated P/T or dNTP) concentrations. As expected for a Michaelian behaviour, the data yielded the typical saturation curves. Double reciprocal plots of these data are presented in Figure 4, and from these, K_m (and V_{max}) values for both β -DNA and dNTP were calculated to be 0.19 nM ($V_{max} = 0.35$ min⁻¹) and 0.12 μ M ($V_{max} = 0.3$ min⁻¹), respectively.

DISCUSSION

In this paper, we show clearly that upon interaction with β subunit, native Pol IV forms a stable initiation complex with DNA with a half-life of ~2.3 min. Complex formation increases the processivity of Pol IV from 1 to an average of 300–400 nt. Pol IV also acquires a high affinity for its substrates (i.e. $K_{mDNA} = 0.19$ nM; $K_{mdNTP} = 0.12$ μ M). Compared with most DNA polymerases, the rate of processive DNA synthesis by Pol IV (k_{pol}) is relatively low (3–5 nt/s). At odds with these results, Goodman and colleagues recently found that the β clamp increased the processivity of Pol IV only to a minor extent, reaching 6–8 nt (Tang *et al.*, 2000). Moreover, they also reported a much higher K_{mdNTP} value (5 versus 0.12 μ M here). This 40-fold difference in both K_{mdNTP} and processivity values most probably reflects the use by Tang *et al.* (2000) of a maltose binding protein (MBP)–DinB fusion instead of the native DinB protein. The large

MBP domain (42.7 kDa) may strongly interfere and weaken the interaction of Pol IV (39.5 kDa) with the β subunit. The 50-fold lower V_{max} value ($V_{max} = 0.3$ min⁻¹) of the native protein (Figure 4) as compared with the MBP–Pol IV fusion protein ($V_{max} = 17$ min⁻¹; Tang *et al.*, 2000) also shows that the weakened interaction of the fusion protein with the β clamp increases its cycling rate compared with that of the native protein.

When Pol III stalls and dissociates from its template at lesion sites, the β clamp that remains bound to DNA may then facilitate the recruitment of Pol IV. In view of the relatively high processivity acquired by Pol IV following its interaction with the β clamp, the length of the track it will synthesize (~400 nt) appears to be longer than initially expected. Indeed, given the reduced fidelity of these specialized polymerases, they were thought to synthesize only small tracks. However, 400 nt long tracks are still small compared with the size of the chromosome. A simple calculation shows that even if Pol IV is 1000-fold less accurate than Pol III, to produce the same amount of errors as Pol III, Pol IV may synthesize 10 such tracks per chromosome replication. Experimental evidence suggests that DinB is indeed responsible for one third to one half of the spontaneous errors made in a wild-type strain (S.-R. Kim, K. Matsui and T. Nohmi, unpublished results).

In conclusion, when Pol III dissociates at a lesion site it is formally possible that an elaborate system controls the access to the primer template and regulates the length of the synthesis track made by the translesional DNA polymerases. However, we favour a model involving a simple competition among the various DNA polymerases for access to the free primer template.

METHODS

Purification of native Pol IV. Native Pol IV protein was over-produced from the *E. coli* strain BL21(DE3)/pLysS harbouring the pET-16b (Novagen) based plasmid expressing the *dinB* coding sequence. The soluble protein fraction was sequentially passed through a Q Sepharose Fast Flow (Pharmacia) column, precipitated with ammonium sulfate added to 40% saturation, applied to a Phenyl Sepharose High Performance (Pharmacia) column, developed with a decreasing ammonium sulfate gradient, applied to a HiTrap Heparin column (Pharmacia) and eluted with a NaCl gradient, subjected to gel filtration using the Superdex 75 prep. grade XK 16/60 column (Pharmacia) equilibrated with 50 mM HEPES pH 8.0, 150 mM NaCl, and concentrated by ion exchange chromatography on the ResourceS column (Pharmacia) in the same buffer system. Finally, the NaCl concentration was reduced to 50 mM by dialysis and the purified Pol IV protein was flash frozen in liquid nitrogen. The purity was >99% as judged by Coomassie G250 staining.

DNA substrates and traps. 5' end radiolabelling, purification and annealing of synthetic primers were performed as previously described (Wagner *et al.*, 1999). The 30/90mer synthetic construct was obtained by annealing the 30 mer primer (5'-GTAAACCGACGGCCAGTGCCCAAGCTTAGTC) with the 90 mer template (5'-CCATGATTACGAATTACGATCATCACCGGCCGACAGAC-TAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGG-GAAAACCCTGG) to form a double-stranded structure with 5' and 3' ssDNA overhangs 25 and 35 nt long, respectively. The first base to be inserted using this 30/90 (P/T) as substrate is T. The 24mer primer (5'-TGTGCTGCAAGGCGATTAAGTTGG)

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was used for replication of pUC118 ssDNA. The -40 (17mer) M13/pUC sequencing primer was used for replication of M13mp18 ssDNA (NEB).

Replication assays. All replication experiments were carried out at room temperature in EDB buffer (50 mM HEPES pH 7.5, 100 mM potassium glutamate, 200 µg/ml bovine serum albumin, 20% glycerol, 0.02% NP-40, 10 mM dithiothreitol) supplemented with 300 µM ATP, 7.5 mM MgCl₂ and 100 µM dNTP. When specified, SSB (USB), RecA (NEB), γ complex and β subunit were pre-incubated with the DNA for 10 min at room temperature prior to addition of the polymerase and dNTP(s). When present, SSB was added at 90-, 600- and 3000-fold molar excess with respect to the 90mer, pUC118 and M13 templates, respectively. γ complex and β subunit were added at equimolar and 10-fold molar excess over P/T, respectively. Unless specified, reactions were quenched by EDTA, heat-denatured and analysed by gel chromatography on 12% denaturing polyacrylamide gels. Radiolabelled products were visualized and quantified using a PhosphorImager 445 SI (Molecular Dynamics) and ImageQuant software.

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