The processivity factor β controls DNA polymerase IV traffic during spontaneous mutagenesis and translesion synthesis *in vivo*

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The *dinB*-encoded DNA polymerase IV (Pol IV) belongs to the recently identified Y-family of DNA polymerases. Like other members of this family, Pol IV is involved in translesion synthesis and mutagenesis. Here, we show that the C-terminal five amino acids of Pol IV are essential in targeting it to the β -clamp, the processivity factor of the replicative DNA polymerase (Pol III) of *Escherichia coli*. *In vivo*, the disruption of this interaction obliterates the function of Pol IV in both spontaneous and induced mutagenesis. These results point to the pivotal role of the processivity clamp during DNA polymerase trafficking in the vicinity of damaged-template DNA.

INTRODUCTION

Genetic information is continuously subject to attack by endogenous and exogenous chemical and physical agents that damage the DNA template, thus compromising its biochemical functions. Replication of damage-containing DNA, a process termed translesion synthesis (TLS), is a major source of point mutations. Recently, this process has gained much understanding as the specialized DNA polymerases involved in TLS have been characterized (reviewed by Friedberg *et al.*, 2000). All organisms contain several such DNA polymerases in order to deal with the large variety of existing DNA damages (Napolitano *et al.*, 2000). How these polymerases are recruited to the lesion sites remains to be understood. The *dinB*-encoded *Escherichia coli* DNA polymerase IV (Pol IV) (Wagner *et al.*, 1999), which belongs to the recently identified Y-family of DNA polymerases (Ohmori *et al.*, 2001), has been shown to be involved in

spontaneous mutagenesis (Brotcorne-Lannoye and Maenhaut-Michel, 1986; Kim *et al.*, 1997; Wagner *et al.*, 1999; Strauss *et al.*, 2000; Wagner and Nohmi, 2000; McKenzie *et al.*, 2001) and in TLS (Napolitano *et al.*, 2000). Here, we show that Pol IV interacts with the sliding clamp β , the replicative DNA polymerase processivity factor, by means of a small five-amino-acid C-terminal peptide. This interaction is essential for targeting Pol IV to the primer terminus *in vitro* and for allowing Pol IV to participate in spontaneous and induced mutagenesis *in vivo*.

RESULTS

The C-terminal end of Pol IV interacts with the processivity factor β

We have shown recently that, *in vitro*, the presence of the γ -complex and β -subunits of the *E. coli* replicative DNA Pol III holoenzyme strongly enhances the activity of DNA Pol IV (Wagner *et al.*, 2000). This study led us to suggest that the β -clamp may target Pol IV to its DNA substrate. We used the yeast two-hybrid system to study the interaction between Pol IV and the β -clamp. As shown in Figure 1, a two-hybrid interaction was detected between wild-type Pol IV and β . We generated a library of *dinB* fragments and screened it for β -interacting peptides. The shortest fragment isolated in such a screen was the C122 C-terminal peptide of Pol IV (Figure 1). Based on this result and secondary structure predictions, we decided to test the hydrophobic C16 C-terminal peptide for interaction with the processivity clamp

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Fig. 1. Identification of the binding domain of Pol IV with the β -clamp by the two-hybrid system. **(A)** Description of the different Pol IV fragments used in the two-hybrid assays shown in **(B)**. The five amino acids deleted in the constructed $\Delta C5$ mutant appear in larger letters within the C16 fragment. **(B)** When the wild-type, C122, C16 or $\Delta C5$ pGBKT7 constructs were co-transformed with the empty pGADT7 vector ($-\beta$ in the figure), no growth occurred on the selective medium, indicating the absence of transcriptional auto-activation by these fusion proteins. When these constructs were co-transformed with the *dnaN*-containing pGADT7 vector ($+\beta$ in the figure), growth on the selective medium was observed with wild-type, C122 and C16 pGBKT7 constructs, thus identifying the 16 last amino acids of Pol IV as containing the β -interaction domain of Pol IV. The lack of growth of the $\Delta C5$ -GBKT7/*dnaN*-pGADT7 co-transformants on selective medium suggests that the C-terminal pentapeptide of Pol IV is strictly required for efficient interaction.

and found that this small peptide is actually proficient for such an interaction (Figure 1). This peptide includes a stretch of five hydrophobic amino acids (C5 = LVLGL) at its C-terminal end (Figure 1A). The importance of this C5 hydrophobic cluster was analysed by constructing a C5 deletion mutant of Pol IV, $\Delta C5$ (Figure 1A). The mutant protein fails to interact with the processivity clamp in the two-hybrid assay (Figure 1B). To demonstrate this property *in vitro*, we purified both the wild-type and $\Delta C5$ mutant Pol IV proteins. The intrinsic polymerase activity (femtomoles of dNTP incorporated per minute) of the $\Delta C5$ Pol IV mutant is not affected by the deletion of the pentapeptide (Figure 2B, and lanes 6 and 2 in Figure 2A). Similarly, in the presence of the single-strand DNA-binding protein (SSB), the activity of both proteins increases ~ 4 -fold (Figure 2B, and lanes 7 and 3 in Figure 2A). Stimulation by the SSB is most likely achieved by preventing unspecific binding of Pol IV to single-stranded DNA (Wagner *et al.*, 2000). Most interestingly, the $\Delta C5$ mutation totally obliterates the stimulatory effect of the β -subunit on Pol IV activity observed with the wild-type protein (Figure 2B, and lanes 8 and 4 in Figure 2A), confirming the two-hybrid results.

In vivo, the functioning of Pol IV in spontaneous mutagenesis and TLS requires its interaction with the processivity factor β

When overexpressed from a multi-copy plasmid, Pol IV confers a mutator phenotype to its recipient host (Kim *et al.*, 1997; Wagner *et al.*, 1999; Wagner and Nohmi, 2000). We used the rifampicin resistance (*Rif^R*) chromosomal mutation assay (*rpoB* gene) to investigate whether the *dinB* mutator phenotype was dependent upon the capacity of Pol IV to interact with the processivity factor. Expression of wild-type Pol IV from a multi-copy plasmid increased the spontaneous *Rif^R* mutation frequency ~ 15 -fold (Table I; Wagner and Nohmi, 2000). In

Table I. Spontaneous mutation frequencies measured using the *Rif^R* assay (*rpoB* gene) in strains overexpressing the wild-type (*p_{dinB}*) or $\Delta C5$ (*p $\Delta C5$*) derivative Pol IV protein

Expression plasmid	<i>Rif^R</i> /10 ⁸ cells ($\pm$ SD) ^a	Relative mutation frequency
–	8.7 ($\pm$ 5.1)	1
<i>p_{dinB}</i>	146 ($\pm$ 92.2)	16.8
<i>p$\Delta C5$</i>	6.3 ($\pm$ 4.1)	0.72

^aIndicated average values result from at least six independent experiments.

contrast, no increase over background was observed when the $\Delta C5$ mutant was similarly overexpressed, thus demonstrating the functional importance of the Pol IV– β -clamp interaction *in vivo*. Wild-type Pol IV and its $\Delta C5$ derivative were shown, by western blot analysis, to be expressed to a similar extent *in vivo* (data not shown).

In addition to its role in spontaneous mutagenesis, Pol IV was shown recently to be involved in TLS at a single benzo(a)pyrene (BaP) guanine adduct *in vivo* (Napolitano *et al.*, 2000). When located at the 3'-end of a run of three guanines, SOS-dependent TLS of a BaP adduct yields both error-free and -1 frameshift bypass products. Both bypass pathways require the *umuDC* (Pol V) and *dinB* (Pol IV) gene products (Lenne-Samuel *et al.*, 2000; Napolitano *et al.*, 2000). To test whether the interaction between Pol IV and β is functionally relevant for the TLS process *in vivo*, wild-type and mutant $\Delta C5$ *dinB* genes under the control of their natural promoter/operator sequences were cloned into a pSC101-derived plasmid (pGB2). These low-copy-number plasmids were then introduced into the *dinB*-deficient *E. coli* strain MGZ Δ *dinB* (Napolitano *et al.*, 2000). The resulting strains

Fig. 2. Effect of the β -clamp/Pol-IV interaction on its DNA polymerase activity *in vitro*. (A) DNA polymerase activity of Pol IV (200 fmol) and its $\Delta C5$ derivative (200 fmol) were assayed on a 30/90 DNA substrate (20 fmol) in a 10 μ l final volume for 1 min at room temperature as previously described (Wagner *et al.*, 2000). Both enzymes exhibit a similar basal activity (lanes 2 and 6). An equivalent stimulation in activity is also seen upon addition of the SSB (1.22 pmol; lanes 3 and 7). However, while the presence of the β -clamp and the γ -complex (60 and 20 fmol, respectively) efficiently stimulates the activity of wild-type Pol IV (Wagner *et al.*, 2000), no such increase is seen for the $\Delta C5$ derivative (lanes 4 and 8). The structure of the primer template exhibits 3' and 5' single-stranded overhangs in order to prevent β from sliding off the template (Bloom *et al.*, 1997). (B) DNA polymerase activity under the different experimental conditions is quantified and expressed as the amount of dNTP incorporated by 200 fmol of enzyme per minute. A 4-fold stimulation of the activity of both enzymes is found with SSB. In the presence of the β -clamp, the activity of wild-type Pol IV is increased an additional 4-fold, while the activity of the $\Delta C5$ derivative remains unaffected.

were tested for their ability to perform error-free and mutagenic TLS under SOS-induced conditions using a plasmid containing a single BaP guanine adduct, as described previously (Napolitano *et al.*, 2000). The strain expressing the wild-type *dinB* gene complements efficiently the defect of the $\Delta dinB$ strain, restoring both error-free and frameshift TLS to the level measured in a wild-type strain (Table II). In contrast, the strain expressing the $\Delta C5$ *dinB* mutant failed to restore either error-free or frameshift TLS (Table II). Thus, the results clearly demonstrate that, in order to participate in the bypass of the BaP adduct, Pol IV needs to be targeted to the stalled replication intermediate via its interaction with the processivity factor β .

DISCUSSION

Besides the essential role of the β -clamp as the processivity factor of the replicative DNA Pol III (Stukenberg *et al.*, 1991), the present work shows that the β -subunit plays a crucial role in targeting a translesion DNA polymerase to its substrate, i.e. a blocked replication intermediate. Targeting of Pol IV is achieved by a direct interaction between a small hydrophobic C-terminal peptide of the polymerase and the processivity clamp. A similar interaction between phage RB69 DNA polymerase and the corresponding processivity clamp has been characterized at the structural level and shown to occur via a small peptide (KKASLFDMFDF) that acts as a flexible tether between the two proteins (Shamoo and Steitz, 1999). During the preparation of the present paper, a consensus sequence that mediates binding to the eubacterial β -clamps was identified as QL(S/D)LF (Dalrymple *et al.*, 2001). This sequence motif is present in most sequenced members of the eubacterial DnaE, PolC, PolB, DinB and UmuC families of DNA polymerases and in the mismatch repair protein MutS1 (Dalrymple *et al.*, 2001). Moreover, the direct interaction of the δ -subunit of the clamp

loader complex of *E. coli* DNA Pol III (i.e. the γ -complex) with the β -clamp has been resolved at the structural level (Jeruzalmi *et al.*, 2001). The sequence of the β -interacting peptide of δ thus identified (QAMSLF) is very close to the aforementioned consensus sequence. It should be noted that the actual DinB (Pol IV) motif (QLVLGL) identified in the present work exhibits a relatively poor match to the consensus sequence (QL(S/D)LF). As processivity clamps are multimeric structures (reviewed by Bruck and O'Donnell, 2001), it is conceivable that several proteins (DNA polymerases) may connect simultaneously to a single clamp. In *E. coli*, it turns out that the activity of all five DNA polymerases is stimulated by the processivity factor *in vitro* (Stukenberg *et al.*, 1991; Bonner *et al.*, 1992; Tang *et al.*, 2000; Wagner *et al.*, 2000; Lopez De Saro and O'Donnell, 2001). Whether all these interactions are of functional significance in regulating the access of DNA polymerases to the primer template *in vivo* remains to be established. During the preparation of this paper, a similar physical and functional interaction between the eukaryotic processivity clamp PCNA and DNA polymerase η in *Saccharomyces cerevisiae* and human cells was described, highlighting the evolutionary conservation of this important targeting mechanism (Haracska *et al.*, 2001a,b).

Speculation

This work directly demonstrates the essential role of a translesion DNA-polymerase/sliding-clamp interaction at the physiological level: abolishing this interaction obliterates the TLS activity of the polymerase and, as a consequence, its mutagenic potential. The apparent universality of the interaction between DNA polymerases and processivity clamps may allow for the design of inhibitors that affect the binding of specific translesion DNA polymerases to blocked replication forks, thus permitting the

Table II. Bypass of a single BaP adduct *in vivo* requires the functional interaction between Pol IV and the β -clamp

MGZ strain / complementation plasmid	UV (J/m ²)	Error-free TLS ^a (%)	−1 frameshift TLS ^b (%)
wt / pGB2	50	3.11 (18/579)	0.16 (6/3680)
Δ dinB / pGB2	45	0.67 (4/597)	<0.028 (0/3613)
Δ dinB / pGB2- <i>dinB</i>	45	5.06 (32/633)	0.24 (8/3368)
Δ dinB / pGB2- Δ C5	45	0.97 (6/615)	<0.034 (0/2929)

^aNumber of colonies giving a positive hybridization signal with the strand containing the BaP adduct / total number of colonies analysed.

^bNumber of blue colonies (−1 frameshift) / total number of colonies analysed.

modulation of TLS and mutagenesis. Such approaches may lead to new tools in medical fields such as cancer therapy.

METHODS

Two-hybrid screen. All the two-hybrid fusion proteins were constructed by cloning polymerase chain reaction (PCR) amplification products of *dinB* and *dnaN* (the gene coding for the β -subunit) into the multiple cloning site of pGBKT7 or pGADT7 vectors (Clontech), respectively. A library of *dinB* fragments fused to the Gal4 DNA-binding domain was constructed by ligation into the filled *Bam*HI site of pGBKT7. *dinB* fragments were obtained by digestion of the PCR amplification product of the *dinB* coding sequence using DNase I (0.03 U for 800 ng of DNA at 15°C for 4 min, 50 mM Tris–Cl pH = 7.5, 10 mM Mn²⁺) followed by a T4 DNA polymerase treatment to repair ends. The ligation mixture was transformed into the *E. coli* XL1-blue strain, and plasmidic DNA was prepared. Pair combinations of two-hybrid plasmids were introduced into the yeast strain AH109 (MAT α , *trp1-901*, *leu2-3*, *112 ura3-52*, *his3-200*, *gal4 Δ* , *gal80 Δ* , *UASGAL2-ADE2*, *LYS2::UASGAL1-HIS3*, *URA3::UASMEI1-lacZ*; Clontech) following the lithium-acetate method (Agatep *et al.*, 1998). Co-transformants were selected on synthetic dropout plates minus leucine and tryptophan (non-selective medium) and checked for two-hybrid interaction on plates depleted in leucine, tryptophan, histidine and adenine (selective medium).

In vitro primer extension assays. The coding sequence of *dinB* was amplified by PCR and cloned into the *Nde*I and *Bam*HI sites of pET-15b vector (Novagen) available for overproducing DinB (Pol IV) with an N-terminal His₆ tag. Mutant sequence Δ C5 was cloned in exactly the same way using an appropriate C-terminal oligonucleotide for PCR amplification. Small-scale purifications of both recombinant proteins were performed under native conditions using Ni-NTA spin columns (Qiagen). Similar yields and purity were obtained for both proteins. Primer extension assays were performed at room temperature for 1 min, as described previously (Wagner *et al.*, 2000).

Rif^R assays. Low-copy-number plasmids expressing wild-type (*p_{dinB}*) or Δ C5 (*p Δ C5*) Pol IV from the *Plac* promoter were constructed by replacement of the *Sna*BI–*Bam*HI fragment of plasmid pYG782 (Kim *et al.*, 1997) containing the wild-type *dinB* sequence by the 900 and 885 C-terminal base pairs of wild-type and Δ C5 *dinB* sequences, respectively. These plasmids were introduced into the wild-type MG1665 strain and the frequencies of spontaneous Rif^R colonies were measured as described previously (Wagner and Nohmi, 2000).

Construction of low-copy-number vectors for TLS assays. Using plasmid pYG751 as a source of the *dinB*⁺ gene (Kim *et al.*, 1997), the wild-type and the Δ C5 *dinB* sequences were PCR amplified with their natural operator/promoter sequence and cloned into the *Pst*I–*Bam*HI restricted pSC101-based low-copy-number plasmid, pGB2.

TLS assay *in vivo*. TLS was monitored *in vivo* using a single adducted plasmid carrying a genetic marker that allows the analysis of strand segregation (Koffel-Schwartz *et al.*, 1996; Lenne-Samuel *et al.*, 2000). Specifically, the plasmid used in the present study carries a single BaP adduct located at the 3′-end of a run of three guanines (5′-GGG^{BaP}-3′; Koffel-Schwartz *et al.*, 1996; Lenne-Samuel *et al.*, 2000). This construction was transformed by electroporation into *E. coli* strains MGZ (S1521: *lacZ* Δ M15, *lacY*::Tn10) and its *dinB*-deficient derivative (S1521: *lacZ* Δ M15, *lacY*::Tn10, *dinB*::Tn5; Napolitano *et al.*, 2000) containing either the wild-type *dinB* gene or its Δ C5 derivative expressed from the low-copy-number plasmid pGB2. The strains were SOS-induced prior to the transformation step by irradiation with UV light at a dose yielding survival levels of ~10% (Napolitano *et al.*, 2000). Transformants were plated on LB medium containing ampicillin (100 μ g/ml), X- β -Gal (40 μ g/ml) and IPTG (0.1 mM). Error-free TLS was determined by colony hybridization using specific oligonucleotide probes as described elsewhere (Napolitano *et al.*, 2000). Mutagenic TLS (−1 frameshift) was quantified using a phenotypic assay (Napolitano *et al.*, 2000). Indeed, the construction exhibits a LacZ[−] phenotype (forming white colonies on a medium containing IPTG and X- β -Gal) that can be reverted to the LacZ⁺ phenotype (forming dark-blue colonies on the same indicator medium) by a −1 frameshift mutation (5′-GGG^{BaP}-3′→GG).

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