Architecture of the Pol III–clamp–exonuclease complex reveals key roles of the exonuclease subunit in processive DNA synthesis and repair

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DNA polymerase III (Pol III) is the catalytic α subunit of the bacterial DNA Polymerase III holoenzyme. To reach maximum activity, Pol III binds to the DNA sliding clamp β and the exonuclease ε that provide processivity and proofreading, respectively. Here, we characterize the architecture of the Pol III–clamp–exonuclease complex by chemical crosslinking combined with mass spectrometry and biochemical methods, providing the first structural view of the trimeric complex. Our analysis reveals that the exonuclease is sandwiched between the polymerase and clamp and enhances the binding between the two proteins by providing a second, indirect interaction between the polymerase and clamp. In addition, we show that the exonuclease binds the clamp via the canonical binding pocket and thus prevents binding of the translesion DNA polymerase IV to the clamp, providing a novel insight into the mechanism by which the replication machinery can switch between replication, proofreading, and translesion synthesis.

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Introduction

The E. coli DNA polymerase III holoenzyme (DNA Pol III HE) is a large macromolecular machine that contains 10 proteins (α, β, ε, θ, γ, δ, χ, and ψ) with a combined molecular weight close to one megadalton (Johnson and O’Donnell, 2005). Its catalytic centre is the α subunit, the third DNA polymerase identified in E. coli, termed as Pol III (Gettler et al., 1971). In isolation Pol III is a rather inefficient enzyme compared to other DNA polymerases. It has a low affinity for DNA and as a result the isolated Pol III is only capable of synthesizing short stretches of DNA (Fay et al., 1981; Bloom et al., 1997). The processivity of Pol III is greatly enhanced upon binding to the DNA sliding clamp (β subunit) a ring-shaped molecule that encircles the DNA (LaDuca et al., 1986; Stukenberg et al., 1991). In addition, the exonuclease (ε subunit) is also required for optimal activity (Studwell and O’Donnell, 1990; Kim and McHenry, 1996a). Once assembled into the holoenzyme, Pol III transforms into a highly efficient enzyme synthesizing DNA at a remarkable speed of up to 1000 bp/s and >80 000 bp synthesized per binding event (Georgescu et al., 2012). As a result, during replication a single holoenzyme is sufficient to complete the entire genome (McInerney and O’Donnell, 2004; Reyes-Lamothe et al., 2010). However, due to the opposite polarity of the two DNA strands, synthesis at the lagging strand is discontinuous, requiring repositioning of Pol III every 1–3 kb (McInerney et al., 2007; Georgescu et al., 2012). In addition, errors introduced during DNA synthesis are removed by the exonuclease subunit (Scheurmann et al., 1983) upon which Pol III temporarily releases the DNA. Furthermore, chemically modified bases form a block for the high fidelity Pol III and require the action of translesion DNA polymerases. These low fidelity DNA polymerases are capable of DNA synthesis over the lesion after which normal DNA replication can resume (Sutton and Walker, 2001). Interestingly, some controversy has arisen over the switching of the replicative polymerase Pol III and translesion polymerase Pol IV at the site of a lesion. Initial reports suggested a model in which the DNA sliding clamp functions as a ‘molecular toolbelt’ than can bind two polymerases simultaneously (Indiani et al., 2005; Furukohri et al., 2008; Wagner et al., 2009). However, more recent work challenges the toolbelt model and instead suggests that only one polymerase can bind to the clamp at one point and that therefore Pol IV directly competes of Pol III (Heltzel et al., 2009). Regardless of this, it is clear that while on one hand Pol III needs to bind efficiently to the clamp and exonuclease in order to synthesize long stretches of DNA, it also needs to be able to dissociate from the DNA frequently during the different stages of the replication and repair. The mechanisms by which these switches operate are currently not known.

The interactions between the Pol III, clamp, and exonuclease have been studied extensively and atomic structures have been solved for each of the individual subunits (Kong et al., 1992; Hamdan et al., 2002; Lamers et al., 2006). However, no structural information is available on how Pol III interacts with the other two subunits. Therefore, to structurally analyse the interactions between Pol III, clamp, and exonuclease, we made use of chemical crosslinking combined with mass spectrometry to map the interaction sites between the three proteins. We find that in addition to the known direct interaction between Pol III and clamp, the exonuclease provides a second, indirect interaction to the clamp. By doing so, it enhances the interaction between Pol III and clamp and provides the exonuclease with a more efficient access to the DNA. In addition, we find that by occupying the second binding pocket of the clamp the
exonuclease prevents binding of the translesion DNA polymerase Pol IV, providing the first structural insight into the control of translesion synthesis in bacteria.

Results

The exonuclease enhances binding between Pol III and clamp

DNA Pol III is not a processive enzyme and depends on the sliding clamp to enhance its processivity during DNA synthesis (Stukenberg et al., 1991). However, the affinity between Pol III and clamp is rather low, with reported values for $K_D$ of $\sim 1\, \mu M$ (Kim and McHenry, 1996b; Dohrmann and McHenry, 2005; Lamers et al., 2006), which contrasts with the observation that once assembled into the holoenzyme Pol III can synthesize DNA of $\sim 80\, \text{kb}$ per binding event (Georgescu et al., 2012). It has been reported that in addition to the clamp the exonuclease also has an effect on the processivity of Pol III (Studwell and O’Donnell, 1990; Kim and McHenry, 1996a). Therefore, we wondered if the exonuclease could affect the interaction between Pol III and the clamp. To further investigate this, we used analytical size-exclusion chromatography to analyse the interactions between the three proteins (Figure 1A). (Chromatograms of exclusion chromatography to analyse the interactions of the individual proteins as well as molecular weight standards are shown in Supplementary Figure S1A). Different concentrations ($1.5$ to $10\, \mu M$) of dimeric Pol III–clamp complexes and trimeric Pol III–clamp–exonuclease were injected onto a Superdex 200 column and small fractions collected during elution of the protein. The fractions were analysed by SDS gel electrophoresis and protein band intensities measured (Figure 1B–D). At low concentration ($1.5\, \mu M$), Pol III and clamp migrate independently on the size-exclusion column due to the weak interaction between the two proteins. At higher concentrations ($5\, \mu M$), almost half of the clamp co-migrates with the Pol III, while at $10\, \mu M$ the two proteins form an almost single complex. Strikingly, in the presence of the exonuclease (Figure 1, bottom row) the interaction between Pol III and clamp is enhanced, as shown by the co-migration of the clamp in the Pol III–exonuclease complex even at the lowest concentration ($1.5\, \mu M$). At higher concentrations ($5$ to $10\, \mu M$), the complex is further enhanced, ultimately creating a robust complex. We also tested the effect of $\theta$, a small non-essential protein that binds to the exonuclease. We find that the addition of $\theta$ has no effect on the migration pattern of the Pol III–clamp–exonuclease complex even though it co-migrates with the complex (Supplementary Figure S1B).

To obtain an estimate of the affinity between the different complexes, we fitted a ‘sum of two Gaussians’ to the migration profile of the clamp (see Supplementary Figure S1C). From the ratio of the volume of the two Gaussians, representing bound and unbound clamp, we can calculate a $K_D$ value using the equation $K_D = [\text{Pol}][\text{Clamp}]/[\text{Pol-Clamp}]$. Additionally, we applied a correction factor for the $\sim$ five-fold dilution that takes place on the column (loading volume 50 $\mu l$, elution volume 250 $\mu l$). Doing so, we find that the $K_D$ for Pol III–Clamp is $1.2 \pm 0.2\, \mu M$, which is similar to values observed before (Kim and McHenry, 1996b; Dohrmann and McHenry, 2005; Lamers et al., 2006). Addition of the exonuclease reduces the $K_D$ to $\sim 4$-fold to $0.3 \pm 0.1\, \mu M$. Hence, this shows that the exonuclease indeed stabilizes the Pol III–clamp complex and provides an explanation for its stimulating effect on processive DNA synthesis (Studwell and O’Donnell, 1990; Kim and McHenry, 1996a).

Chemical crosslinking maps the interactions between Pol III, clamp, and exonuclease

To define the organization of the trimeric Pol III–clamp–exonuclease complex in more detail, we employed a chemical crosslinking approach similar to as described before (Leitner et al., 2010). Individual proteins or different protein complexes were incubated with the lysine

![Figure 1](image-url)
crosslinker bis(sulphosuccinimidyl) glutarate (BS2G) or bis(sulphosuccinimidyl) suberate (BS3) after which the samples were purified by size-exclusion chromatography to remove any non-specific crosslinked products (Supplementary Figure S2A). Next, the purified crosslinked samples were double digested with trypsin and Glu-C protease, fractionated by cation exchange chromatography and analysed by nano-scale reversed phase liquid chromatography coupled to a tandem mass spectrometer for detection and identification of crosslinked peptides. All mass spectra were analysed with an in-house developed program ‘Crosslinker’ (Andrew N. Holding, manuscript in preparation). This resulted in a total number of 27 unique crosslinks, with the majority of crosslinks measured multiple times (Table I). A detailed list of crosslinked peptides as well as fragmentation spectra are given in Supplementary Figure S2B and C.

For the isolated polymerase (Figure 2A and B), we find a good correlation between the crosslinks and the known crystal structure of *E. coli* Pol III (residues 1–917) (Lamers et al., 2006). Notably, a strong cluster of crosslinks is present between residues 29 and residues 714/715/716. Although these are distant in sequence, they are in close vicinity of each other in the protein structure, showing that the crosslinking accurately represents the structure. The average observed distance between the Cα atom of two crosslinked lysines is ~22 Å, which is well within the predicted distance of 24 Å (2 × length of a lysine side chain (6.4 Å) + length of the crosslinker BS3 (11.4 Å)). We also find a few longer crosslinks, with some distances reaching 28 Å. However, these distances are measured on a static crystal

| Crosslink Residue 1 | Crosslink Residue 2 | Protein complex | Pol | PE | PC | PCE | Total |
|---------------------|---------------------|----------------|-----|----|----|-----|-------|
| 29                  | 714/5/6             | Pol Pol        | 16  | 10 | 4  | 8   | 38    |
| 29                  | 722                 | Pol Pol        | 10  | 3  | 0  | 5   | 18    |
| 229                 | 1009                | Pol Pol        | 3   | 0  | 1  | 0   | 4     |
| 316                 | 595                 | Pol Pol        | 1   | 0  | 0  | 0   | 1     |
| 439                 | 1009                | Pol Pol        | 1   | 1  | 0  | 0   | 2     |
| 461                 | 1009                | Pol Pol        | 3   | 1  | 0  | 1   | 5     |
| 500                 | 510                 | Pol Pol        | 1   | 0  | 0  | 0   | 1     |
| 500                 | 1009                | Pol Pol        | 9   | 1  | 0  | 0   | 10    |
| 510                 | 1009                | Pol Pol        | 1   | 0  | 0  | 0   | 2     |
| 561                 | 983                 | Pol Pol        | 2   | 0  | 0  | 0   | 2     |
| 621                 | 983                 | Pol Pol        | 7   | 1  | 0  | 0   | 8     |
| 621                 | 992                 | Pol Pol        | 1   | 0  | 0  | 0   | 1     |
| 855                 | 872                 | Pol Pol        | 14  | 6  | 1  | 3   | 24    |
| 983                 | 992                 | Pol Pol        | 2   | 0  | 0  | 2   | 4     |
| Exo                 | Pol                 | 120             | 1009| —  | 1  | 0   | 1     |
| 136                 | 229                 | Pol Pol        | 1   | 1  | 0  | 1   | 2     |
| 136                 | 510                 | Pol Pol        | 1   | 1  | 0  | 1   | 2     |
| 136                 | 1009                | Pol Pol        | 2   | 1  | 0  | 1   | 4     |
| 141                 | 229                 | Pol Pol        | 1   | 1  | 0  | 1   | 2     |
| 141                 | 1009                | Pol Pol        | 1   | 1  | 0  | 1   | 3     |
| 158                 | 1009                | Pol Pol        | 3   | 0  | 0  | 0   | 3     |
| 235                 | 29                  | Pol Pol        | 4   | 0  | 0  | 0   | 4     |
| 235                 | 714                 | Pol Pol        | 4   | 0  | 0  | 2   | 6     |
| Clamp               | Pol                 | 277             | 872 | —  | 2  | 6   | 8     |
| 277                 | 1009                | Pol Pol        | —   | 1  | 4  | 5   | 5     |
| Clamp               | Exo                 | 277             | 136 | —  | 1  | 4   | 5     |
| 277                 | 141                 | Pol Pol        | —   | 2  | 2  | 2   | 5     |

Numbers reflect total number of independent crosslinks found over multiple experiments. A dash (—) indicates not applicable. Polymerase (P), Exonuclease (E), and Clamp (C).

Figure 2. Chemical crosslinking indicates that the exonuclease is sandwiched between Pol III and clamp. (A) Top view of Pol III with crosslinks in blue dashed lines. Arrow indicates view point in (B). (B) Front view showing the crosslinks between the known part of Pol III and the modelled tail in dark brown. (C) Top view of the model of the Pol III–clamp–exonuclease complex, with close-up view of clamp–exonuclease crosslinks (in circle). Dashed lines indicate inter-protein crosslinks. Cyan: Pol III–exo, magenta: Pol III–clamp, black: clamp–exo (internal Pol III crosslinks not shown). See text for more details. Arrow indicates view point for (D). (D) Bottom view of Pol III (viewed along arrow in C). Truncations indicated with yellow spheres, residues 255–320 in dark grey, PHP domain in grey circle, and remainder of polymerase (residues 321–917) in light orange. (E) Both N-terminal fragments of Pol III (residues 1–285 and 1–270) shift upon binding of the exonuclease. Pol III1–270: blue open circles, Pol III1–285: red open squares, exonuclease: solid black line, Pol III1–270 + exonuclease: solid blue circles, Pol III1–285 + exonuclease: red solid squares.
structure and are not taking into account any molecular motions of the protein, which are substantial in Pol III and DNA polymerases in general (see Steitz and Yin, 2004; Evans et al., 2008; Wing et al., 2008). Similarly, distances of up to 28 Å were also observed in Chen et al. (2010). Interestingly, we also find crosslinks to the tail domain of Pol III (residues 918–1160) that was not included in the crystal structure of E. coli Pol III (Lamers et al., 2006). To visualize these crosslinks, we created a model of the E. coli Pol III tail with the program ‘Modeller’ (Eswar et al., 2006) using the crystal structure of full-length Thermus aquaticus Pol III (Bailey et al., 2006) as a template. The generated model fits well with the crosslinks that we find (Figure 2A and B), suggesting that the tail of Pol III adopts a similar position in both E. coli and Taq Pol III.

In addition to the internal Pol III crosslinks, we also find a large number of crosslinks between Pol III and clamp, Pol III and exonuclease, as well as crosslinks between clamp and exonuclease (see Table 1). To visualize these crosslinks, we created a model of the Pol III–clamp–exonuclease complex using the obtained crosslinks as a guide (Figure 2C). Between Pol III and clamp we find two crosslinks (coloured magenta). The first crosslink (labelled with ‘1’ in Figure 2C) brings the internal clamp binding motif of Pol III (residues 920–924) close to the canonical binding pocket of the clamp. This fits well with the previously reported role of the internal motif of Pol III that is essential for the interaction between the two proteins (Dohrmann and McHenry, 2005). The second crosslink between Pol III and the clamp (labelled with ‘2’) positions the clamp in line with the exit path of the DNA (shown in light grey). Between Pol III and the exonuclease, we find two clusters of crosslinks (coloured cyan) separated by >60 Å. The first cluster (labelled with ‘3’) is between the catalytic domain of the exonuclease (residues 1–180) and the ‘polymerase and histidinol phosphatase’ (PHP) domain (Aravind and Koonin, 1998) of Pol III (residues 1–270). The second cluster of crosslinks (labelled with ‘4’) places the very C-terminus of the exonuclease at the other side of PHP domain, thus wrapping its tail around the polymerase. In addition, and most interestingly, we also find two crosslinks between clamp and exonuclease (coloured black, see zoom). This firmly places the catalytic domain of the exonuclease between the PHP domain of Pol III and the clamp.

Hence, our crosslinking data provide a first structural view of the trimeric Pol III–clamp–exonuclease complex. It confirms the known interactions between Pol III and clamp, but also reveals for the first time how the exonuclease binds to Pol III by wrapping the exonuclease tail around the PHP domain. Furthermore, the crosslinking results suggest a potential direct interaction between the exonuclease and the clamp. These interactions between the exonuclease and the polymerase and clamp are further analysed below.

The location of the exonuclease next to the Pol III PHP domain correlates well with our finding that the first 270 residues of the polymerase are sufficient for exonuclease binding. Previously, it was reported that the first 320 residues of Pol III are sufficient for exonuclease binding (Wieczorek and McHenry, 2006). Yet, this region stretches across the entire length of Pol III and therefore does not provide a detailed map of the exonuclease binding site (Figure 2D). Therefore, we made three additional truncations at residues 255, 270, and 285. The first truncation renders the protein insoluble and could therefore not be purified. In contrast, the truncations to residues 270 and 285 yield well-behaved proteins that retain full exonuclease binding (Figure 2E). Taken together, our findings show that the tail of the exonuclease binds to the PHP domain of Pol III by wrapping itself around it and placing the catalytic domain adjacent to the exit path of the DNA. This position is similar to the position of the exonuclease domain in the homologous Pol C from Geobacillus kaustophilus (Evans et al., 2008), but different from the position of the exonuclease domain in Pol I (Beece et al., 1993) and Pol II (Wang and Yang, 2009; Supplementary Figure S3).

### Exonuclease binds the clamp using a canonical clamp binding motif

The location of the exonuclease catalytic domain also places it in an ideal position to interact with the clamp. Indeed, upon closer examination, we find a short sequence immediately downstream of the catalytic domain (QTSMAF, residues 182–187) that form a canonical clamp binding motif (Qxx(L/M)xF) found in other proteins that bind the clamp such as Pol II, Pol III, Pol IV, and others (Dalrymple et al., 2001). The position of this motif immediately after the catalytic domain fits well with the model predicted from the crosslinking results. The clamp binding motif can easily be modelled into the binding pocket of clamp without violating any of the crosslinking results (i.e., all Ca-Ca distances of crosslinked lysines are kept within 28 Å). Next, to verify that this is indeed a bona fide binding motif, we mutated the two conserved hydrophobic residues methionine 185 and phenylalanine 187 to alanine. Following this, we first tested the direct interaction between the exonuclease and the clamp (Figure 3). This interaction is rather weak as even at 30 μM only a fraction of the exonuclease co-migrates with the clamp. In contrast, the mutant exonuclease(185/187) has lost all affinity for the clamp and travels unaltered at all three concentrations, providing proof that the exonuclease binds to the clamp using a canonical binding motif.

### The exonuclease–clamp interaction is required for optimal proofreading activity

To further investigate the role of the direct interaction between exonuclease and the clamp, we made use of a real-time primer extension assay (Song et al., 2009). In this assay, the fluorescence intensity of a carboxyfluorescein (FAM) dye at the 5’ end of the template strand is strongly reduced through the extension of the primer strand (Figure 4A). In the presence of Pol III, the signal is rapidly reduced, which is modestly enhanced by the presence of the clamp or the exonuclease alone (Figure 4B). Interestingly, when the exonuclease is present, the fluorescence quickly returns back to starting values due to the activity of the exonuclease that can remove the primer strand once the polymerase runs out of nucleotides. Importantly, the return rate is faster when both exonuclease and clamp are present, while mutation of the clamp binding motif in the exonuclease (M185A+F187A) abolishes the stimulation to levels identical to exonuclease alone. Hence, the direct interaction between the clamp and the exonuclease does not only enhance the interaction between the Pol III and the clamp, but also positions the exonuclease in a conformation that is more favourable for DNA access. This may be explained by the observation that the catalytic domain of the exonuclease is tethered via a
concentrations, the exonuclease co-migrates with the clamp. The mutant exonuclease 185/187 shows no interaction with the clamp and migrates separate from the clamp even at 30 μM protein concentration.

Previously, it has been reported that Pol III has two clamp binding motifs (Qx2[L/M]x0/1F) that are located at either end of the tail of Pol III: an internal clamp binding motif spanning residues 920–924 (Dalrymple et al, 2001; Dohrmann and McHenry, 2005) and a second motif located at the very C-terminus (residues 1154–1159) (López de Saro et al, 2003; Georgescu et al, 2008). However, as the clamp is composed of two β monomers, there are only two binding pockets per clamp, not enough to bind the two potential binding motifs from Pol III and a third motif from the exonuclease. It has been shown that the C-terminal clamp binding motif is not required for replication (Kim and McHenry, 1996b; Dohrmann and McHenry, 2005). Here too, we find that the C-terminal binding motif of Pol III does not appear to contribute to clamp binding: we find crosslinks between the clamp and the internal clamp binding motif of Pol III, but none to the C-terminal clamp binding motif. Further evidence that the C-terminal motif does not contribute to clamp binding can be found in the structure of Taq Pol III (Bailey et al, 2006), where the motif is buried in between the oligonucleotide/oligosaccharide binding (OB) domain and the C-terminal domain of the polymerase tail and therefore inaccessible for clamp binding (Supplementary Figure S6A and B). Moreover, while the internal clamp binding motif is found conserved in 25 out of 30 bacterial Pol III sequences, the C-terminal motif is only found in 3 species (Supplementary Figure S6C).
interaction sites. All of the deletion constructs could be expressed delimited by domain structures and potential binding motifs with the clamp we made a series of deletion constructs (Figure 5A). The tail of Pol III has multiple interactions with the clamp. (Figure 6A) Gel filtration analysis of the different proteins. Note the aggregated protein for the deletion at residue 1072. (C) SDS–PAGE analysis of the purified proteins.

Hence, we can expect that in the trimeric Pol III–clamp–exonuclease complex, both binding pockets of the clamp are occupied, one by the internal clamp binding motif of Pol III and one by the exonuclease clamp binding motif. To address the relative contributions the internal clamp binding motif of Pol III and the clamp binding motif of the exonuclease, we mutated the conserved [L/M] and F to an alanine in each motif (i.e., exonuclease M185A + F187A, Pol III M923A + F924A). We already showed that the mutation of the clamp binding motif in the exonuclease results in a loss of clamp binding (Figure 6B), but does not abolish it, suggesting that the tail of Pol III has indeed multiple interactions with the clamp. For the deletion constructs 975 and 956 the loss in affinity can be rescued to different degrees by the addition of exonuclease, suggesting that this construct indicates that the tail of Pol III has multiple interactions with the clamp, with the exonuclease providing an additional contact. Multiple clamp interactions have also been described for other clamp binding proteins: E. coli Pol IV (Bunting et al., 2003), the archaeal RB69 DNA polymerase (Mayanagi et al., 2011), and the T4 phage clamp loader complex (Kelch et al., 2011). The nature of the additional Pol III–clamp interactions awaits structural characterization.
The Pol III tail and exonuclease prevent binding of the translesion DNA polymerase IV to the clamp

With both binding pockets of the clamp occupied by Pol III and the exonuclease, as well as additional interactions of the Pol III tail with the clamp, most of the clamp is protected in the trimeric Pol III–clamp–exonuclease complex. Yet, it has been reported that Pol III and the translesion (TLS) DNA polymerase Pol IV can bind to the clamp simultaneously (Indiani et al., 2005) and that the two polymerases switch positions on the DNA during stalling of the replication machinery, but not during active replication (Indiani et al., 2005; Furukohri et al., 2008; Heltzel et al., 2009; Wagner et al., 2009). In addition, Pol IV binds to the clamp using the canonical binding motif (Bunting et al., 2003). Hence, we wondered if the exonuclease and the tail of Pol III play a part in the regulation of the access of Pol IV to the clamp. Therefore, we analysed the interactions of the clamp, Pol III, and Pol IV, in absence and presence of the exonuclease (Figure 7). Alone, Pol IV readily forms a complex with the clamp, which is not disrupted by addition of the exonuclease (Figure 7A, bottom panel). In contrast, addition of Pol III effectively competes off Pol IV, even with a two-fold higher concentration of the latter (Figure 7B). Addition of the exonuclease further displaces Pol IV from the clamp (bottom panel). When using increasing deletions of the Pol III tail, Pol IV can access the clamp again (Figure 7C–E). Addition of the exonuclease to the complex displaces Pol IV again in deletion 975, to a lesser extent in the 956 deletion construct while no rescue is seen for the 917 deletion construct (bottom row). Hence, our findings show that the exonuclease subunit plays an important role in preventing access of the Pol IV polymerase to the replication machinery by occupying the second binding pocket of the clamp. In addition, the whole of the Pol III tail (residues 917–1160) is required to bind sufficiently tight to the clamp and compete off Pol IV, providing further evidence that the tail of Pol III has multiple interactions with the clamp. Reversely, it has been found that Pol IV too has multiple contacts to the clamp: one via the canonical clamp binding pocket and a second contact on the side of the clamp (Bunting et al., 2003; Heltzel et al., 2009).

Discussion

During replication of the genome, the replicative DNA polymerase Pol III needs to associate tightly with the clamp in order to synthesize very long stretches of DNA. At the same time, Pol III needs to be able to quickly change its position on, or dissociate from the clamp in response to different events such as: (i) handing over the DNA to the exonuclease after incorporation of the wrong nucleotide, (ii) making place for a translesion DNA polymerase upon encountering of the chemically modified base, or (iii) repositioning of Pol III at the end of an Okazaki fragment. Thus, rather than a simple tether to the DNA, the interaction between Pol III and the clamp is complex, requiring a substantial degree of control. This is further exemplified by the different reports on the polymerase switch between the Pol III holoenzyme and Pol IV. At low concentrations, Pol IV appears to bind to the clamp simultaneously with Pol III (Indiani et al., 2005; Furukohri et al., 2008). Low concentrations of Pol IV appear also to be required for translesion synthesis past nitrofurazone-induced DNA lesions (Wagner et al., 2009). At higher concentrations, Pol IV is capable of displacing a stalled Pol III holoenzyme from the primer junction in vitro, ultimately resulting in a complete inhibition of replicative DNA synthesis (requiring a 25- to 100-fold molar excess of Pol IV) (Indiani et al., 2005; Furukohri et al., 2008; Heltzel et al., 2009; Wagner et al., 2009). Interestingly, intracellular Pol IV levels during normal growth are estimated to be ~10-fold higher than that of Pol III holoenzyme, while during the SOS response, Pol IV levels increase to ~100 times that of Pol III levels (Kim et al., 2001), while even higher levels of Pol IV result in lethality in vivo (Uchida et al., 2008). Hence, it appears that Pol IV has potentially two roles, one where it can act in consort with the replication machinery to do ‘on the fly repair’ whereas at high concentrations during the SOS

Figure 7 Exonuclease prevents binding of DNA Pol IV to the clamp. (A) Pol IV (dashed line with crosses) readily forms a complex with the clamp (open circles). Addition of exonuclease does not disrupt the complex (bottom panel). Grey box indicates peak fractions 13 and 14 of Pol IV–clamp complex. Protein concentration used: Pol IV 20 μM, clamp 10 μM (dimer), and exonuclease 10 μM. (B) Addition of Pol III (10 μM) displaced most of Pol IV from the clamp, which is further enhanced in the presence of the exonuclease (bottom panel). (C, D) Partial deletion of the Pol III tail enables Pol IV to regain access to the clamp. Note that the migration of Pol IV–clamp complex overlaps with the migration of the Pol III deletions constructs and does not indicate a trimeric complex between Pol III–Pol IV–clamp. Such a trimeric complex would elute at fractions 10 and 11 similar to the trimeric Pol III–clamp–exonuclease complex (B). Addition of the exonuclease can restore much of the binding between Pol III and clamp in deletion 975, but only marginally for the deletion at residue 956 (bottom row). (E) All binding between Pol III and clamp is lost in the deletion at residue 917, allowing Pol IV full access to the clamp, with the exonuclease unable to rescue binding (bottom panel).
response Pol IV displaces the replication machinery from the DNA template, thus acting as a cellular checkpoint (Uchida et al., 2008).

Having a modular system in which multiple sites interact with the clamp allows for a finer control of the binding between Pol III and the clamp. Our observation that the exonuclease forms a second, indirect interaction between Pol III and the clamp provides a simple yet elegant mechanism by which the access of the mutagenic TLS polymerase Pol IV that is able to synthesize over the lesion is allowed access to the clamp and DNA. As Pol IV is not very processive, Pol III will ultimately regain access to the DNA and reinitiate high-speed DNA synthesis where the exonuclease repositions itself between Pol III and clamp and thus displacing Pol IV. See also Supplementary Figure S7.

Materials and methods

Materials

All chemicals were purchased from Sigma unless stated otherwise. All reactions were performed in 50 mM Hepes pH 7.5, 150 mM NaCl, and 2 mM DTT.

Protein purification

All proteins were expressed in *E. coli* BL21(DE3) or *E. coli* BL21(DE3) pLYS for Pol IV and purified as described before with some alterations (Maki and Kornberg, 1985; Kong et al., 1992; Miller and Perrino, 1996; Beuming et al., 2006). In brief, N-terminally His-tagged (N-His₆) Pol III (α subunit) was purified using a Histrap, Resource Q, and a Superdex 200 column (all columns from GE Healthcare). N-His₆ clamp (β subunit) was purified using a Histrap column, followed by a Superdex 200 column. N-His₆ Exonuclease (ε subunit) was purified from inclusion bodies in 6 M Urea using a Histrap column. The protein was refolded by overnight dialysis to 0 M Urea and concentrated using a Resource Q column. Pol IV was purified using a Capto S column, Hitrap Phenyl column, and Superdex 200 column. All proteins were flash frozen in liquid nitrogen and stored at −80°C. For non-tagged protein, Pol III was purified by Source Q, Heparin, Resource Q, and Superdex 200 columns. Non-tagged clamp was purified using Phenyl sepharose, Hitrap Q, and Hitrap SP columns. Non-tagged exonuclease was obtained after removal of the His₆ tag by a 3-h incubation at room temperature with human rhinovirus 3C protease. His-tagged and non-tagged proteins did not show a difference in their migration pattern on a gel filtration column (see Supplementary Figure S4 for comparison).

Size-exclusion chromatography analysis of exonuclease binding

Samples of the different complexes were prepared at 1.5, 5.0, 10.0, and 30.0 μM and 50 μl injected onto a PC3.2/30 (2.4 ml) Superdex 200 gel filtration column (GE Healthcare) pre-equilibrated in 50 mM Hepes pH 7.5, 150 mM NaCl, and 2 mM DTT. A superdex 75 column was used for the clamp-exonuclease complex (Figure 3). In all, 50 μl fractions were collected and analysed by SDS–PAGE using 4–12% NuPage Bis-Tris precast gels (Life Technologies). Gels were stained with Coomassie brilliant blue and protein band intensities measured using ImageJ (Schneider et al., 2012). For estimation of *Kₚ* between the clamp and Pol III ± exonuclease, we used only the measured band intensities of the clamp to fit a ‘sum of two Gaussians’ using GraphPad Prism (version 5 for Mac OSX, Graphpad Software, San Diego, CA, USA). The ratio of the area under the curve of the two Gaussians was then used to calculate the concentration of bound and free clamp, using the starting concentration for total concentration of clamp. A correction factor of 5 was applied to compensate for the dilution of the proteins on the gel filtration column (input volume 50 μl, elution volume 250 μl). Calculated values for *Kₚ* are the average of nine experiments (three repeats of three concentrations).

Real-time DNA primer extension assay

Activity of Pol III was measured using a 38-nt long DNA substrate annealed to a 30-nt primer strand. The carboxyfluorescein moiety was located at the 5’ end of the template strand. Template strand: 5’/56-FAM/-CC CCC CCC CCC CCC ACC TAA AGT TGG GAG TCC TTC GTC GTA TTA-3’. Primer strand: 5’/5-Tag GAC GAA GGA CTC CCA ACT TTA GGT GC-3’. Reactions were performed by mixing different concentrations of dGTP (from 0 to 27 μM) with 100 nM labelled DNA, and 1 μM unlabelled DNA in a final volume of 20 μl in buffer

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Figure 8 Model for polymerase switching at the clamp. During normal replication, both binding pockets of the clamp are occupied: one by the Pol III directly and one by the exonuclease forming a second, indirect interaction between Pol III and clamp. When Pol III inserts a wrong base, DNA synthesis slows down allowing the exonuclease access to the DNA resulting in removal of the wrongly incorporated base. However, when the error in the DNA is on the template strand (in grey), the exonuclease has no access to the lesion (working solely on 3’ end of the DNA). Then, to bypass the lesion, the low fidelity translesion DNA polymerase Pol IV that is able to synthesize over the lesion is allowed access to the clamp and DNA. As Pol IV is not very processive, Pol III will ultimately regain access to the DNA and reinitiate high-speed DNA synthesis where the exonuclease repositions itself between Pol III and clamp and thus displacing Pol IV. See also Supplementary Figure S7.
10 mM Tris–HCl pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 6 mg/ml BSA. Reactions were initiated by addition of 270 nM protein (Pol III, clamp, exconuclease) and 10 mM MgCl\(_2\) (final concentrations) and measured in a 348-well plate using a BMG Labtech Pherastar FS plate reader during 30 min with 7 s intervals at 25°C. Data were analysed in GraphPad Prism and the kinetics was calculated by using the initial linear section of the curve (approximately the first 30 s) as shown in Supplementary Figure S5.

**Crosslinking and mass spectrometry analysis**

Protein complexes were crosslinked at 40 μM in 50 μl using either 2 mM bis[1-sulfoethyl]glutamate (BSG) or bis[1-sulfoethyl]glycine (BSG) (Pierce) using a 50/50% mix of deuterated (d4) and non-deuterated crosslinking reagent. Reactions were incubated for 15 min at room temperature and quenched with 50 mM NH\(_4\)HCO\(_3\). Samples were subsequently injected onto a PC3.2/30 (2.4 ml) Superdex 200 gel filtration column (GE Healthcare). In all, 50 μl fractions were collected and analysed by SDS-PAGE using 4–12% NuPage Bis-Tris precast gels (Life Technologies) (Supplementary Figure S2A).

Selected fractions were made up to 100 μl using 100 mM NH\(_4\)HCO\(_3\) and reduced with 5 μl DTT at 10 mM in 100 mM NH\(_4\)HCO\(_3\) and alkylated with 4 μl iodoacetamide at 55 mM in 100 mM NH\(_4\)HCO\(_3\) before digestion with porcine sequencing grade trypsin (Promega) at a protein-to-enzyme ratio of 20:1 (w:w) in 3 M Urea/100 mM NH\(_4\)HCO\(_3\)). The digest protein was de-salted using Sep-Pak Light C18 (Waters) as directed by manufacturer and then lyophilized. The resultant peptide mixture resolved into the initial buffer for fractionation by strong ion exchange (SCX) performed using a PolyLC Poly SULPHOTHYRL A column (5 μm, 300 Å, 50 mm × 1.0 mm). Peptides were eluted using a linear gradient from 30% acetonitrile in 5 mM KH\(_2\)PO\(_4\) to 70% acetonitrile in 10 mM KH\(_2\)PO\(_4\) 100 mM NH\(_4\)HCO\(_3\) over 75 min at 80 μl/min, before sub-digestion with endoproteinase Glu-C (Promega) divided equally between fractions such as the total amount is to a ratio of 20:1 (w:w) to initial protein amount.

Selected fractions were desalted by ZipTip C18 (Millipore) and analysed using a Dionex U3000 HPLC machine coupled to a Thermo-Scientific LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). The reversed-phase LC separations were performed using a Dionex Acclaim PepMap100 column (C18, 3 μm, 100 Å, 75 μm × 150 mm). Peptides were eluted using a linear gradient from 5% acetonitrile in 0.1% formic acid to 40% acetonitrile in 0.1% formic acid over 110 min at 200 nl/min. A cycle of one full FT scan mass spectrum (m/z 350–1800, resolution of 60 000 at 400, lock mass at 445.120025) was followed by 10 data-dependent MS/MS spectra of any MS doubles found to match within 2 p.p.m. of the mass of a theoretical crosslinked peptide were then confirmed manually. See also Supplementary Figure S2B and C.

**Modelling of the Pol III tail**

The model of the E. coli polA polymerase tail was generated with the program Modeller (Eswar et al, 2006) using the crystal structure of Taq Pol III (Bailey et al, 2006) as a template. A sequence alignment of E. coli and T. aquaticus Pol III used for the modelling was calculated with Clustal (Larkin et al, 2007) using Pol III sequences from 35 different bacterial species. The model was further manually adjusted in PyMol (Schrodinger, 2010) and Coot (Emsley et al, 2010) using the OB domain from G. kausophilus Pol C (Evans et al, 2008). Figure 2A–D was prepared with PyMol.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Author contributions**

ATR, ANH, and MHL designed, performed, and analysed experiments, HK performed experiments. ATR, ANH, and MHL wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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