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Interactions of replication versus repair DNA substrates with the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*

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ABSTRACT

Different DNA polymerases partition differently between replication and repair pathways. In this study we examine if two Pol I family polymerases from evolutionarily distant organisms also differ in their preferences for replication versus repair substrates. The DNA binding preferences of Klenow and Klentaq DNA polymerases, from *Escherichia coli* and *Thermus aquaticus* respectively, have been studied using a fluorescence competition binding assay. Klenow polymerase binds primed-template DNA (the replication substrate) with up to 50× higher affinity than it binds to nicked DNA, DNA with a 2 base single-stranded gap, blunt-ended DNA, or to a DNA end with a 3′ overhang. In contrast, Klentaq binds all of these DNAs almost identically, indicating that Klenow has a stronger ability to discriminate between replication and repair substrates than Klentaq. In contrast, both polymerases bind mismatched primed-template and blunt-ended DNA tighter than they bind matched primed-template DNA, suggesting that these two proteins may share a similar mechanism to identify mismatched DNA, despite the fact that Klentaq has no proofreading ability. In addition, the presence or absence of 5′- or 3′-phosphates has slightly different effects on DNA binding by the two polymerases, but again reinforce Klenow's more effective substrate discrimination capability.

1. Introduction

Pol I family DNA polymerases are involved in both replication and repair activities within the prokaryotic cell. Pol I is an essential enzyme both in excision repair and in the processing of Okazaki fragments generated during lagging strand DNA replication [1]. During DNA repair, Pol I plays an important role in the gap filling synthesis involved in nucleotide excision repair (NER) [1,2]. Both Klenow and Klentaq completely fill ssDNA gaps of various sizes in duplex DNA [3,4]. The thermodynamic analysis of Klenow and Klentaq binding to gapped DNA, however, has not been previously addressed. In this study we examine these two Pol I polymerases from evolutionarily distant organisms to ask if they differ in their preferences for interacting with replication versus repair substrates. The initial binding preferences of the polymerases for different types of "damaged DNAs" versus primed-template DNA (the reference replication substrate) should reflect the partitioning of the polymerases into repair versus replication pathways in vivo.

Klenow and Klentaq are the "large fragment domains" of the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*, respectively, and both are fully functional DNA polymerases on their own. Evolutionarily, *T. aquaticus* is more than a billion years older than *E. coli*, and this study asks if the DNA selectivity of the enzyme has changed over that evolutionary time. The most obvious difference between these two polymerases is the presence of 3′ exonuclease activity in Klenow and its absence in Klentaq, despite the fact that the two proteins retain high structural and sequence homology [1,5–10]. Herein, we have characterized the interactions between Klenow or Klentaq and primed-template DNA versus different damaged DNA substrate analogs. Damaged DNA takes many forms. In this study we specifically examine gapped DNA, nicked DNA, and DNA with different DNA end structures that mimic potential DNA double-strand breaks. The role of the 5′- or 3′-phosphate in the binding of gapped DNA by both proteins was also examined.

It is found that Klenow polymerase has the ability to discriminate between primed-template DNA and different repair substrates during the initial DNA recognition step, while Klentaq barely distinguishes among the different potential substrates. Klenow has not evolved toward a strongly biased preference for one type of substrate over another, however, but has developed a discriminatory binding capability that Klentaq does not have.

Interestingly, for primed-template and blunt-ended substrates, both polymerases bind mismatched DNA about 2–3× tighter than they bind matched DNA, suggesting that these two proteins may share a similar mechanism to identify mismatched DNA, despite the fact that Klentaq has no proofreading ability. Such a capability may be related to a proposed role of DNA polymerases in protecting DNA ends after double-strand breaks [11,12].

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2. Materials and methods

2.1. Preparation of oligonucleotides

Oligo-(deoxyribo)-nucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotide concentrations were determined by measuring the absorbance at 260 nm and using extinction coefficients provided by manufacturer. Hairpin DNAs and gapped DNAs were prepared by self-annealing, by heating at 95 °C for 5 min and then slowly cooling to room temperature. The DNA sequences of constructs used for experiments are shown in Table 1.

2.2. Preparation of Klenow and Klentaq polymerases

Klenow Fragment (KLN) was purified as described previously [13,14]. The Klenow clone used in this study contains the D424A mutation (Klenow exo-) and was provided by Catherine Joyce of Yale University. This mutant significantly decreases the 3′-5′exonuclease activity, but DNA binding to the proofreading site remains intact [15].

2.3. Fluorescence anisotropy competition experiments

Competition assays were used to study the binding affinity of Klenow and Klentaq to different DNA constructs, as described in detail previously [16,17]. Briefly, a competition assay is initiated by mixing 1 nM rhodamineX-labeled 13/20mer DNA with polymerase at a concentration equal to or slightly higher than the Kd. 13/20mer is labeled at the 5′ end of the primer (i.e. at the blunt-end of the construct). As demonstrated previously, the fluorophore does not affect binding of the polymerase, and polymerase binding only alters anisotropy not steady-state fluorescence [14,16,17]. Unlabeled competitor DNA is then titrated into the mixture, while maintaining the labeled DNA and the polymerase at constant concentrations. The anisotropy decreases as the unlabeled DNA competes with labeled DNA to bind the protein. See reference [17] for a detailed discussion of the competitive binding assay and requisite controls. In this study, competition assays were performed at 25 °C with and without 5 mM $MgCl_2$ in 10 mM Tris, pH 7.9, and in 50 mM KCl for Klentaq and 150 mM KCl for Klenow.

Competition curves are then fit to the equation:

$$\Delta A = \{\Delta A_T([I]/K_I)/(1 + [I]/K_I + E_T/Kd)\} \quad (1)$$

where ΔA is the change in fluorescence anisotropy, ΔA_T is the total change in anisotropy, [I] is the total competitor DNA concentration at each point during the titration, K_I is the inhibition constant for the competitive DNA binding, E_T is the total polymerase concentration, and Kd is the dissociation constant for polymerase DNA binding to the fluorescent 13/20mer DNA. E_T is kept constant along with the concentration of fluorescent 13/20mer DNA by including these components at constant concentration in the solution with the competitor DNA. All competition experiments were replicated at least three times. Nonlinear regression was performed using the program Kaleidagraph (Synergy Software).

Table 1
Sequences of DNA constructs used in this study.

13/20	5′-TCGCAGCCGTCCA-3′ 3′-AGCGTCGGCAGGTTCCCAAA-5′
hp39	AAGGCTACCTGCATGA-3′ AGCCGATGGACGTACTACCCCCC-5′
hp57	AACGGCTATGCTCACCGCCACTACGCAAACC-3′ GAGCCGATACGAGTGGCGGTGATGCG-5′
hp46	AAGGCTACCTGCATGATAATTGG-3′ AGCCGATGGACGTACTATTAACC-5′
hp39m	AAGGCTACCTGCA^{CAG}-3′ AGCCGATGGACGT$_{ACT}$ACCCCCC-5′
hp46m	AAGGCTACCTGCATGATAAT^{CAC}-3′ AGCCGATGGACGTACTATTA$_{ACC}$-5′
nick	5′-GCGTGTGAGGAGTACCTCACACGCCACGCT CTGAGGATGACCTCAGAGCGTG-3′
gap2	5′-GCGTGTGAGGAGTACCTCACACGCCACGCT CTGAGGATGACCTCAGAGCG-3′
gap10	5′-GCCAGTCGAGTACGACTGGCAATATATATT CGGAGACAGCATGAGCTGTCTCCG-3′

3. Results

Most of the DNA molecules used in this study to explore the binding preferences by Klenow and Klentaq have stable terminal hairpin structures flanking the duplex region to prevent blunt-end binding of the polymerase on one or both sides. Hp39 is a primed-template hairpin DNA, which is used throughout as the “reference DNA”, representing a “normal” replication-type substrate. The different DNA substrates that are compared to the binding of Hp39 in this study include: 1) nicked DNA, 2) DNA with a 2-base single-stranded gap, 3) DNA with a 10-base gap, 4) blunt-end DNA, 5) blunt-end DNA with 3-mismatched bases at the end, 6) ptDNA with 3-mismatched bases at the primed-template junction, and 7) DNA with a 3′ overhang instead of a 5′ overhang. In addition to these comparisons, the effects of 5′ or 3′-phosphorylation within gapped and nicked DNA were also investigated.

3.1. Gap and nick binding

Fig. 1 shows representative competition curves for the binding of Klenow and Klentaq to different DNA substrates at 25 °C. Curves are fit with Eq. (1) from Materials and methods to obtain Kd values for each type of DNA construct. It is immediately obvious from Fig. 1 that the affinities of Klenow for the different DNA constructs vary quite a bit, while the affinities of Klentaq for the different constructs are quite similar. Fig. 2B shows the free energies of binding of each of the constructs. In Fig. 2B, it can be seen that Hp39, the reference ptDNA substrate, binds tightest to Klenow, and that the nicked DNA binds the weakest. Both gapped DNAs also bind more weakly to Klenow than does the ptDNA, but as the gap length increases, the binding affinity to Klenow increases. In contrast, Fig. 2B shows that Klentaq polymerase binds ptDNA, gapped-DNA and nicked-DNA all with similar binding affinities.

Fig. 2C illustrates the results as ΔΔG values, where each DNA construct is directly compared to the Hp39 reference ptDNA. All subsequent figures will utilize this same ΔΔG format, always using Hp39 ptDNA as the reference against which all other DNA substrates are compared. Fig. 2 thus shows that Klenow polymerase readily distinguishes these different DNA constructs, while Klentaq polymerase cannot really distinguish among them. If gapped DNA is representative of a repair-type substrate and ptDNA represents a replication-type substrate, these data indicate that Klenow both distinguishes between replication and repair substrates, and prefers replication-type substrates by up to 2.4 kcal/mole. Translating the ΔΔG values in Fig. 2C into relative affinity differences (e.g. K_a^{ptDNA}/K_a^{nick}) indicates that Klenow's affinity for ptDNA is 50× greater than its affinity for nicked-DNA, 25× greater than its affinity for gap2-DNA, and 4× greater than its affinity for gap10-DNA, while the ΔΔG values for Klentaq binding show its negligible affinity difference among these different DNAs. All relative binding relationships are the same in experiments done in the absence of Mg^{+2} (data not shown). Klenow's increase in affinity as the gap length increases indicates that gaps slightly longer than 10 bases will likely appear equivalent to ptDNA for Klenow.

Another aspect of Figs. 1 and 2, which we have also noted in previous studies, is that they show how readily the fluorescence anisotropy assay detects even very small differences in binding free energies among different substrates [16,17]. For example, the widely spaced titration curves illustrated for Klenow binding to different DNA

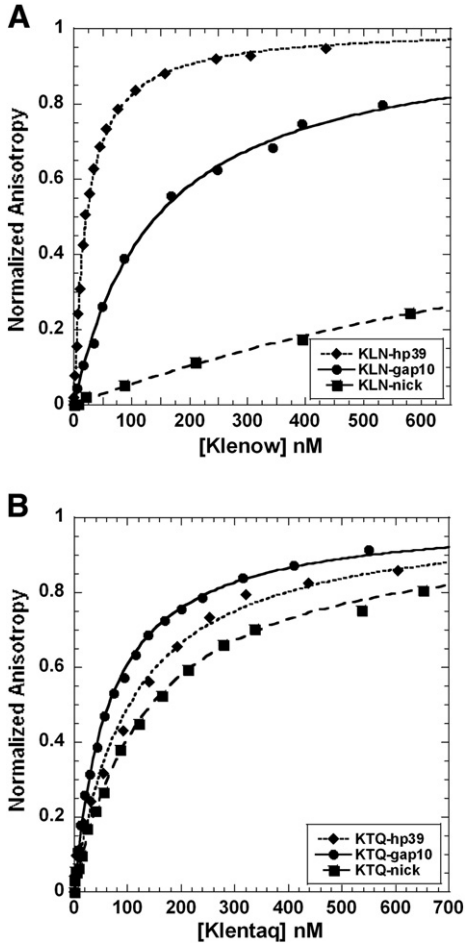

Fig. 1. Equilibrium competition titrations for binding of Klenow (KLN, panel A) and Klentaq (KTQ, panel B) to different DNA structures. In both panels, primed-template DNA (Hp39) is shown with diamonds, gap10 DNA is shown with circles, and nicked DNA is shown with squares. Klenow titrations were performed at 25 °C in 10 mM Tris, 5 mM $MgCl_2$ and 150 mM KCl at pH 7.9. Klentaq titrations were performed at 25 °C in 10 mM Tris, 5 mM $MgCl_2$ and 50 mM KCl at pH 7.9. In order to plot each set of titrations on the same plot, only part of the collected data is shown for some of the constructs. Lines show the fit to Eq. (1) as described in Materials and methods.

constructs in Fig. 1A span a free energy range of 2.4 kcal/mole (as shown in Fig. 2), while the still resolvable different titration curves for Klentaq binding to different DNAs in Fig. 1B span a range of ≤0.15 kcal/mole.

3.2. Effect of 3′-phosphorylation on binding of Pol I polymerases to a 10-base gap

Depending on how a DNA gap or break is generated, the 3′ end of the DNA may or may not be extendable by polymerase. To gain an understanding of the binding of gapped DNA with a nonextendable 3′-terminus, we determined the binding affinities of Klenow and Klentaq for gap10-DNA with a 3′-phosphate within the gap, in the presence and absence of magnesium (Fig. 3). The 3′-phosphate decreases the binding affinity of gap10 by Klenow in the presence (ΔΔG of 0.9 kcal/mol) and absence of magnesium (ΔΔG of 1.5 kcal/mol). This makes reasonable physiological sense, since the polymerase cannot extend this DNA until the phosphate is removed. In contrast, however, addition of the 3′-phosphate only slightly decreases the binding affinity of gap10 by Klentaq (Fig. 3), again suggesting that Klenow has an enhanced ability to discriminate among these different DNA structures relative to Klentaq.

It has previously been shown that removing the 3′-OH from the primer (replacing it with a 3′-H) did not affect the binding strength of Klenow to primed-template DNA [18]. This result provided

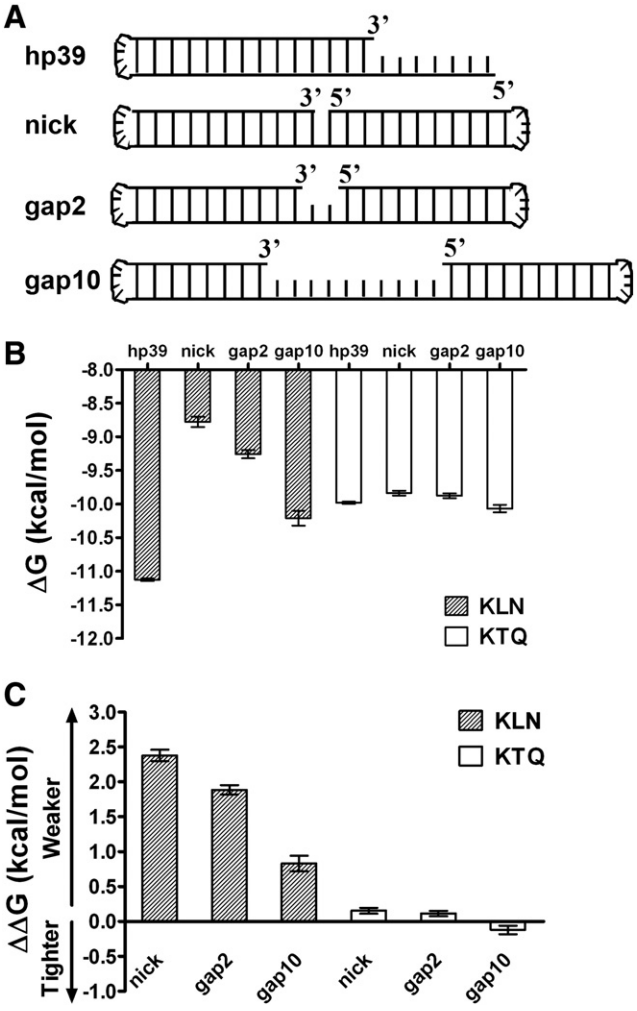

Fig. 2. Affinities of Klenow (KLN) and Klentaq (KTQ) for primed-templates, nicks, and gaps. Panel A shows schematics of the DNA constructs (DNA sequences are given in Table 1). Panel B shows the ΔG of binding for each construct. Error bars are the standard deviations on three titrations. Panel C shows the relative difference in binding free energy for each DNA construct, using the ptDNA (Hp39) as a reference: so for example, $\Delta\Delta G_{nick} = \Delta G_{nick} - \Delta G_{ptDNA}$. Positive ΔΔG values indicate that the compared construct binds more weakly than ptDNA.

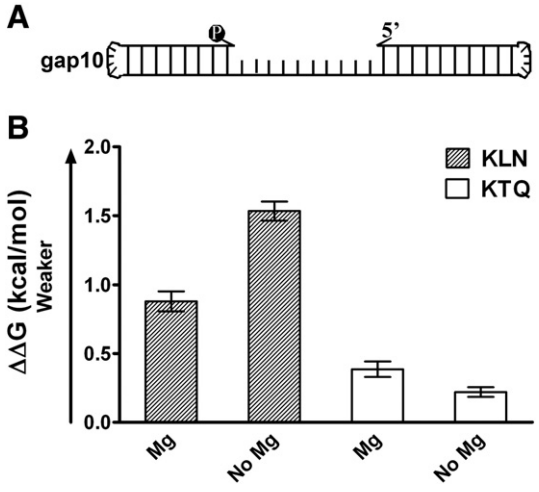

Fig. 3. The effect of 3′-phosphorylation on the binding of a 10-base gap to Klenow (KLN) and Klentaq (KTQ) polymerases in the presence and absence of Mg^{+2}. Data are shown as ΔΔG values compared to the same DNA without a 3′-phophate: $\Delta\Delta G = \Delta G_{with\text{-}3'p} - \Delta G_{no\text{-}3'p}$. Positive ΔΔG values indicate weaker binding of the phosphorylated gapped DNA.

experimental evidence that the 3′-OH does not contribute to the initial binding free energy, while our results show that a 3′-phosphate decreases the binding affinity of Klenow for gap10. The inhibition by 3′-phosphate may be due to steric conflict within the active site. Previous studies showed that the three catalytically important carboxylates (Asp 882, Glu 883 and Asp 705 in Klenow; Asp 785, Glu 786 and Asp 610 in Klentaq) are 2–3 Å away from the 3′ terminus of the primer strand in the binary complex with DNA [10], but addition of phosphate group to the primer terminus will add about 4 Å.

3.3. 5′-phosphate effect on binding of gapped DNAs to Pol I DNA polymerases

The 5′ end of DNA in vivo is frequently phosphorylated, hence we also evaluated how the presence or absence of 5′-phosphorylation within gapped and nicked DNA might alter binding affinity. For longer gaps, the 5′ side of the gap will be too far away to even interact with the polymerase, but for nicks and these shorter gaps, the 5′ side of the gap could easily be within interaction distance. Joyce and associates have shown that for ptDNA constructs where the 5′ overhang is very short (1–2 bases), that 5′-phosphorylation of the overhang can tighten binding affinity to Klenow by about 1 kcal/mole [19].

Fig. 4 shows small but differing 5′-phosphorylation effects on the binding of Klenow versus Klentaq. The addition of a 5′-phosphate enhanced the binding of Klenow to the nicked DNA, but not to gapped DNA. In contrast, 5′-phosphorylation shows a small unfavorable effect on binding of gapped DNA by Klentaq. Interestingly, the effects of 5′-phosphorylation on gapped DNA binding of both polymerases differs from its effect on the binding of eukaryotic Pol β polymerase, where 5′-phosphorylation moderately increases binding affinity for longer gaps, but not for shorter gaps [20,21].

The 5′-phosphate effects on the affinity of both polymerases for gapped DNA were also examined in the absence of magnesium (data not shown) and the results were nearly identical for Klentaq, and slightly dampened for Klenow (nicked DNA bound 0.35 kcal/mole tighter to Klenow in the absence of magnesium). This relative insensitivity of either polymerase to the presence or absence of Mg^{+2} again contrasts with Pol β, where Mg^{+2} and 5′-phosphorylation have antagonistic effects on binding affinity of that polymerase to gapped DNA [20].

It is also notable that the effects of 5′- and 3′-phosphorylation of a 10 base gap on the binding of Klentaq are almost identical, in contrast to the somewhat different effects that phosphorylating opposite sides of the gap has on Klenow. This could indicate that Klentaq binds equivalently to either side of the gap (and thus is similarly inhibited by phosphorylating either side of the gap), while Klenow binds preferentially to the productive 3′-side of the gap. The data on the binding of the two polymerases to DNA end-structures with 5′ versus 3′ overhangs, which is presented below, also supports this possibility.

3.4. Binding of different DNA end-structures by Klenow and Klentaq

Fig. 5 shows the affinity differences between ptDNA and the binding of two different unproductive DNA end-structures to Klenow and Klentaq. Pol I polymerases have been suggested to act in protecting DNA ends after double-strand breaks [11,12]. Fig. 5 shows again that Klenow discriminates against the non-replicative structures more effectively than Klentaq does. In fact, if two-stranded constructs are used instead of hairpin constructs, the difference in affinity between ptDNA binding and blunt-end binding is even smaller for Klentaq (≤0.3 kcal/mole), whereas it remains larger for Klenow [16]. However, the differences in affinities between the replicative DNA structures and the blunt-ended or inverted-template-primer DNA structures are not large for either polymerase (≤1.1 kcal/mole for Klenow, ≤0.6 kcal/mole for Klentaq): a result that certainly supports a protective end-binding capability for both polymerases.

3.5. Binding of mismatched DNA end-structures by Klenow and Klentaq

Fig. 6 shows the binding of Klenow and Klentaq to DNA constructs with mismatched ends. Our laboratory and others have collected a large body of data on the binding of Klenow and Klentaq to different DNAs, but no laboratory has previously characterized a duplex DNA that binds with higher affinity than the “normal” primed-template substrate (although single-stranded DNA, under high salt concentrations, will bind more tightly than ptDNA). In Klenow, mismatches at

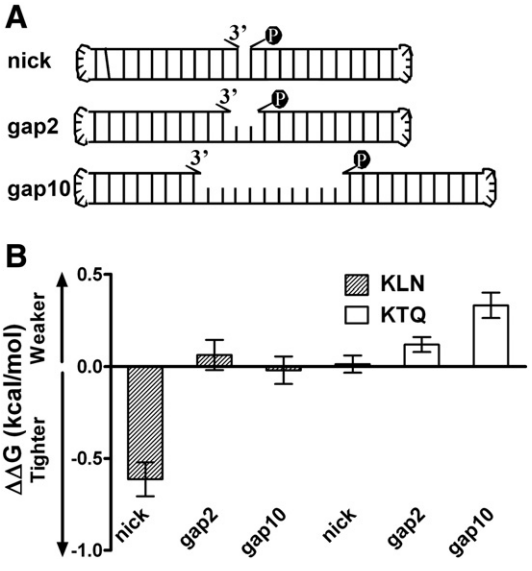

Fig. 4. The effect of 5′-phosphorylation on the binding of nicks and gaps to Klenow (KLN) and Klentaq (KTQ) polymerases. Schematics of DNA constructs are shown in the top panel. Data are shown as ΔΔG values compared to the same DNA without a 5′-phophate: $\Delta\Delta G = \Delta G_{\text{with-5'p}} - \Delta G_{\text{no-5'p}}$. Negative values indicate stronger binding and positive ΔΔG values indicate weaker binding of the phosphorylated DNA relative to the unphosphorylated DNA.

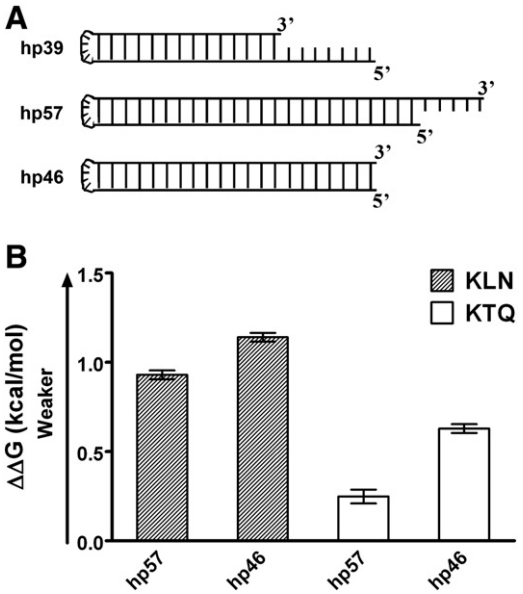

Fig. 5. Binding of different DNA end-structures by Klenow and Klentaq polymerases. The top panel shows schematics of the different DNA structures. Data are shown as ΔΔG values relative to binding of ptDNA (Hp39): $\Delta\Delta G = \Delta G_{\text{structureA}} - \Delta G_{\text{ptDNA}}$. Positive ΔΔG values indicate weaker binding of the compared DNA relative to ptDNA binding.

the 3′ primer terminus favors the partitioning of the binding to the exo site [22,23], and three consecutive mismatches at the 3′ primer terminus cause Klenow to bind almost entirely in the editing mode [22,23]. Thus, when binding to Klenow, duplex DNAs containing three consecutive mismatches will bind with the duplex pulled open and the 3′ end of the primer pulled down into the exonuclease active site. The only existing protein-DNA co-crystal of Klenow polymerase shows the DNA binding in this "editing mode" orientation [6,8].

Clearly mismatched DNA binds to the proofreading site of Klenow more tightly than matched DNA binds to the polymerization site, but Fig. 6 unexpectedly shows almost the same enhancement of affinity when mismatched DNA binds to Klentaq polymerase. In fact, this is the only non-replicative DNA in this study that Klentaq appears able to distinguish from ptDNA. Klentaq, however, does not have proofreading ability, nor an exonuclease site, and has only ever been observed to bind DNA in the polymerization mode [9,24]. In fact, the key amino acids identified as being somehow involved in Klenow's proofreading activity [25] are all missing in Klentaq. Again the free energy differences are rather small, both Klenow and Klentaq bind the mismatched DNA ≤0.7 kcal/mol tighter than the matched DNA, but this difference would predict that if these two proteins encountered equal concentrations of matched and mismatched DNA, they would both be 2–3 times more likely to bind to the mismatched DNA.

4. Discussion

In this study, we have examined the thermodynamics of binding of two different Pol I DNA polymerases to different replicative or repair-type DNA substrates, and found that the evolutionarily older polymerase (*Taq*) does not really discriminate among the different potential DNA substrates, while the evolutionarily younger polymerase (*E. coli*) has the capability of distinguishing among these potential intracellular binding sites. The polymerase from *E. coli* generally binds preferentially to replicative-type substrates (ptDNA>nicks, gaps, or unusual end-structures), but the preferences are only on the order of up to 2.4 kcal/mole. This indicates that while the *E. coli* polymerase

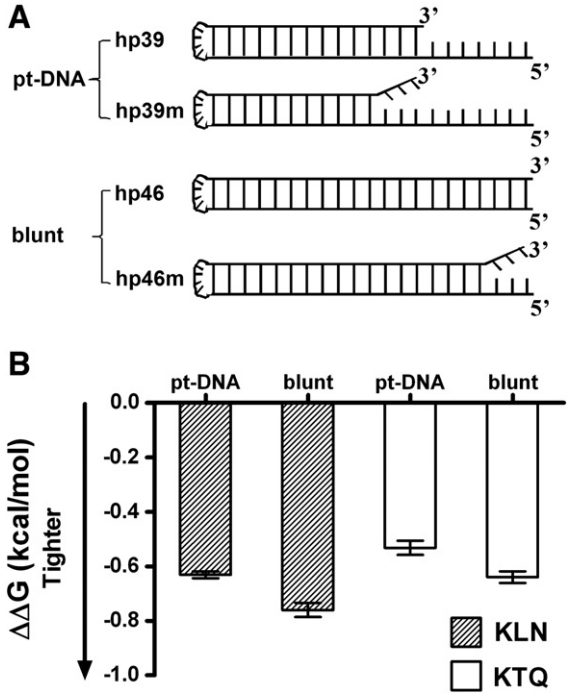

Fig. 6. Binding of mismatched DNA to Klenow and Klentaq polymerasese. The top panel shows schematics of the different DNA structures. Data are shown as ΔΔG values relative to the same construct without mismatches: $\Delta\Delta G = \Delta G_{mismatched} - \Delta G_{matched}$. Negative values indicate tighter binding of the mismatched DNA constructs.

definitely prefers replicative (or long gap) substrates, it does not effectively (thermodynamically) exclude any of these substrates. The gradual decrease in the affinity of Klenow for shorter gaps leading to the nick also makes some sense from a physiological point of view, since after filling a gap Klenow is likely to dissociate from the nick and allow ligase to close the nick (alternatively, Klenow also has the capability to continue forward via displacement synthesis at the nick).

Another conclusion from this data is that the single-stranded portion of the DNA is more important for Klenow binding than for Klentaq binding. This fact has been demonstrated in previous studies of primer-template DNA with different overhang lengths [16,19]. The data in this study extend these findings to include the single-stranded portion of the DNA within a gap, which also clearly contributes to the total binding free energy for Klenow, but not for Klentaq.

While the data clearly indicate that Klenow has a more sophisticated ability to discriminate among different DNAs than Klentaq does, a key question in the further interpretation of the data is: how much of this difference is due to Klenow's proofreading site? The Klenow exo- mutant used in these studies (and nearly all studies of Klenow in the past two decades) does not have exonuclease activity, but still binds DNA in the exonuclease site [15]. The easiest/quickest answer to this question is the assumption that these differences must simply be due to the proofreading site, however, all but one set of DNA substrates are DNAs with fully matched duplex regions, where the partitioning into the proofreading site will be minimal (between 5 and 15%) [22,26,27, Brown & LiCata, unpublished]. In addition, the presence or absence of Mg^{+2}, which is believed to alter the partitioning into the proofreading site [23], does not alter most of the quantitative results nor any of the qualitative results of this study (e.g. the data on 3′-phosphorylated gap binding in Fig. 3 show the largest Mg^{+2} effect observed in this study, but Klenow still shows greater substrate discrimination than Klentaq in both the presence and absence of Mg^{+2}). Despite these arguments, however, we cannot definitively rule out the potential participation of the proofreading site in Klenow's greater discrimination among these substrates.

Related to this discussion of the potential role of the proofreading site in Klenow, possibly the most unusual result in this study is the finding that both Klenow and Klentaq bind mismatched DNA more tightly than matched DNA, and by about the same amount. Yet Klentaq does not have a proofreading site, nor has it ever been observed to bind DNA outside the polymerization site. This finding suggests that DNA end protection is an extremely important function for both polymerases. Protection of unmatched end structures has been implicated in non-homologous end-joining [11,12]. We chose to examine DNA with a three-base mismatch because Klenow is known to bind such a DNA completely in the editing mode, by pulling the primer strand into the proofreading site [6,8,22,26,27], whereas the relative partitioning of editing-mode to polymerization mode binding of 1- and 2-mismatched DNA to Klenow remains controversial. The topology of binding of mismatched DNA to Klentaq has not been previously addressed.

The importance of the end-binding/end-protection role of the polymerases is also supported by the finding that both polymerases bind quite well to a DNA to an inverted end-structure: an overhanging primer instead of an overhanging template. Many DNA binding proteins are known to exhibit "non-specific" binding to DNA ends, but what is unusual in these results is that the affinities of the polymerases for the different non-extendable ends are so close to the affinities for the productive substrates: close enough that competition among all of these different DNA "targets" for the 400 or so Pol I molecules within a prokaryotic cell would be very effective.

4.1. Comparisons with other polymerases

The characterization of eukaryotic polymerase Pol β by Bujalowski and associates constitute the most extensive and detailed thermodynamic studies of polymerase binding to gapped-DNA carried out in

any system to date [20,28,29]. Some of the differences between Pol I and Pol β gap binding were discussed above in Results. Bujalowski and associates have also studied gap binding by Pol X [30], and enzymatic studies of gap filling activity have been carried out for a number of DNA polymerases [31–34]. Among all of these studies (present studies included), one thing is clear: there are no universal rules for polymerase gap binding. Pol X only functions on gaps where the single-stranded DNA portion is less than 4 nucleotides long [30]. Both Pol β and Pol λ processively fill short gaps (less than 5 or 6 nucleotides) with a requirement for a 5′-phosphate at the 5′ end of the gap [33,34]. Pol α prefers long gaps with >30 nucleotides of single-stranded DNA in the gap [32–34]. The presence of a 5′-phosphate stimulates the extension of the primer by PaePOL when the gap is more than 2 nucleotides long [32]. For Pol β, the affinity of the polymerase is not especially dependent on the size of the gap, unless the gap is 5′-phosphorylated — then longer gap binding will be enhanced, and 5′-phosphorylation within the gap and the presence of Mg^{+2} are antagonistic for Pol β [20].

Attempting to integrate the findings for these different polymerases quickly leads to the conclusion that characterizing gap binding behavior in one polymerase has virtually no predictive capability for any other polymerase. Gap binding characteristics clearly change among polymerases with different specializations and between prokaryotes and eukaryotes. In that sense the Klenow/Klentaq pair represents a unique comparison at the evolutionary onset of gap recognition: a comparison between two Pol I family polymerases where one has not yet developed the capability to distinguish gaps from other DNA structures, and one that is just beginning to make such distinctions.

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References

[1] A. Kornberg, T.A. Baker, DNA Replication, 2nd Edition ed. Freeman, New York, 1992.
[2] U. Sibghat, A. Sancar, J.E. Hearst, The repair patch of *E. coli* (A)BC excinuclease, Nucleic Acids Res. 18 (1990) 5051–5053.
[3] K.R. Tindall, T.A. Kunkel, Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase, Biochemistry 27 (1988) 6008–6013.
[4] K.A. Eckert, T.A. Kunkel, High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase, Nucleic Acids Res. 18 (1990) 3739–3744.
[5] L.S. Beese, J.M. Friedman, T.A. Steitz, Crystal structures of the Klenow fragment of DNA polymerase I complexed with deoxynucleoside triphosphate and pyrophosphate, Biochemistry 32 (1993) 14095–14101.
[6] L.S. Beese, V. Derbyshire, T.A. Steitz, Structure of DNA polymerase I Klenow fragment bound to duplex DNA, Science 260 (1993) 352–355.
[7] C.M. Joyce, T.A. Steitz, Polymerase structures and function: variations on a theme? J. Bacteriol. 177 (1995) 6321–6329.
[8] P.S. Freemont, J.M. Friedman, L.S. Beese, M.R. Sanderson, T.A. Steitz, Cocrystal structure of an editing complex of Klenow fragment with DNA, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 8924–8928.
[9] S. Korolev, M. Nayal, W.M. Barnes, E. Di Cera, G. Waksman, Crystal structure of the large fragment of *Thermus aquaticus* DNA polymerase I at 2.5-A resolution: structural basis for thermostability, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 9264–9268.
[10] S.H. Eom, J. Wang, T.A. Steitz, Structure of Taq polymerase with DNA at the polymerase active site, Nature 382 (1996) 278–281.
[11] J.S. King, C.F. Fairley, W.F. Morgan, DNA end joining by the Klenow fragment of DNA polymerase I, J. Biol. Chem. 271 (1996) 20450–20457.
[12] J. King, C. Fairley, W. Morgan, The joining of blunt DNA ends to 3′-protruding single strands in *Escherichia coli*, Nucleic Acids Res. 26 (1998) 1749–1754.
[13] C.M. Joyce, V. Derbyshire, Purification of *Escherichia coli* DNA polymerase I and Klenow fragment, Methods Enzymol. 262 (1995) 3–13.
[14] K. Datta, V.J. LiCata, Salt dependence of DNA binding by *Thermus aquaticus* and *Escherichia coli* DNA polymerases, J. Biol. Chem. 278 (2003) 5694–5701.
[15] V. Derbyshire, P.S. Freemont, M.R. Sanderson, L. Beese, J.M. Friedman, C.M. Joyce, T.A. Steitz, Genetic and crystallographic studies of the 3′,5′-exonucleolytic site of DNA polymerase I, Science 240 (1988) 199–201.
[16] A.J. Wowor, K. Datta, H.S. Brown, G.S. Thompson, S. Ray, A. Grove, V.J. LiCata, Thermodynamics of the DNA structural selectivity of the Pol I DNA polymerases from *Escherichia coli* and *Thermus aquaticus*, Biophys. J. 98 (2010) 3015–3024.
[17] V.J. LiCata, A.J. Wowor, Applications of fluorescence anisotropy to the study of protein–DNA interactions, Methods Cell Biol. 84 (2008) 243–262.
[18] L. Dzantiev, L.J. Romano, Interaction of *Escherichia coli* DNA polymerase I (Klenow fragment) with primer-templates containing N-acetyl-2-aminofluorene or N-2-aminofluorene adducts in the active site, J. Biol. Chem. 274 (1999) 3279–3284.
[19] R.M. Turner Jr., N.D. Grindley, C.M. Joyce, Interaction of DNA polymerase I (Klenow fragment) with the single-stranded template beyond the site of synthesis, Biochemistry 42 (2003) 2373–2385.
[20] M.J. Jezewska, R. Galletto, W. Bujalowski, Rat polymerase beta gapped DNA interactions: antagonistic effects of the 5′ terminal PO4 — group and magnesium on the enzyme binding to the gapped DNAs with different ssDNA gaps, Cell Biochem. Biophys. 38 (2003) 125–160.
[21] R. Prasad, W.A. Beard, S.H. Wilson, Studies of gapped DNA substrate binding by mammalian DNA polymerase beta. Dependence on 5′-phosphate group, J. Biol. Chem. 269 (1994) 18096–18101.
[22] M.F. Bailey, E.J. van der Schans, D.P. Millar, Thermodynamic dissection of the polymerizing and editing modes of a DNA polymerase, J. Mol. Biol. 336 (2004) 673–693.
[23] K. Datta, N.P. Johnson, V.J. LiCata, P.H. von Hippel, Local conformations and competitive binding affinities of single- and double-stranded primer-template DNA at the polymerization and editing active sites of DNA polymerases, J. Biol. Chem. 284 (2009) 17180–17193.
[24] Y. Li, S. Korolev, G. Waksman, Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I: structural basis for nucleotide incorporation, EMBO J. 17 (1998) 7514–7525.
[25] L.S. Beese, T.A. Steitz, Structural basis for the 3′–5′ exonuclease activity of *Escherichia coli* DNA polymerase I: a two metal ion mechanism, EMBO J. 10 (1991) 25–33.
[26] T.E. Carver Jr., R.A. Hochstrasser, D.P. Millar, Proofreading DNA: recognition of aberrant DNA termini by the Klenow fragment of DNA polymerase I, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 10670–10674.
[27] E.H. Thompson, M.F. Bailey, E.J. van der Schans, C.M. Joyce, D.P. Millar, Determinants of DNA mismatch recognition within the polymerase domain of the Klenow fragment, Biochemistry 41 (2002) 713–722.
[28] M.J. Jezewska, R. Galletto, W. Bujalowski, Tertiary conformation of the template-primer and gapped DNA substrates in complexes with rat polymerase beta. Fluorescence energy transfer studies using the multiple donor-acceptor approach, Biochemistry 42 (2003) 11864–11878.
[29] S. Rajendran, M.J. Jezewska, W. Bujalowski, Recognition of template-primer and gapped DNA substrates by the human DNA polymerase beta, J. Mol. Biol. 308 (2001) 477–500.
[30] M.J. Jezewska, P.J. Bujalowski, W. Bujalowski, Interactions of the DNA polymerase X from African swine fever virus with gapped DNA substrates. Quantitative analysis of functional.
[31] H. Zhu, S. Shuman, Gap filling activities of Pseudomonas DNA ligase D (LigD) polymerase and functional interactions of LigD with the DNA end-binding Ku protein, J. Biol. Chem. 285 (2010) 4815–4825.
[32] R.K. Singhal, S.H. Wilson, Short gap-filling synthesis by DNA polymerase beta is processive, J. Biol. Chem. 268 (1993) 15906–15911.
[33] M. Garcia-Diaz, K. Bebenek, R. Sabariegos, O. Dominguez, J. Rodriguez, T. Kirchhoff, E. Garcia-Palomero, A.J. Picher, R. Juarez, J.F. Ruiz, T.A. Kunkel, L. Blanco, DNA polymerase lambda, a novel DNA repair enzyme in human cells, J. Biol. Chem. 277 (2002) 13184–13191.
[34] D.W. Mosbaugh, S. Linn, Gap-filling DNA synthesis by HeLa DNA polymerase alpha in an in vitro base excision DNA repair scheme, J. Biol. Chem. 259 (1984) 10247–10251.