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Replication through an abasic DNA lesion: structural basis for adenine selectivity

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Abasic sites represent the most frequent DNA lesions in the genome that have high mutagenic potential and lead to mutations commonly found in human cancers. Although these lesions are devoid of the genetic information, adenine is most efficiently inserted when abasic sites are bypassed by DNA polymerases, a phenomenon termed A-rule. In this study, we present X-ray structures of a DNA polymerase caught while incorporating a nucleotide opposite an abasic site. We found that a functionally important tyrosine side chain directs for nucleotide incorporation rather than DNA. It fills the vacant space of the absent template nucleobase and thereby mimics a pyrimidine nucleobase directing for preferential purine incorporation opposite abasic residues because of enhanced geometric fit to the active site. This amino acid templating mechanism was corroborated by switching to pyrimidine specificity because of mutation of the templating tyrosine into tryptophan. The tyrosine is located in motif B and highly conserved throughout evolution from bacteria to humans indicating a general amino acid templating mechanism for bypass of non-instructive lesions by DNA polymerases at least from this sequence family.

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Introduction

DNA is constantly damaged by endo- and exogenous agents. The most frequent DNA damage observed under physiological conditions are abasic sites resulting from spontaneous hydrolysis of the bond that connects the sugar and the nucleobase in DNA (Lindahl, 1993). It has been estimated that ~10 000 abasic sites are formed in a human cell per day (Lindahl and Nyberg, 1972; Loeb and Preston, 1986; Lindahl, 1993). Guanine and adenine nucleobase residues are cleaved most efficiently resulting in the abasic sugar moiety (AP, Figure 1A) with the loss of the genetic information stored in the nucleobase (Loeb and Preston, 1986). As these lesions are devoid of the genetic information they are potentially mutagenic. The bulk of this damage is removed by DNA repair systems, which use the sister strand to guide incorporation of the right nucleotide in places of the lesion. However, undetected lesions or those which are formed during S phase pose a challenge to DNA polymerases. Indeed, abasic sites are strong blocks for bypass DNA synthesis catalysed by DNA polymerases (Goodman, 2002; Hübscher *et al*, 2002). Additionally, several studies indicated the mutagenic potential of these lesions in translesion synthesis, which is more pronounced in animal compared with bacterial cells presumably because of higher translesion synthesis in eukaryotes (Schaaper *et al*, 1983; Avkin *et al*, 2002; Pagès *et al*, 2008). Although abasic sites are considered being non-instructive, *in vitro* and *in vivo* studies of the abasic site or the stabilized tetrahydrofuran analogue F (Figure 1B) have shown that adenine, and to a lesser extent guanine, is most frequently incorporated opposite the lesion. DNA polymerases are categorized into seven families according to their sequence homology (Ito and Braithwaite, 1991; Braithwaite and Ito, 1993; Ohmori *et al*, 2001). The strong preference of DNA polymerase for adenine incorporation is mainly found for DNA polymerases from family A (including human DNA polymerases γ and θ) and B (including human DNA polymerases α, ε, and δ) and has been termed 'A-rule' (Sagher and Strauss, 1983; Schaaper *et al*, 1983; Loeb and Preston, 1986; Lawrence *et al*, 1990; Goodman *et al*, 1994; Shibutani *et al*, 1997; Avkin *et al*, 2002; Strauss, 2002; Pagès *et al*, 2008). The apparent selectivity for incorporation of purines ultimately results in transversion mutations commonly found in human cancers (Hoeijmakers, 2001).

What are the determinants of enzymatic specificity for purines? The mechanistic basis for the A-rule is still under debate (Randall *et al*, 1987; Matray and Kool, 1999; Kool, 2002; Taylor, 2002; Freisinger *et al*, 2004; Hogg *et al*, 2004; Seki *et al*, 2004; Reineks and Berdis, 2004; Ling *et al*, 2004a; Fiala *et al*, 2007; Zahn *et al*, 2007; Chen *et al*, 2008; Beard *et al*, 2009; Nair *et al*, 2009). Structural and functional studies have added significantly to our understanding of the basic mechanisms of translesion synthesis by DNA polymerases (Yang and Woodgate, 2007). Intrinsic properties of purines like superior base-stacking ability of the incoming purine nucleotide to the nucleobase π-system at the primer end were proposed as being the driving forces for preferential purine selection when incorporating a nucleotide opposite an abasic site. Interestingly, it was found that DNA polymerases from diverse sequence families also catalyse non-templated nucleotide addition to the 3′-termini of blunt-ended DNA with a strong preference for incorporation of adenine that parallels the incorporation tendency opposite non-instructive abasic sites (Clark *et al*, 1987; Clark, 1988; Peliska and Benkovic, 1992; Hwang and Taylor, 2004; Fiala *et al*, 2007b). Here again

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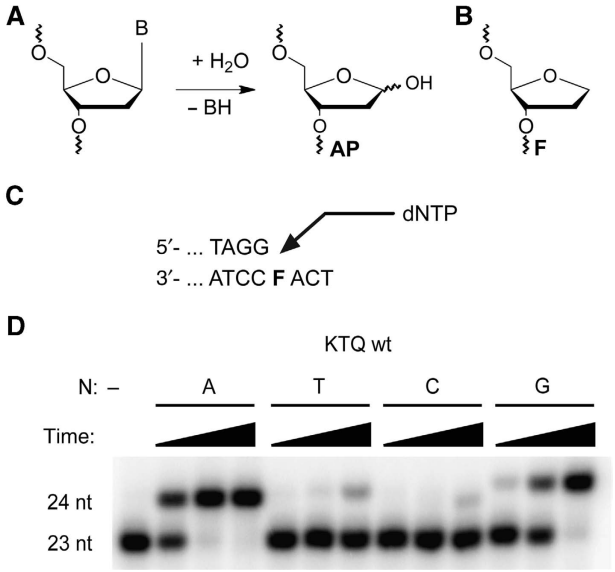

Figure 1 Nucleotide incorporation opposite an abasic site. (**A**) Hydrolysis of the glycosidic bond leading to nucleobase loss and formation of abasic site AP. B: nucleobase. (**B**) Structure of the abasic site analogue F. (**C**) Partial primer template sequence used in primer extension experiments. (**D**) Single nucleotide incorporation of *KlenTaq* opposite F for 2, 10, or 60 min, respectively. The respective dNTP is indicated.

superior base-stacking ability of adenine was suggested as a driving force for the enzymes to select an adenine nucleotide. Several structures from DNA polymerases belonging to DNA polymerase families X and Y in complex with a primer template duplex containing the abasic site analogue F and incoming 2′-deoxynucleoside-5′-triphosphate (dNTP) were recently reported (Ling *et al*, 2004a; Beard *et al*, 2009; Nair *et al*, 2009). DNA polymerases from these families use different, sequence-depending mechanisms that might compete with the A-rule when bypassing abasic sites. However, DNA polymerases from family A and B, which are involved in the majority of DNA synthesis in DNA replication and repair, follow the A-rule when bypassing abasic sites. Up to now only structures of RB69 DNA polymerase from family B were reported including one structure in complex with an abasic site lesion and an incoming dGTP (Freisinger *et al*, 2004). This structure as well as another structure of RB69 DNA polymerase that captures an artificial 5-nitro-1-indoyl-nucleotide, which is unable to undergo hydrogen bonds like natural nucleotides but is more efficiently incorporated opposite abasic sites than dAMP because of increased stacking ability, suggest that base stacking is likely to have a paramount role in the selective incorporation of dAMP opposite abasic sites (Zahn *et al*, 2007).

In this study, we present two structures of the large fragment of *Thermus aquaticus* DNA polymerase that follows the A-rule. In these structures, the enzyme is caught while incorporating either an adenine opposite an abasic site or at the 3′-terminus of a blunt-end primer template duplex. These structures provide insights into the mechanistic origin for purine selectivity of this DNA polymerase in absence of nucleobase information. Interestingly, in both structures we found the incoming triphosphate positioned in a way that obviates stacking interactions to the primer template duplex. Instead, a tyrosine protein side chain fills the space of the absent template nucleobase at the site of the nascent nucleobase pair and thereby supports hydrogen bond networks with the incoming adenine nucleotide. Furthermore, the shape and size of the adenine tyrosine pair mimics the geometries of canonical base pairs indicating that geometric complementarity to the active site of the enzyme adds to the selection of purines in the absence of templating nucleobase information. This tyrosine is highly conserved throughout evolution in DNA polymerase family A from bacteria to humans suggesting that this 'amino acid side chain templating' is a general mechanism for bypass of non-instructive lesions by DNA polymerases from this sequence family. The mechanistic model of 'amino acid side chain templating' is further corroborated by the results of mutagenesis studies in which the selectivity for purine incorporation was switched to pyrimidines on substituting the six-membered aromatic phenol of the templating tyrosine to the bicyclic indole of tryptophan.

Results

KlenTaq follows the A-rule

Significant mechanistic insights of nucleotide incorporation during DNA polymerization were derived from high-resolution structures of *KlenTaq*, an N-terminally truncated form of *T. aquaticus* (*Taq*) DNA polymerase (Korolev *et al*, 1995; Li *et al*, 1998; Li and Waksman, 2001; Rothwell *et al*, 2005). *KlenTaq* is a member of family A DNA polymerases that are involved in prokaryotic and eukaryotic repair of DNA lesions (Goodman, 2002; Hübscher *et al*, 2002; Seki *et al*, 2004). It has been shown that DNA polymerases from this family follow the A-rule (Sagher and Strauss, 1983; Shibutani *et al*, 1997; Seki *et al*, 2004), which we found for *KlenTaq* as well (Figure 1C and D). Single nucleotide incorporation opposite F was conducted with all four dNTPs and preferential incorporation of dAMP was observed (Figure 1D). Quantification of these findings by pre-steady-state kinetics confirmed that indeed dAMP is most efficiently incorporated opposite the abasic site F followed by dGMP (Table I). Consistently with earlier findings of A family enzymes pyrimidines are significantly less efficiently processed opposite the lesion.

Crystallization and structure determination of KlenTaq in complex with incoming nucleoside triphosphate opposite abasic site

To show the structural basis for the ability of DNA polymerases to promote abasic site bypass according to the A-rule we crystallized *KlenTaq* in complex with relevant substrates. We were able to obtain several crystals and could solve the structure of a ternary complex of *KlenTaq* bound to an abasic site, containing a primer, template, and an incoming 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) opposite the lesion (henceforth termed $KlenTaq_{AP}$ for simplicity). The crystals were obtained by a strategy used earlier for *KlenTaq* (Li and Waksman, 2001) bound to non-damaged substrates. The structure was solved by difference Fourier techniques and provides a snapshot of nucleotide incorporation opposite an abasic site F in the template strand at a resolution of 2.3 Å (Supplementary Table I).

Table I Transient kinetic analysis of nucleotide incorporation opposite abasic site F by *KlenTaq* and mutants

Enzyme	Template	dNTP	k_{pol} ($s^{-1} \times 10^{-2}$)	K_d (μM)	k_{pol}/K_d ($\mu M^{-1} s^{-1} \times 10^{-4}$)
Wild type	dT	A	516 ± 44	15.3 ± 4.7	3373
Wild type	F	A	2.73 ± 0.23	149 ± 35	1.83
Wild type	F	G	0.43 ± 0.01	65.5 ± 5.2	0.66
Wild type	F	T	0.03 ± 0.006	299 ± 122	0.01
Wild type	F	C	0.02 ± 0.001	294 ± 17	0.007
Y671A	dT	A	1.06 ± 0.06	168 ± 26	0.63
Y671A	F	A	0.02 ± 0.002	304 ± 68	0.007
Y671A	F	G	0.05 ± 0.002	182 ± 23	0.03
Y671A	F	T	NA	NA	NA
Y671A	F	C	NA	NA	NA
Y671F	dT	A	2265 ± 161	54.1 ± 12.0	4187
Y671F	F	A	1.16 ± 0.06	233 ± 30	0.50
Y671F	F	G	0.33 ± 0.03	294 ± 54	0.11
Y671F	F	T	0.02 ± 0.002	245 ± 64	0.008
Y671F	F	C	0.01 ± 0.001	262 ± 65	0.004
Wild type	dT	d3A	59.6 ± 3.3	95.4 ± 13.4	62.5
Wild type	F	d3A	0.12 ± 0.01	572 ± 67	0.02
Y671W	dT	A	91.6 ± 15.2	363 ± 104	25.2
Y671W	F	A	0.10 ± 0.02	514 ± 182	0.02
Y671W	F	G	NA	NA	NA
Y671W	F	T	0.35 ± 0.03	382 ± 76	0.09
Y671W	F	C	0.92 ± 0.12	845 ± 161	0.11

NA, Not accessible because the turnover after 1 h using 600 μM dNTP was <20%.

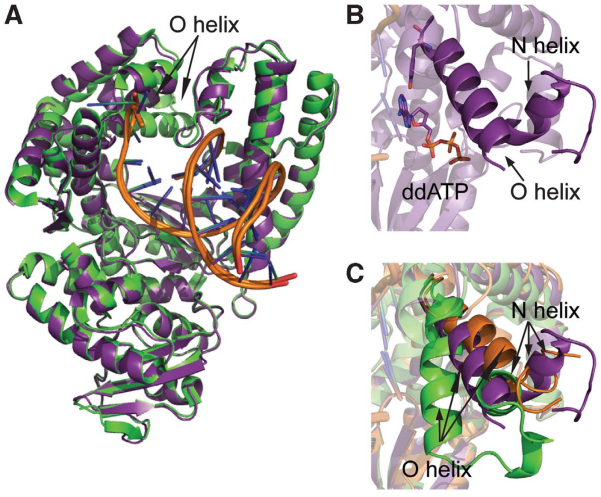

Figure 2 Structures of *KlenTaq* in complex with substrates. (**A**) Structure of $KlenTaq_{AP}$ (purple) superimposed with *KlenTaq* (green, PDB 1QSY) bound to undamaged DNA. The location of the O helix is indicated. (**B**) Close-up view highlights the location of the O and N helices in $KlenTaq_{AP}$. (**C**) Superimposition of $KlenTaq_{AP}$, *KlenTaq* (green, PDB 1QSY, closed conformation) and *KlenTaq* (orange, PDB 2KTQ, open conformation).

Overall DNA polymerase structure in complex with abasic site template

KlenTaq in complex with ddATP opposite an abasic site template adopted an overall conformation very similar to the one in a ternary complex of the enzyme bound to non-damaged DNA and ddATP (PDB 1QSY; Li and Waksman, 2001) described earlier resulting in a root mean square deviation for Cα of 0.45 Å (Figure 2A). However, remarkable structural changes are found in the finger domain of the enzyme especially in the orientation of the O helix near the primer terminus and the dNTP-binding site (Figure 2B and C). Previously described structures of the ternary complexes of *KlenTaq* bound to undamaged DNA and ddNTP in an open (PDB 2KTQ, Li *et al*, 1998) and a closed (PDB 1QSY) conformation show that switching from the open form to the closed conformation causes a structural change of 46° of the finger domain. In the open conformation the orientation of the O helix resembles the binary structure of *KlenTaq* in complex with the primer template (PDB 4KTQ). The positioning of the O helix allows binding of the substrates in the open conformation, whereas in the closed conformation the O helix packs against the templating nucleobase and incoming dNTP and thereby closes the active site. However, in the $KlenTaq_{AP}$ structure, the O helix is in a conformation that leaves the active site more open compared with the closed structure containing the undamaged primer template complex. The overall orientation of the O helix is somehow between the open and closed conformations, illustrated in the superimposition of the structures (Figure 2C; Supplementary Figure S2).

Protein side chain and substrate conformations at the abasic site

The abasic site is intrahelically placed within the DNA substrate and the tetrahydrofuran moiety rotated by about 90° in comparison to the respective sugar conformation found in canonical *KlenTaq* structures (Supplementary Figure S1). The incoming ddATP is stabilized by a network of hydrogen bond interactions. Arg587 adopts a conformation that allows the formation of hydrogen bonds between the primer strand and the incoming nucleotide (Figure 3A). The distances found indicate bonding of the Arg587 guanidinium

group to the 3′-terminal nucleotide in the primer strand by binding to N7 of the adenine and the phosphodiester as well as to N7 of the adenine of the incoming ddATP. The conformation of Arg587 in the *KlenTaq*$_{AP}$ structure differs from the conformations described in the earlier structures where such interactions were not found (Figure 3A and B). Furthermore, the nucleobase of the incoming ddATP is positioned in a cleft formed by Phe667 and Tyr671 (Figures 3A and 4A). The distance between the hydroxyl group of Tyr671 and the N3 of adenine indicates a hydrogen bonding interaction between these residues (Supplementary Figure S3). Thereby, the aromatic side chain of Tyr671 fills the vacant space that has been left by the missing nucleobase and may act as a positioning device to substitute for the missing nucleobase (Figure 4A). These interactions place the incoming ddATP opposite the abasic site in a position where stacking interactions to the nucleobase π-system at the primer end, which are found in the non-damaged complexes, are lost (Figure 4C and D). The Tyr671, on the other hand, is positioned in a way that it nicely stacks to the template nucleobase positioned 3′ of the abasic site (Figure 4C and D).

Compared with the earlier *KlenTaq* structure bound to canonical substrates (PDB 1QSY) the incoming nucleotide aligns in a different manner in the *KlenTaq*$_{AP}$ structure. The distance from the primer 3′-terminus to the α-phosphate of the ddATP is larger by 2.3 Å (Figure 3C and D). However, the distance from the oxygen that bridges α- and β-phosphate groups of the triphosphate moiety and Lys663, which was previously discussed to act as a general acid in catalysis of nucleotide bond formation (Castro *et al*, 2009), is found to be shorter by 0.9 Å (Figure 3C and D). DNA polymerases use a general two-metal ion mechanism (Fothergill *et al*, 1995; Steitz, 1998; Yang *et al*, 2006) to catalyse the nucleophilic substitution of pyrophosphate by the 3′-hydroxyl group of the primer. However, in the *KlenTaq*$_{AP}$ structure only one Mg^{2+} is found coordinating to the triphosphate moiety and involved in interaction with two water molecules (Figure 3C and D). The two other water molecules form several hydrogen bonds to the amino acid residues known to be essential for catalysis. This altered arrangement may account for the observed 1840-fold decline in dAMP incorporation efficiency (k_{pol}/K_d) opposite F compared with incorporation opposite dT (Table I) and stalling of the DNA polymerase when encountering the lesion.

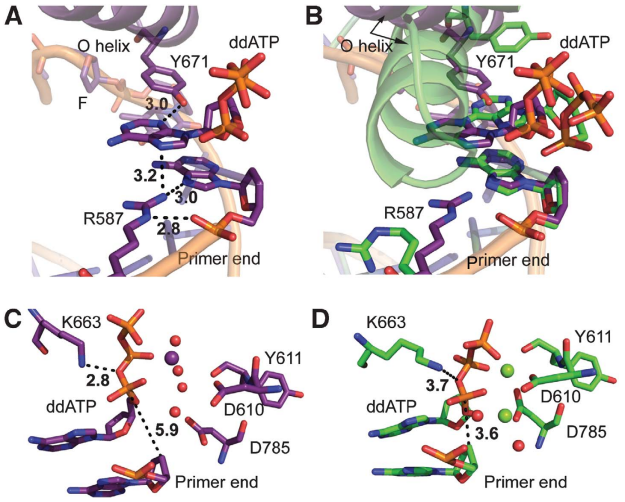

Figure 3 Interaction network of incoming ddATP opposite abasic site F. (**A**) Hydrogen bond network stabilizing ddATP. Labelled are the amino acid side chains R587 and Y671. (**B**) Superimposed structures of *KlenTaq*$_{AP}$ (purple) and *KlenTaq* (green, PDB 1QSY) show the difference in orientation of residues R587 and Y671. (**C**) Inner coordination spheres showing metal ion in *KlenTaq*$_{AP}$. A Mg^{2+} ion (purple sphere) is coordinated by the triphosphate moiety of ddATP and two water molecules (red spheres). Two additional water molecules form hydrogen bonds to residues D610, Y611, and D785. Residue K663, recently, discussed to act as general acid in catalysis, is shown. (**D**) *KlenTaq* (PDB 1QSY) showing the same residues as in (**C**). All distances are in Å.

Tyrosine 671 mimics the absent nucleobase in the template strand

The positioning of Tyr671 opposite the incoming ddATP at the place that is usually occupied by the templating nucleobase suggests that the protein side chain is a templating device and mimics the shape and size of a six-membered pyrimidine nucleobase in the template strand. These active site constraints might determine the preferential incorporation of adenine by a close geometric fit to the enzyme active site

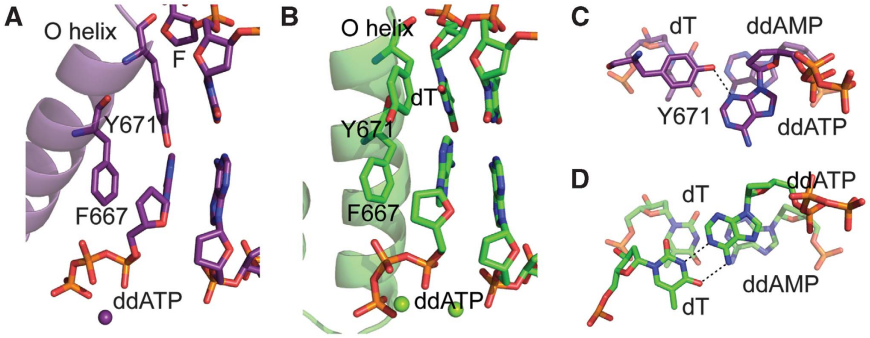

Figure 4 Active site and nascent base pair assemblies. (**A**) Close-up view of *KlenTaq*$_{AP}$ active site processing ddATP opposite abasic site F. Shown are O helix, residues Y671, and F667, the respective template residues and incoming ddATP. (**B**) Structure of *KlenTaq* (PDB 1QSY) active site processing ddATP opposite template dT. (**C**) Top view to the nascent base pair opposite F. In the front the incoming ddATP opposite Y671 is depicted. The hydrogen bond between the hydroxyl group of Y671 and N3 of adenine is indicated as dashed line. In transparent the first nucleobase pair of the primer template terminus is shown. (**D**) Top view of the nascent base pair in *KlenTaq* (PDB 1QSY). The hydrogen bonding between the incoming ddATP and the templating dT is shown in dashed lines. The primer template terminus is illustrated in transparent.

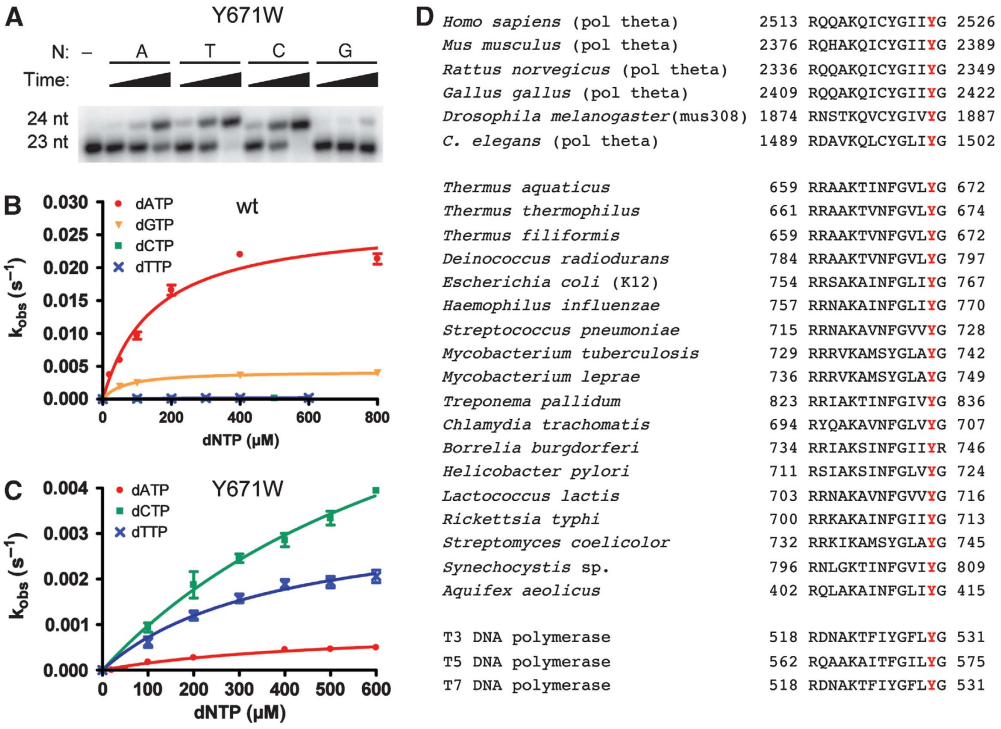

Figure 5 Nucleotide incorporation opposite abasic site F. (**A**) Single nucleotide incorporation of *KlenTaq* Tyr671Trp mutant opposite F for 2, 10, or 60 min, respectively. The respective dNTP is indicated. (**B**, **C**) Pre-steady-state kinetics of single nucleotide incorporation opposite F catalysed by *KlenTaq* wild type (**B**) and Tyr671Trp mutant (**C**), respectively. The curves show dependence of the observed pre-steady-state rates (k_{obs}) on dNTP concentration. The k_{obs} values were plotted versus the concentration of the used dNTP and fitted to a hyperbolic equation. (**D**) Amino acid sequence alignment of DNA polymerases highlighting the conserved position equivalent to Y671 in *KlenTaq*.

(Goodman, 1997; Kool, 2002). To study the functional role of Tyr671 in abasic site lesion bypass we constructed mutants at this position. The Tyr671Ala mutant was constructed to test the requirement of an aromatic residue at this position. Indeed, this mutation led to an enzyme that has significantly reduced activity in general. The efficiency of dAMP incorporation opposite canonical template dT is more than 5350-fold reduced (Table I). Incorporation efficiency opposite an abasic site is further reduced and we were able to measure the incorporation of dAMP and dGMP only. Interestingly, the enzyme lost its preference of incorporation of dAMP opposite an abasic site and instead incorporates dGMP four-fold more efficient. To probe the effects of the hydroxyl group in Tyr671 on enzyme activity we generated the Tyr671Phe mutant next. We found that this enzyme is highly active when non-damaged substrates are processed. The Tyr671Phe mutant follows the A-rule; however, in comparison to the wild type, this enzyme incorporates dAMP opposite an abasic site with four-fold reduced efficiency resulting from both, an increased K_d and a reduced k_{pol}, indicating the requirement of the hydroxyl group for efficient catalysis.

To further analyse the interaction between the hydroxyl group of Tyr671 and N3 of adenine in the incoming nucleotide an adenine analogue in which the N3 is substituted by a non-polar CH (namely 3-deaza-2′-deoxyadenosine-5′-triphosphate (d3DATP)) was used in primer extension studies (Supplementary Figure S4). These kinds of purine analogues were intensively studied to access the role of N3–hydrogen bond acceptors in DNA polymerase function (Spratt, 2001; Washington *et al*, 2003; Meyer *et al*, 2004; Moore *et al*, 2004; McCain *et al*, 2005; Wolfle *et al*, 2005; Beckman *et al*, 2007; Cavanaugh *et al*, 2009; Trostler *et al*, 2009). In accordance with earlier reports (Trostler *et al*, 2009) we found that incorporation efficiency of the analogue was decreased by 54-fold compared with the natural substrate (Table I). Interestingly, incorporation efficiency of d3DAMP opposite the abasic site was decreased by more than 90-fold compared with dAMP incorporation opposite the lesion. These studies further highlight the importance of the interaction of the hydroxyl group of Tyr671 and N3-adenine in the incoming nucleotide.

Next, the mutation Tyr671Trp was constructed. As this mutation will transform the six-membered phenol ring of Tyr671 into a bicyclic indole consisting of a six-membered ring fused to a five-membered ring, the amino acid side adopts an approximate size of a purine. We assumed that, if the amino acid side chain at this position indeed inherited the templating role of the absent nucleobase, the smaller pyrimidine nucleotides thymidine and cytidine should be more preferentially incorporated opposite an abasic site because of the closer geometric fit of the tryptophane side chain to purines. Indeed, this was observed in primer extension studies and quantified by measurement of the kinetics (Figure 5A–C; Table I). Although the wild type enzyme incorporates dTMP and dCMP with 183- and 261-fold reduced efficiency opposite the abasic site compared to dAMP, respectively, the contrary is observed for Tyr671Trp. In the Tyr671Trp mutant enzyme the preference for dATP vanished and instead the pyrimidines dTMP and dCMP are about five-fold more efficiently incorporated than dAMP (Figure 5A–C; Table I).

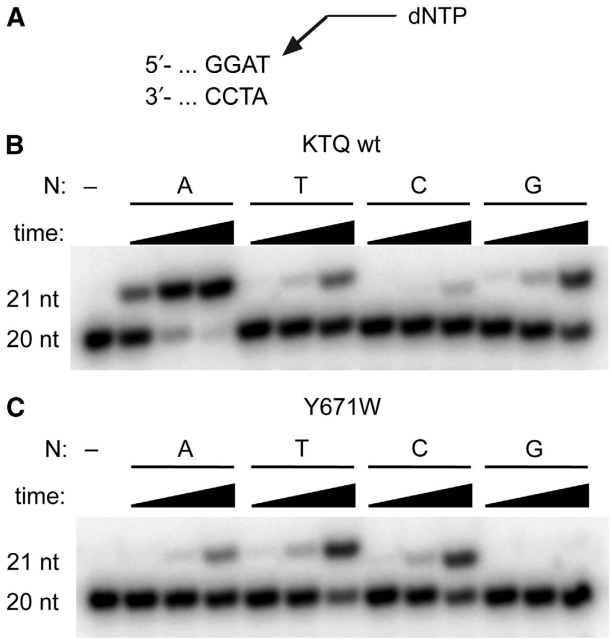

Figure 6 Nucleotide incorporation at a blunt-end primer template duplex. (**A**) Partial sequence used in primer extension experiments. (**B**) Single nucleotide incorporation of *KlenTaq* by extension of blunt-end primer template duplex for 2, 10, or 60 min, respectively. The respective dNTP is indicated. (**C**) As in (**B**) for *KlenTaq* mutant Tyr671Trp in presence of a blunt-end primer template duplex.

Template-independent extension of blunt-end primer templates by KlenTaq

Many DNA polymerases catalyse non-templated nucleotide addition to the 3′-termini of blunt-ended DNA with a strong preference for incorporation of adenine (Clark *et al*, 1987; Clark, 1988; Peliska and Benkovic, 1992; Hwang and Taylor, 2004; Fiala *et al*, 2007b). The same holds true for *KlenTaq* as our analyses show (Figure 6; Table II). *KlenTaq* extends the blunt-end primer terminus by incorporation of dAMP about 19-fold more efficient than of dGMP. The pyrimidines are even less efficiently incorporated compared with dAMP (38-fold and 280-fold reduced efficiency for dTMP and dCMP, respectively). To study a potential templating effect of Tyr671 we analysed the Tyr671Trp mutant in blunt-end extension, next. Again, the mutation caused a shift of the incorporation specificity to preferential incorporation of pyrimidines (Figure 6; Table II). Thus, dTMP and dCMP are about two-fold more efficiently incorporated than dAMP by the mutated enzyme. We could not observe any significant incorporation of dGMP even after prolonged incubation times.

KlenTaq structure in complex with blunt-end primer template duplex and incoming nucleoside triphosphate

To generate structural insights into template-independent nucleotidyl transferase activity of *KlenTaq* we crystallized the enzyme in presence of the same primer used before, a template and ddATP. By the incorporation of ddAMP at the primer terminus a blunt-end primer template duplex lacking a 3′-terminal hydroxyl group is formed and a ddATP captured at the active site for template-independent elongation of the primer end. This structure (henceforth termed $KlenTaq_{BE}$) was determined with a resolution of 2.2 Å (Supplementary Table I).

Table II Transient kinetic analysis blunt-end DNA duplex extension by *KlenTaq* and mutants

Enzyme	dNTP	k_{pol} ($s^{-1} \times 10^{-2}$)	K_d (μM)	k_{pol}/K_d ($\mu M^{-1} s^{-1} \times 10^{-4}$)
Wild type	A	3.11 ± 0.08	275 ± 13	1.13
Wild type	G	0.21 ± 0.01	371 ± 39	0.06
Wild type	T	0.13 ± 0.01	503 ± 98	0.03
Wild type	C	0.07 ± 0.02	1729 ± 620	0.004
Y671W	A	0.12 ± 0.01	450 ± 42	0.03
Y671W	G	NA	NA	NA
Y671W	T	0.52 ± 0.03	747 ± 69	0.07
Y671W	C	0.44 ± 0.04	746 ± 108	0.06

NA, Not accessible because the turnover after 1 h using 600 μM dNTP was <20%.

Comparison of the $KlenTaq_{BE}$ and $KlenTaq_{AP}$ structures shows high similarity resulting in a very low root mean square deviation for Cα of about 0.26 Å (Supplementary Figures S3 and S5). The close-up view of the O helix in $KlenTaq_{BE}$ and its superimposition to $KlenTaq_{AP}$ highlight the similarity (Figure 7). Both structures show the same orientation of the O helix and the interaction patterns of the amino acids Arg587 and Tyr671 are very much alike. The same holds true for the position of the incoming ddATP that is again placed in a way obviating significant stacking interactions to the nucleobase π-system at the primer end (Figure 7).

Discussion

Several studies show that adenine is most frequently incorporated opposite abasic site lesions in cells resulting in transversion mutations. Indeed, it has been shown that DNA polymerases from the sequence families A and B, which are involved in the majority of DNA synthesis in a cell, follow the A-rule (Sagher and Strauss, 1983; Randall *et al*, 1987; Shibutani *et al*, 1997; Matray and Kool, 1999; Taylor, 2002; Freisinger *et al*, 2004; Hogg *et al*, 2004; Seki *et al*, 2004). On the other hand, DNA polymerases from other sequence families like X and Y use different, sequence-depending mechanisms that might compete with the A-rule. Structures of these DNA polymerases complexed to abasic site containing DNA duplexes were reported for human DNA polymerases ι and β, and DNA polymerase IV (Dpo4) of *Sulfolobus Solfataricus* (Ling *et al*, 2004a; Beard *et al*, 2009; Nair *et al*, 2009). Human DNA polymerase ι incorporates a nucleotide opposite an abasic site with relatively small preference for G, T, and A over C. Structures of this enzyme in complex with an abasic site lesion and several dNTPs suggest that the enzyme forms an active site that stabilizes the incoming dNTP by distinct networks of hydrogen bonds (Nair *et al*, 2009). In contrast, DNA polymerase β and Dpo4 show obviation of the A-rule by predominantly inserting a nucleotide that is complementary to the first downstream templating nucleobase (Efrati *et al*, 1997; Ling *et al*, 2004a; Fiala *et al*, 2007; Beard *et al*, 2009). The structural data of Dpo4 show that the tendency for using the nucleobase downstream to the lesion stems from the ability of the enzyme to bulge out the lesion in an extrahelical position. In contrast, structures of RB69 DNA polymerase from family B that obeys the A-rule suggest that intrinsic properties of the purine nucleobase of the incoming dNTP like base stacking and

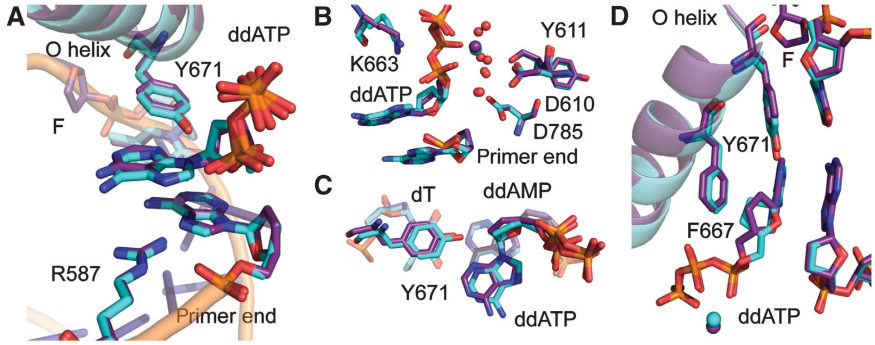

Figure 7 Comparison of $KlenTaq_{BE}$ (cyan) and $KlenTaq_{AP}$ (purple). (**A**) Superimposed structures of $KlenTaq_{BE}$ and $KlenTaq_{AP}$. (**B**) Comparison of the inner coordination sphere of metal ion in $KlenTaq_{BE}$ and $KlenTaq_{AP}$. (**C**) Superimposition of the top view of the nascent nucleobase pair of $KlenTaq_{BE}$ and $KlenTaq_{AP}$. (**D**) Close-up views of the active sites of $KlenTaq_{BE}$ and $KlenTaq_{AP}$. Labelled are O helix, residues F667, and Y671.

solvation properties are likely crucial for selection of dATP opposite the abasic site by this DNA polymerase sequence family (Freisinger *et al*, 2004; Hogg *et al*, 2004; Zahn *et al*, 2007).

Sequence family A members *Taq* DNA polymerase and *Escherichia coli* DNA polymerase I have been studied extensively in the past in their structure–function relationship (Patel *et al* 2001; Loh and Loeb 2005, Loh *et al*, 2007). We present here the first structure of a DNA polymerase that follows the A-rule and is complexed with an abasic site lesion and an incoming adenine opposite the lesion. The structure and mutagenesis studies show that the amino acid side chain of Tyr671, which is placed opposite the incoming ddATP at the location that is usually occupied by the templating nucleobase, confers the templating ability of the missing nucleobase at least to some extent. Previous studies on Tyr671 of *Taq* DNA polymerase and the corresponding Tyr766 residue in DNA polymerase I from *E. coli* have shown the involvement of this tyrosine residue in discriminating ribonucleotides and non-canonical nucleotides (Suzuki *et al*, 1996; Bell *et al*, 1997; Minnick *et al*, 1999). However, the role in translesion synthesis of this tyrosine has not been shown before. That amino acid templating of Tyr761 is involved in selecting a purine nucleotide is further corroborated by the finding that the respective tryptophan mutant lost its purine specificity and instead incorporates pyrimidines more efficiently. The result can be rationalized by assuming that the bicyclic indole consisting of a six-membered ring fused to a five-membered ring will mimic the approximate size and shape of a purine, and as a consequence, will direct for pyrimidine incorporation because of enhanced geometric fit to the enzyme active site. These findings corroborate the model of amino acid templating for abasic site bypass and highlight the importance of geometric fit of the substrates to the active site of the enzyme for DNA polymerase activity. In agreement with these observations, it has been shown that DNA polymerases are able to process non-natural nucleobase surrogates placed in template strands that mimic the shape and size of the natural nucleobase but have decreased hydrogen bonding capabilities (Kool, 2002).

Amino acid sequence alignment (Figure 5D) of A-family DNA polymerases discloses that the respective tyrosine is within motif B (Delarue *et al*, 1990) and is highly conserved throughout evolution from bacteria to humans. Owing to the high conservation of amino acids structure and sequence at these positions in DNA polymerases from prokaryotes to eukaryotes it is likely that the depicted mechanism of abasic site bypass is general and applies to other DNA polymerases in this sequence family as well. The mechanism of using protein residues to direct nucleotide incorporation is reminiscent of a transfer RNA CCA-adding enzyme (Xiong and Steitz, 2004) and the specialized DNA polymerase Rev1 that exclusively incorporates cytosine nucleotides (Nair *et al*, 2005). Unlike other DNA lesions abasic sites are devoid of any structural information that is at least residual in damaged nucleobases. Residual information is used by DNA polymerases to catalyse translesion synthesis through aberrant hydrogen bonding patterns of the incoming dNTP to the damaged template nucleobase. Bypass of DNA lesions such as thymidine dimers (Ling *et al*, 2003), 8-oxidized guanosine residues (Brieba *et al*, 2004; Hsu *et al*, 2004; Rechkoblit *et al*, 2006), or nucleobase adducts (Ling *et al*, 2004b; Nair *et al*, 2006) is promoted in this fashion.

Interestingly, all studied DNA polymerases that obviate the A-rule when encountering an abasic site show preference for incorporation of dAMP when catalysing the template-independent extension of blunt-end 3′ termini of DNA duplexes (Clark, 1988; Fiala *et al*, 2007b). These enzymes include DNA polymerases β, η, and Dpo4. A structure of Dpo4 in complex with a blunt-end DNA duplex and ddATP shows that the nucleobase and the sugar of the incoming ddATP stack to the nucleobase π-system of the blunt-end DNA duplex (Fiala *et al*, 2007b). This result corroborates earlier conclusions drawn from functional studies that intrinsic stacking interactions and solvation properties are driving forces for selection of adenine. However, our structural data presented here do not provide any evidence for stacking interactions of the incoming ddATP with the primer template terminus and rather indicate that amino acid templating applies for selection of adenine for DNA polymerases from sequence family A. This conclusion is corroborated by the findings that (a) no significant stacking of the incoming ddATP to the π-system of the DNA duplexes is observed in the structures and (b) mutation of the templating tyrosine to tryptophan switches the selectivity for purines to pyrimidines. Nevertheless, it cannot be excluded that stacking interactions might have a role in processes that are unresolved by the structural data, for example, at states prior or later on the reaction coordinate than the one resolved here.

Both structures presented here show different protein and substrate arrangements in the active site in the absence of

any templating nucleobase in comparison to the canonical complexes. They differ from the corresponding structure of ddATP in the *KlenTaq* structure with undamaged template DNA, which exhibits more favourable distances for formation of the new bond from the α-phosphate of the incoming nucleotide to the 3′-hydroxyl end of the primer (Korolev *et al*, 1995; Li *et al*, 1998; Li and Waksman, 2001). Moreover, the location of the α-phosphate of the incoming ddATP in *KlenTaq*$_{AP}$ obviates tight complexation of a Mg^{2+} ion by coordination together with D785 as found in the *KlenTaq* structure (Figure 3C and D) and, as a consequence, only one Mg^{2+} ion was found in the active site. This indicates that in the absence of the nucleobase larger conformational fluctuations as well as binding of a second Mg^{2+} ion would be required for phosphodiester bond formation, in accord with the observed decline in nucleotide incorporation efficiency and the strong block of the abasic site lesion. Bypass of the non-instructive abasic site by DNA polymerases is intrinsically error-prone. Thus, pausing resulting from the lesion might allow the DNA polymerase to be replaced by repair systems that use the sister strand for error-free repair. However, recently it was reported that the A-family member human DNA polymerase θ is able to bypass abasic sites with high activity and thereby incorporates dAMP opposite the lesion preferentially (Seki *et al*, 2004). DNA polymerase θ is involved in the diversification of immunoglobulin (Ig) genes during somatic hypermutation (Masuda *et al*, 2007). Hence, the efficient but intrinsically error-prone bypass of abasic sites by DNA polymerase θ following the A-rule might be of advantage for physiological mutation in somatic hypermutation of Ig genes.

Although our structural data show interaction patterns that stabilize purines in favour of pyrimidines as incoming nucleoside triphosphates, they do not directly allow drawing conclusions on the preference of adenine over guanine opposite an abasic site as well as at the blunt-end duplex. Intrinsic properties of the respective nucleobase like stacking and solvation properties may likely account for the preference of adenine. Modelling of a guanine in the position that is occupied by adenine in the structure indicates that the exocyclic C_2–NH_2 group of guanine shortens the distance to Tyr671 in comparison to the C_2–H in adenine (Supplementary Figure S6). This restriction of the active site might perturb conformational changes required for catalysis of nucleotide incorporation, and thereby adds to lowering the efficiency of dGMP incorporation by the enzyme.

Materials and methods

Proteins and oligonucleotides

Protein mutation and purification were conducted as described (Li *et al*, 1998). All oligonucleotides were purchased from Purimex, Germany and double HPLC purified. The nucleotide d3DATP was synthesized starting from d3DA purchased from Berry & Associates Inc., USA, according to published procedures (Di Pasquale *et al*, 2008).

Primer extension assays

Incorporation opposite dT or F: 20 μl of the *KlenTaq* reactions contained 100 nM primer (5′-d(CGT TGG TCC TGA AGG AGG ATA GG)-3′), 130 nM template (5′-d(AAA TCA TCC TAT CCT CCT TCA GGA CCA ACG TAC)-3′), or F-containing template (5′-d(AAA TCA FCC TAT CCT CCT TCA GGA CCA ACG TAC)-3′), respectively, 100 μM dNTPs in buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, and 2 mM $MgCl_2$) and 500 nM of the respective *KlenTaq* polymerase. Reaction mixtures were incubated at 37°C. Incubation times are provided in the respective figure legends. Primer was labelled using [γ^{32}P]-ATP according to standard techniques. Reactions were stopped by addition of 45 μl stop solution (80% [v/v] formamide, 20 mM EDTA, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol) and analysed by 12% denaturing PAGE. Visualization was performed by phosphoimaging. Blunt-end extension experiments were performed as described above using DNA primer (5′-d(CGT TGG TCC TGA AGG AGG AT)-3′) template (5′-d(ATC CTC CTT CAG GAC CAA CGA AA)-3′) strands that form a blunt-end only at one terminus.

Enzyme kinetics

The rate of single turnover, single nucleotide incorporation was determined by rapid quench flow kinetics using a chemical quench flow apparatus (RQF-3, KinTek Corp., University Park, PA) as described earlier (Di Pasquale *et al*, 2008). For reaction times longer than 5 s, a manual quench was performed. In brief, 15 μl of radiolabelled primer/template complex (200 nM) and DNA polymerase (2 μM) in reaction buffer were rapidly mixed with 15 μl of a dNTP solution in buffer at 37°C. Quenching was achieved by adding 0.3 M EDTA solution at defined time intervals before mixing with the previously described stop solution. For the analysis of dNTP incorporation opposite to the abasic site primer (sequences see above) and templates (sequences see above) were applied. Quenched samples were analysed on a 12% denaturing PAGE followed by phosphoimaging. For kinetic analysis experimental data were fit by nonlinear regression using the program GraphPad Prism 4. The data were fit to a single exponential equation: $[\text{conversion}] = A^*(1-\exp(-k_{\text{obs}}t))$. The observed catalytic rates (k_{obs}) were then plotted against the dNTP concentrations used and the data were fitted to a hyperbolic equation to determine the K_d of the incoming nucleotide. The incorporation efficiency is given by k_{pol}/K_d. Kinetic experiments for blunt-end measurements were performed under the same conditions. The identical primer/template duplex was used as described above.

Crystallization and structure determination

KlenTaq$_{AP}$. The crystallization was set up using a solution containing *KlenTaq* (102 μM) in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, DNA template (5′-d(AAA FTG CGC CGT GGT C)-3′ (300 μM), DNA primer (5′-d(GAC CAC GGC GC)-3′ (300 μM), ddATP (4 mM), 20 mM $MgCl_2$ and mixed in 1:1 ratio with the reservoir solution (0.05 M sodium cacodylate, pH 7, 0.2 M ammonium acetate, 0.01 M magnesium acetate, and 30% PEG 8000). Crystals were produced by the hanging drop vapour diffusion method by equilibrating against 1 ml of the reservoir solution for 5 days at 18°C.

KlenTaq$_{BE}$. The crystallization was set up with *KlenTaq* (113 μM) in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, template (5′-d(TG CGC CGT GGT C)-3′ (340 μM), primer (5′-d(GAC CAC GGC GC)-3′ (340 μM), ddATP (2.3 mM), 20 mM $MgCl_2$ and mixed in 1:1 ratio with the reservoir solution containing 0.05 M Tris–HCl, pH 8, 0.2 M ammonium chloride, 0.01 M calcium chloride, and 35% PEG 4000. Crystals were produced by the sitting drop vapour diffusion method by equilibrating against 1 ml of the reservoir solution for 5 days at 18°C. The crystals were frozen and analysed. Datasets were collected at the beamline PXIII (X06DA) at the Swiss Light Source of the Paul Scherrer Institute (PSI) in Villigen, Switzerland, at a wavelength 1.0 Å and using a Mar225 CCD detector. Data reduction was performed with the XDS package (Kabsch, 1993). The structures were solved by difference Fourier techniques using *KlenTaq* wild type (PDB 1QSY) as model. Refinement was performed with PHENIX (Adams *et al*, 2002) and model rebuilding was done with COOT (Emsley and Cowtan, 2004). The structures were solved and refined to a resolution of 2.3 Å in *KlenTaq*$_{AP}$ and 2.2 Å in *KlenTaq*$_{BE}$. Both structures were in the same space group $P3_121$, with cell dimensions *a, b* = 110.2 Å, *c* = 91.3 Å for *KlenTaq*$_{AP}$ and *a* ,*b* = 109.6 Å, *c* = 91.0 Å for *KlenTaq*$_{BE}$, respectively. The Mg^{2+} ion in the enzyme active sites of both structures was coordinated characteristic for a bivalent metal ion. Figures were made with PyMOL (DeLano, 2002).

Accession numbers
The coordinates and structure factors have been deposited in the Protein Data Bank with the accession numbers 3LWL and 3LWM.

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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