Published in final edited form as:

Biochim Biophys Acta. 2010 May ; 1804(5): 1113–1123. doi:10.1016/j.bbapap.2009.07.004.

Variations on a theme: eukaryotic Y-family DNA polymerases

M. Todd Washington^{a,*}, Karissa D. Carlson^b, Bret D. Freudenthal^a, and John M. Pryor^a

^a Department of Biochemistry, University of Iowa College of Medicine, Iowa City, IA 52242

^b Department of Chemistry, Northwestern College, Orange City, IA 51041

Abstract

Most classical DNA polymerases, which function in normal DNA replication and repair, are unable to synthesize DNA opposite damage in the template strand. Thus in order to replicate through sites of DNA damage, cell are equipped with a variety of non-classical DNA polymerases. These non-classical polymerases differ from their classical counterparts in at least two important respects. First, non-classical polymerases are able to efficiently incorporate nucleotides opposite DNA lesions while classical polymerases are generally not. Second, non-classical polymerases synthesize DNA with a substantially lower fidelity than do classical polymerases. Many non-classical polymerases are members of the Y-family of DNA polymerases, and this article focuses on the mechanisms of the four eukaryotic members of this family: polymerase eta, polymerase kappa, polymerase iota, and the Rev1 protein. We discuss the mechanisms of these enzymes at the kinetic and structural levels with a particular emphasis on how they accommodate damaged DNA substrates. Work over the last decade has shown that the mechanisms of these non-classical polymerases are fascinating variations of the mechanism of the classical polymerases. The mechanisms of polymerases eta and kappa represent rather minor variations, while the mechanisms of polymerase iota and the Rev1 protein represent rather major variations. These minor and major variations all accomplish the same goal: they allow the non-classical polymerases to circumvent the problems posed by the template DNA lesion.

Keywords

Translesion synthesis; DNA replication; DNA repair; Mutagenesis; enzyme kinetics; protein-DNA interactions

1. Introduction

Classical DNA polymerases, those that participate in DNA replication and repair and use non-damaged templates, synthesize DNA with remarkably high efficiency and high fidelity. They can achieve rates of as high as 1,000 nucleotide-incorporation events per second and achieve error rates as low as one error per 10^6 incorporations. What most of these enzymes cannot do, however, is incorporate nucleotides efficiently opposite template DNA lesion. Such lesions can be formed either spontaneously or by the exposure of DNA to various chemical agents and forms of radiation. Consequently, these lesions prevent nucleotide incorporation by classical DNA polymerases thus resulting in replication fork stalling.

*To whom correspondence should be addressed: M. Todd Washington, Department of Biochemistry, 4-403 Bowen Science Building, University of Iowa, Iowa City, IA 52242-1109. Phone: 319-335-7518, Fax: 319-335-9570, todd-washington@uiowa.edu.

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To overcome these replication blocks, cells possess a variety of non-classical DNA polymerases, which are capable of efficiently incorporating nucleotides opposite template DNA lesions. Most of these non-classical polymerases belong to the Y-family of DNA polymerases. Since the discovery of Y-family polymerases in the late 1990s, much work has been done to understand their cellular functions and their mechanisms of action. This article focuses on mechanisms of the four eukaryotic members of the Y-family: DNA polymerase eta (pol η), DNA polymerase kappa (pol κ), DNA polymerase iota (pol ι), and the Rev1 protein. We explore the similarities and differences between non-classical and classical polymerases at the kinetic and structural levels, and we show that these non-classical polymerases actually closely resemble their classical counterparts in terms of overall structure and chemical mechanism. However, interesting differences are present that account for the ability of these non-classical polymerases to accommodate damaged DNA templates. Thus if one thinks of the mechanisms of classical DNA polymerases as being the theme, the mechanisms of non-classical polymerases represent rather interesting variations on this theme.

2. The basic theme: mechanisms of classical DNA polymerases

Despite having diverse amino acid sequences and cellular functions, classical DNA polymerases share many structural and mechanistic features that contribute to their high fidelity of DNA synthesis [1–7]. The polymerase domains of classical polymerases all have a similar architecture that resembles a right hand. This architecture includes “palm”, “fingers”, and “thumb” sub-domains, which have conserved functions. The structures of the palm sub-domains of many DNA polymerases are similar overall, but their topologies differ across the various DNA polymerase families. The palm sub-domains are usually a β sheet comprised of four to six β strands supported on one side by several α helices. The β strands of this sub-domain contain several conserved glutamate or aspartate residues that coordinate two Mg^{2+} ions, which are critical for catalysis. The structures of the fingers sub-domains vary considerably across the DNA polymerase families. This subdomain constitutes part of the binding pocket for the incoming dNTP and therefore plays an important role in maintaining polymerase fidelity. The thumb sub-domains are primarily comprised of α helices, although their structures differ greatly across DNA polymerase families. In general, the thumb sub-domains contact the minor groove of the DNA substrate.

The structures of classical DNA polymerases bound to various DNA and dNTP substrates have revealed a great deal about the structural basis of DNA polymerase fidelity [8,9]. When a polymerase binds to the DNA substrate to form a binary complex, it adopts an “open” conformation. Upon binding the correct incoming dNTP to form a ternary complex, the fingers sub-domain rotates so that the protein achieves a “closed” conformation. In the closed conformation, residues of the fingers sub-domain help to constrain the active site containing the nascent base pair (*i.e.*, the pair comprised of the template base and the incoming dNTP). Only the four correct Watson-Crick base pairs will fit in the active site of classical polymerases in the closed conformation, and only in this conformation are the 3'-oxygen atom of the primer terminus, the α -phosphate of the incoming nucleotide, and the two Mg^{2+} ions in the proper positions for efficient catalysis. Structures of classical DNA polymerases bound to incorrect incoming dNTPs in ternary complexes have been difficult to obtain presumably because of the unstable nature of such complexes. Several structures approximating this, however, have been determined, and these polymerases adopt a “partially open” conformation, which does not appear to be conducive to catalysis [10,11].

The catalytic cycle of classical DNA polymerases contains at least six distinct steps (Fig. 1). First, the polymerase binds the DNA to form an enzyme-DNA binary complex (step 1). This binary complex is in the pre-insertion state. Next, the binary complex binds an incoming dNTP to form an enzyme-DNA-dNTP ternary complex (step 2). The bound nucleotide is then added

to the 3' end of the primer strand through the formation of a phosphodiester bond (step 3). Pyrophosphate is released (step 4); the remaining enzyme-DNA binary complex is now in the post-insertion state. Finally, either the polymerase translocates one nucleotide along the DNA converting from the post-insertion state to the pre-insertion state to allow for another round of catalysis (step 5) or the polymerases dissociates from the DNA (step 6). This scheme represents a simplified, minimal mechanism for the polymerase catalytic cycle, and several of these steps are likely composites of additional elementary steps. For example, step 2 (the formation of the ternary complex) probably involves both the nucleotide-binding step and the open-to-closed transition of the fingers sub-domain that occurs very rapidly after nucleotide binding. Similarly, step 3 (the incorporation of the bound nucleotide) likely involves both the catalytic step (*i.e.*, phosphodiester bond formation) and a partially or fully rate limiting conformational change step.

Pre-steady state kinetic studies of correct and incorrect nucleotide incorporation by classical polymerases have revealed a great deal about the kinetic basis of DNA polymerase fidelity. With respect to step 2 (formation of the ternary complex), some classical DNA polymerases, such as the bacteriophage T7 polymerase bind the incoming dNTP with a significantly higher affinity than they bind the incorrect dNTP [12,13]. Others, including the Klenow fragment of *E. coli* DNA polymerase I, bind correct and incorrect incoming nucleotides with similar affinities [14,15]. With respect to step 3 (incorporation of the bound nucleotide), all classical polymerases incorporate the nucleotide much faster when it is correctly paired with the template base than when it is incorrectly paired. This is likely because the enzyme-DNA-dNTP ternary complex is in the closed conformation in the former case and in the partially open conformation in the latter case.

Kinetic studies with nucleotides containing base analogs have provided further evidence that the principle feature exploited by classical polymerases to discriminate between the correct and incorrect nucleotides is the DNA geometry. First, nucleotides containing difluorotoluene, a base analog with precisely the same shape as thymine but lacking its ability to form Watson-Crick hydrogen bonds, are efficiently utilized by classical DNA polymerases [16]. Moreover, the fidelity of incorporation with nucleotides containing this isosteric base analog remains high. This study shows that the shape of the nascent base pair is important regardless of whether the Watson-Crick hydrogen bonds can be formed. Second, DNA substrates containing base analogs lacking minor groove hydrogen bond acceptors at key positions in the DNA are not good substrates for classical DNA polymerases [17,18]. These studies show that classical DNA polymerases make important hydrogen bonding interactions with the DNA minor groove, and that these are likely important for checking the proper geometry of the nascent base pair.

The dependence on DNA geometry to preferentially incorporate the correct nucleotide has a dramatic impact on the ability of classical polymerases to use damaged templates. Many DNA lesions inherently distort the DNA structure causing damaged DNA substrates to not be tolerated by classical DNA polymerases. Evidence for this comes from structural studies of classical polymerases bound to damaged DNA substrates. For example, a structure of the bacteriophage RB69 DNA polymerase in a ternary complex with dATP and DNA containing a template abasic site shows that the DNA geometry in the vicinity of the lesion is distorted and the fingers sub-domain remains in the open conformation [19]. Similarly, a structure of the bacteriophage T7 DNA polymerase in a binary complex with DNA containing a template thymine dimer shows that the thymine dimer is flipped out of the protein's active site. This precludes closing of the fingers subdomain even after the incoming dATP is added. [20].

3. The variations: mechanisms of Y-family DNA polymerases

Because classical polymerases are blocked by many types of DNA damage in the template strand, cells would die without a means of overcoming these blocks. One such means is brought about by the activity of non-classical DNA polymerases. These non-classical polymerases are thought to replace the classical polymerase that is stalled at sites of DNA damage. The mechanism governing the polymerase switching event is poorly understood, but some of its general features are shown in Fig. 2. First, the classical polymerase is displaced from the stalled replication complex (step 1). Following this, the non-classical polymerase is recruited to the complex (step 2). The non-classical polymerase then catalyzes the incorporation of nucleotides opposite the damaged template and further extension (step 3). Next, the non-classical polymerase dissociates from the replication complex (step 4). Finally, the classical polymerase returns to the complex and continues DNA synthesis (step 5). In the case of certain DNA lesions, two non-classical polymerases are likely required to carry out nucleotide incorporation and further extension (step 3). In such cases, an additional polymerase switching event would be required in which the first non-classical polymerase (which catalyze the incorporation step) is replaced by a second non-classical polymerase (which catalyze the subsequent extension step).

Most of these non-classical DNA polymerases are members of the Y-family of polymerases, which has both prokaryotic and eukaryotic members. The best studied prokaryotic members of this family include pol IV (DinB) and Pol V (UmuC) from the eubacterium *E. coli* and Dpo4 and Dbh from the archaeon *S. solfataricus*. In this review article, we will focus on the eukaryotic members of this family: pol η , pol κ , pol ι , and the Rev1 protein.

3.1. Variation no. 1: DNA polymerase η tolerates distorted templates

Pol η is present in the genomes of all eukaryotes [21]. In yeast, lack of pol η leads to a dramatic increase in the number of mutations induced by ultraviolet radiation [22–24]; in humans, lack of this enzyme is responsible for the variant form of xeroderma pigmentosum, a cancer-prone genetic disorder [25,26]. Of all non-classical DNA polymerases, the biological role of pol η is the most clear; it catalyzes the replication of thymine dimers [24–26] and 8-oxo-G lesions [27]. Purified pol η has the ability to incorporate nucleotides opposite both 8-oxo-G lesions (which partially block classical polymerases) [27,28] and thymine dimers (which completely block classical polymerases) [29–32]. In fact, steady state kinetic studies have shown that pol η incorporates nucleotides opposite these particular DNA lesions with exactly the same catalytic efficiencies (k_{cat}/K_m) as it does opposite the corresponding non-damaged template residue [27,28,30–32]. This enzyme also incorporates nucleotides with high catalytic efficiencies opposite thymine glycols [32] and cisplatin-induced intrastrand crosslinks [33]. In addition, pol η incorporates nucleotides with significantly lower catalytic efficiencies opposite a variety of other lesions, including abasic sites [32,34], O⁶-methyl-G [35], (6–4) photoproducts [36], and a wide range of bulky N²-adducted G residues [32,37–41].

Steady state kinetics is a useful approach to determine the fidelity of DNA synthesis by a DNA polymerase. Polymerase fidelity is usually expressed as an error frequency, which is the ratio of the catalytic efficiency of incorporating the incorrect nucleotide to the catalytic efficiency of incorporating the correct nucleotide. Steady state kinetic studies as well as other complementary approaches have shown that purified yeast and mammalian pol η synthesize DNA with a low fidelity with error frequencies ranging from 10^{-2} to 10^{-3} [30,31,42–44]. While steady state kinetics provides a convenient way to quantify the fidelity of DNA synthesis, it provides little information about its mechanistic basis. Instead, pre-steady state kinetic studies are necessary to determine the contribution of the individual steps of the DNA synthesis reaction (see Fig. 1) toward the enzyme's fidelity. In the case of yeast pol η , pre-steady state kinetic studies showed that the enzyme binds to an incorrect incoming dNTP with a ~5-fold

lower affinity than it binds to a correct one [45]. After the nucleotide binds to the protein, it is incorporated at a ~150-fold slower rate if it is an incorrect nucleotide than if it is a correct one [45]. Thus even though pol η synthesizes DNA with a low fidelity, what limited ability it has to discriminate between correct and incorrect nucleotides occurs primarily at the nucleotide-incorporation step, not the initial nucleotide-binding step.

Further pre-steady state kinetic studies were carried out to determine how template lesions impact the individual steps of the DNA synthesis reaction of yeast pol η . 8-oxo-G is a common DNA lesion that partially blocks classical DNA polymerases, because an 8-oxo-G-dCTP base pair causes a significant, local distortion of the structure of the DNA backbone [46,47]. Many classical DNA polymerases cope with this lesion by rotating the 8-oxo-G base about the N-glycosidic bond from the normal *anti* conformation to the *syn* conformation [46,48]. These polymerases preferentially incorporate an A opposite this lesion because the resulting 8-oxo-G(*syn*)-dATP(*anti*) nascent base is less distorting to the structure of the DNA backbone than is the 8-oxoG-(*anti*)-dCTP(*anti*) base pair. Pre-steady state kinetic studies showed that pol η preferentially incorporates C opposite 8-oxo-G and that the kinetics for incorporating a C opposite an 8-oxo-G and a non-damaged G template are very similar [49]. The incoming dCTP is bound with the same high affinity opposite both damaged and non-damaged templates, and the nucleotide is incorporated at the same rate regardless of whether the template residue is damaged or not. This demonstrates that pol η incorporates nucleotides opposite 8-oxo-G with no kinetic barriers introduced by the distorted DNA structure of the 8-oxo-G(*anti*)-dCTP (*anti*) base pair [49].

Unlike 8-oxo-G lesions which partially block classical DNA polymerases, thymine dimers are complete blocks to classical DNA polymerases. There are two reasons for this. First, the crosslinking of the two thymines introduces a significant distortion in the DNA structure. Second, with non-damaged DNA substrates, classical DNA polymerases flip the unpaired base on the 5' side of the template base out of the active site. This mode of DNA binding cannot accommodate a template thymine dimer, because the two adjacent bases are covalently linked. In fact, when presented with a template thymine dimer, classical polymerases flip both crosslinked bases out of the protein's active site [20]. Given how classical polymerases are unable to deal with thymine dimers, it is remarkable that the kinetics for yeast pol η incorporating an A opposite a thymine dimer and a corresponding non-damaged sequence are so similar [50]. Pre-steady state kinetic studies showed that the incoming dATP is bound with the same high affinity opposite both damaged and non-damaged templates, and the nucleotide is incorporated at the same rate regardless of whether the template residue is damaged or not. This demonstrates that pol η incorporates nucleotides opposite thymine dimers without any kinetic barriers introduced by the presence of this lesion [50]. This also implies that pol η does not flip out the thymine dimer and instead somehow keeps both bases of the lesion within its active site during DNA synthesis.

Insight into the ability of pol η to maintain both bases of a thymine dimer in its active site came from the X-ray crystal structure of the catalytic core of yeast pol η [51]. The catalytic core consists of two domains: a polymerase domain and a polymerase-associated domain (PAD, sometimes called the little fingers domain), which is a domain that is present in and unique to all Y-family polymerases. The overall architecture of the polymerase domain resembles a right hand with palm, fingers, and thumb sub-domains. In this respect, the structure of the polymerase domain is analogous to those of classical DNA polymerases. The conserved acidic residues that are necessary for coordinating two Mg^{2+} ions are in the palm sub-domain, and the positions of these residues indicate that Y-family polymerases utilize the same metal-assisted catalytic mechanism used by classical DNA polymerases. The most striking feature of the structure of pol η is that the fingers sub-domain is smaller than those of classical DNA polymerases. This is significant because it leaves the active site of pol η more spacious and

less geometrically restrictive. This provides the structural basis for the unusual tolerance of this enzyme to distorted DNA geometries, its low fidelity, and its ability to efficiently incorporate opposite certain template lesions.

No structure has currently been determined for pol η bound to a DNA substrate containing a thymine dimer. However, modeling suggests that both residues of the thymine dimer could fit within the active site of pol η without steric clashes (Fig. 3) [51]. Similar modeling of the “open” form of prokaryotic pol I, a classical polymerase, shows that a thymine dimer could not fit in its active site without serious steric clashes [51]. Recently, a structure of pol η has been determined bound to DNA substrates containing a cisplatin GG adduct [52]. Like the thymine dimers, the cisplatin GG adduct is comprised of two adjacent crosslinked bases. This structure of pol η bound to the cisplatin-containing DNA is nearly indistinguishable from the model of pol η bound to the thymine dimer-containing DNA shown in Fig. 3. In both cases, the two bases of the lesions are in the protein’s active site, and a correct incoming dNTP is paired with the first available template residue of the lesion.

Finally, studies with nucleotide base analogs have provided further insights into the mechanism of yeast pol η . For example, steady state kinetic studies with incoming dNTP and DNA substrates containing difluorotoluene – which has the same shape as thymine, but lacks the ability to form Watson-Crick hydrogen bonds – are poor substrates for pol η [53]. These studies demonstrated that pol η is much more dependent on Watson-Crick hydrogen bonding between the template base and the incoming dNTP than are classical polymerases. Moreover, steady state analyses using modified thymine dimers with methyl groups attached to the N3 positions (which eliminates base pairing) showed that A could no longer be efficiently incorporated opposite either base of the lesion [54]. This is important because it provides direct biochemical support for the notion that pol η synthesizes DNA through a template thymine dimer by maintaining the lesion in its active site and directly incorporating nucleotides opposite the lesion utilizing Watson-Crick base pairing.

3.2. Variation no. 2: DNA polymerase κ tolerates distorted primer termini

Pol κ is present in the genomes of many eukaryotes, including mammals, nematodes, and some yeast (such as *S. pombe*), but is absent from those of insects and other yeast (such as *S. cerevisiae*) [21]. Human pol κ synthesizes DNA with a low to moderate fidelity with error frequencies ranging from 10^{-2} to 10^{-4} [55–57]. It is not capable of efficiently incorporating nucleotides opposite some of the lesions opposite which pol η incorporates, such as thymine dimers [55,58,59], cisplatin adducts [58], abasic sites [55], and (6–4) photoproducts [55,58, 59]; it can, however, incorporate A opposite 8-oxo-G lesions [60] and thymine glycols [61]. The cellular role of this enzyme remains unclear, and there are two seemingly conflicting views regarding the function of pol κ in the replication of damaged DNA. One view is that pol κ catalyzes the incorporation of nucleotides opposite bulky minor groove adducts, such as N²-acetylaminofluorene-G lesions [58,59,62] and various stereoisomers of N²-benzo(a)pyrene diolepoxide-G lesions [59,63–66]. The other view is that pol κ catalyzes the extension from aberrant primer-terminal base pairs resulting from the incorporation of nucleotides opposite DNA lesions by another polymerase, usual pol ι [60,67–69].

The notion that pol κ inserts nucleotides opposite bulky minor groove adducts comes from biochemical studies showing that the purified protein is capable of incorporating opposite these lesions [58,59]. Steady state kinetic analyses showed, however, that nucleotide incorporation across from these lesions is very inefficient. For example, pol κ incorporates C opposite an N²-acetylaminofluorene-G lesion with ~800-fold lower catalytic efficiency than it does opposite a non-damaged G [62]. Similarly, depending on the sequence context and the specific stereoisomer of the lesion, pol κ incorporates C opposite an N²-benzo(a)pyrene diolepoxide-G lesion with efficiencies ranging from ~50-fold to ~20,000-fold lower than those for

incorporation opposite a non-damaged G [64–66]. These low efficiencies of nucleotide incorporation raise questions regarding this being a major cellular role for pol κ . It should be pointed out, however, that pol κ has been shown to incorporate nucleotides with reasonably high efficiency opposite somewhat smaller N²-adducted guanines, including N²-methyl-G, N²-ethyl-G, and N²-isobutyl-G [70]. Thus pol κ does have a limited ability to incorporate opposite some minor groove lesions.

The notion that pol κ extends from aberrant primer-terminal base pairs comes from steady state kinetic studies. In the case of non-damaged DNA substrates, pol κ has the highly unusual ability to extend from primer-terminal mismatches. Other DNA polymerases, including classical polymerases and pol η , extend from primer-terminal mismatches with very low efficiencies [71]. These efficiencies are similar to those with which they incorporate incorrect nucleotides. Human pol κ , by contrast, extends from mismatched primer termini with ~10-fold to ~30-fold lower efficiencies than which it extends from properly matched primer termini [67]. Even more striking is the ability of this enzyme to extend from aberrant primer-terminal base pairs containing DNA lesions with nearly the same efficiency with which it extends from the corresponding non-damaged primer-termini. Lesions efficiently extended by pol κ include O⁶-methyl-G [60], 8-oxo-G [60], certain stereoisomers of N²-BPDE-G [72], γ -hydroxy-1,N²-propano-G [68], and certain stereoisomers of 4-hydroxy-2-nonenal-G [69]. These studies strongly suggest that the major cellular role for pol κ is catalyzing this extension step.

Pre-steady state kinetic studies with human pol κ also showed that this enzyme had a slightly impaired ability to extend from mismatched primer termini relative to matched primer terminus [73]. This difference was due almost entirely to a ~60-fold slower rate of incorporation of the bound incoming nucleotides in the presence of the primer-terminal mismatch. The binding affinity for the DNA substrate and the incoming nucleotide were approximately the same for matched and mismatched primer-termini. Active site titrations showed that, unlike other classical and non-classical polymerases, pol κ forms non-productive polymerase-DNA complexes with DNA substrates containing matched primer-terminal base pairs. Surprisingly, however, pol κ does not form non-productive complexes with DNA substrates containing mismatched primer-termini; pol κ is fully active on these substrates. Non-productive polymerase-DNA complexes have been observed previously with other enzymes on damaged DNA substrates [74–76] and on DNA substrates with a high degree of secondary structure [77,78]. In the case of pol κ , this is the first known example of a polymerase forming a non-productive complex on a normal DNA substrate (*i.e.*, the matched DNA), but not on one an abnormal DNA substrate (*i.e.*, the mismatched DNA). This indicates that DNA with aberrant primer-terminal base pairs are the preferred substrates for pol κ and that this enzyme has evolved to specifically function on such substrates.

The structural basis for both the ability of pol κ to extend efficiently from aberrant primer termini and its tendency to form non-productive complexes with normal primer termini are not well understood. Some hints, however, have come from the X-ray crystal structures of the catalytic core of human pol κ alone [79] and in a ternary complex with DNA and an incoming nucleotide (Fig. 4) [80]. The catalytic domain of pol κ is similar to those of other DNA polymerases in that it resembles a right hand with palm, thumb, and fingers sub-domain. Like other Y-family polymerases, it also has a C-terminal PAD domain. The active site of pol κ is more open than those of classical DNA polymerases, but it is not as open as the active site of pol η . This nicely explains why pol κ synthesizes DNA with a lower fidelity than do classical polymerases but with a higher fidelity than does pol η . The active site of pol κ has room for only one template base, and this explains why pol κ is unable to incorporate nucleotides opposite thymine dimers, cisplatin adducts, and (6–4) photoproducts. Unlike other Y-family polymerases, however, pol κ has an extended N-terminal region, referred to as the N-clasp, which extends from the thumb and completely encircles the DNA near the primer terminus

[80]. It has been suggested that this N-clasp plays an important role in stabilizing pol κ when it is bound to a mismatched DNA substrate. In fact, deletion of part of the N-clasp reduces the ability of pol κ to extend from mismatches more than the ability to extend from matches [80]. Finally, the DNA substrate in this structure has a matched primer terminus making it likely that this represents the structure of the non-productive polymerase-DNA complex. Interestingly, there is only one Mg^{2+} ion present in this structure, when two are need for catalysis. It is tempting to speculate that the absence of this second Mg^{2+} ion is the structural basis for the non-productive complex.

3.3. Variation no. 3: DNA polymerase ι uses Hoogsteen base pairing

Pol ι is present in the genomes of many higher eukaryotes, including mammals and insects, but is absent from those of yeast or nematodes [21]. A substantial number of *in vitro* biochemical studies of purified pol ι have suggested a role for this enzyme in the replication of a wide range of DNA lesions including minor groove-adducted purines [68,69,81–84], bulky major groove-adducted purines [85–87], and other types of damage [87–90]. Steady state kinetic studies of human pol ι show that this protein has an unusual biochemical activity. Opposite a non-damaged template A, the efficiency and the fidelity of nucleotide incorporation are moderate with error frequencies ranging from 10^{-4} to 10^{-6} [88,91,92]. By contrast, opposite a template G, the efficiency and fidelity of nucleotide incorporation are reduced significantly with error frequencies ranging from 10^{-1} to 10^{-4} [88,91,92]. Similarly, opposite a template C, the efficiency and fidelity are low with error frequencies ranging 10^{-1} to 10^{-3} [88,91,92]. More striking, however, are the error frequencies for incorporation opposite template T, which are so extraordinarily high that pol ι incorporates an incorrect G with a 3 to 10-fold greater efficiency than it incorporates the correct A [88,91,92].

For nearly all polymerases examined to date, the efficiency and fidelity of nucleotide incorporation on all four non-damaged template bases is roughly the same for a given enzyme. Pre-steady state kinetic studies with human pol ι provided the kinetic basis for the high efficiency/moderate fidelity incorporation opposite a template A and the low efficiency/low fidelity incorporation opposite a template T [93]. Opposite a template A, pol ι binds the correct incoming dTTP with a ~50 to 100-fold greater affinity than it binds an incorrect dNTP. Once bound, pol ι incorporates the correct nucleotide with a ~50 to 300-fold greater rate than it incorporates an incorrect nucleotide. Opposite a template T, by contrast, pol ι actually binds the incorrect incoming dGTP with an ~8-fold greater affinity than it binds the correct dATP. Once bound, all four nucleotides are incorporated by pol ι at approximately the same very slow rate. Given that the kinetics of nucleotide incorporation differs so drastically with a template A versus a template T, it was suggested that this enzyme must use a different base pairing scheme from the usual, symmetrical Watson-Crick base pairing scheme [93].

A series of X-ray crystal structures of human pol ι bound to DNA and dNTP substrates have revealed that this protein indeed uses an alternative base pairing scheme [84,85,94–96], and this provides an elegant explanation for its unusual enzymatic activities. Overall, the structure of the catalytic core of pol ι is very similar to those of other Y-family polymerases; it possesses both a polymerase domain containing the usual fingers, palm, and thumb sub-domains and as well as a PAD domain. When pol ι is bound to the DNA substrate in the absence of an incoming dNTP, the template base is in the normal *anti* conformation with respect to rotation about the N-glycosidic bond [94]. When the incoming dNTP binds, however, this forces the template base to rotate to the *syn* conformation. This is in contrast to the structures of nearly all other DNA polymerases examined to date, in which the template base is in the *anti* conformation. The unique *syn* conformation of the template base in the active site of pol ι allows it to form Hoogsteen base pairs with the incoming dNTP (which remains in the *anti* conformation) [95, 96]. This nicely explains the high efficiency/moderate fidelity incorporation opposite template

A, because the A (*syn*)•dTTP (*anti*) Hoogsteen base pair is able to adopt the appropriate shape required to fit in the active site of pol ϵ [96]. This also explains the low efficiency/low fidelity incorporation opposite template T, because Hoogsteen base pairs cannot be formed when T is in the *syn* conformation; thus the active site of pol ϵ does not allow base pairing with template T [96].

The Hoogsteen base pairing mechanism is essential for the ability of pol ϵ to incorporate nucleotides opposite template DNA lesions in at least two ways. First, it allows for incorporation opposite DNA lesions that cannot form Watson-Crick base pairs. For example, pol ϵ incorporates both T and C opposite a 1,N⁶-etheno-A lesion with only slightly lower efficiencies than it does opposite a non-damaged A [85]. In the normal *anti* conformation, the exocyclic ring of this adduct is in the DNA major groove and sterically prevents the formation of Watson-Crick hydrogen bonds with an incoming dNTP. The X-ray crystal structure of pol ϵ in a ternary complex with a DNA containing a 1,N⁶-etheno-A template shows that this template base is rotated into the *syn* conformation (Fig. 5) [85]. In this conformation, the exocyclic ring of the adduct remains in the major groove, but allows Hoogsteen base pairing between the lesion and an incoming dTTP or dCTP without steric clashes. The Hoogsteen base pairing mechanism likewise explains the ability of pol ϵ to efficiently incorporate C opposite both γ -hydroxy-1,N²-propano-G and 1,N²-propano-G, a stable ring-closed analog of this naturally occurring lesion [68,83]. In these cases, the minor-groove exocyclic ring also sterically interferes with the formation of Watson-Crick hydrogen bonds, while Hoogsteen base pairing is unaffected.

Second, the Hoogsteen base pairing mechanism allows for the incorporation opposite lesions that disrupt the DNA minor groove. For example, pol ϵ incorporates C opposite an N²-ethyl-G with the same efficiency that it does opposite a non-damaged G [84]. Ordinarily, the ethyl group of this lesion projects into the DNA minor groove and interferes with nucleotide incorporation by a wide range of polymerases, which contact the DNA via the minor groove. The X-ray crystal structure of pol ϵ in a ternary complex with a DNA containing an N²-ethyl-G template and an incoming dCTP shows that the adducted template base is rotated into the *syn* conformation, positioning the N²-ethyl moiety into the major groove, where it is readily accommodated [84]. This Hoogsteen base pairing mechanism also explains the ability of pol ϵ to efficiently incorporate nucleotides opposite other minor groove lesions, including the ring-opened form of γ -hydroxy-1,N²-propano-G [68], N²-isopropyl-G [97], *trans*-4-hydroxy-2-nonenal-G [69].

Biochemical studies with nucleotide analogs support the Hoogsteen base pairing model for pol ϵ . The structures of both the A(*syn*)•dTTP(*anti*) and the G(*syn*)•dCTP(*anti*) Hoogsteen base pairs in the active site of pol ϵ show that hydrogen bonds are formed between the incoming dNTP and the N7 atom of the purine template [95,96]. The base analogs 7-deaza-A and 7-deaza-G lack this hydrogen bond acceptor. When these base analogs are used as templates for other DNA polymerases, including the Klenow fragment of *E. coli* pol I, yeast DNA polymerase zeta (pol ζ), yeast pol η , and human pol κ , there is either a slight increase or a slight decrease in the efficiency of nucleotide incorporation (< 5-fold) [98]. With pol ϵ , the decrease in efficiency for correct nucleotide incorporation when using 7-deaza-A and 7-deaza-G as a template residue is ~200-fold and ~30-fold, respectively [98]. This unique sensitivity to removal of a hydrogen bond donor at this position provides direct biochemical support for the Hoogsteen base pairing mechanism.

3.4. Variation no. 4: the Rev1 protein uses an arginine residue as the template

The Rev1 protein, which is present in the genomes of all eukaryotes [21], plays an important role in DNA damage-induced mutagenesis [99]. In this process, it plays both a structural role and an enzymatic role [100,101]. In a structural capacity, the Rev1 protein may act as a scaffold

by interacting with other non-classical polymerases and replication accessory factors [102–109]. In an enzymatic capacity, Rev1 catalyzes the incorporation of C opposite template abasic sites and a variety of damaged G residues [100,110–114]. Here we will focus on the enzymatic role of the Rev1 protein. Steady state kinetic studies show that the substrate specificity of the Rev1 protein is the most unusual among all of the non-classical polymerases [111,112,115,116]. Opposite a non-damaged template G, the Rev1 protein incorporates the correct C with a high efficiency and a moderate fidelity with error frequencies usually ranging from 10^{-3} to 10^{-4} . Surprisingly, opposite non-damaged A, T, and C templates, the Rev1 protein actually incorporates the incorrect C preferentially (albeit with a ~20 to 500-fold lower efficiency than it incorporates C opposite G). In addition, when provided with a DNA substrate containing a run of template G residues, the Rev1 protein has a low processivity, but it is not purely distributive; following each nucleotide incorporation event, ~60% of the Rev1 molecules continue to incorporate additional nucleotides [111].

Overall, steady state kinetic studies show that the Rev1 protein has a strong preference both for an incoming dCTP and for a template G. The X-ray crystal structure of the catalytic core of the yeast Rev1 protein bound to both dCTP and DNA substrates revealed the structural basis for this unique specificity [117]. Like other Y-family polymerases, the Rev1 protein has a polymerase domain (with fingers, thumb, and palm sub-domains) as well as a PAD domain. In addition, the Rev1 protein has a small, mostly α -helical region, called the N-digit, which is adjacent to the polymerase domain. The side chain of Leu-325, from the N-digit, projects into the DNA substrate and flips the template G out of the double helix into a hydrophobic pocket. Here, the N7 and O⁶ atoms of the flipped out G residue form hydrogen bonds with the backbone of residues within an extended loop of the PAD domain called the G loop. Such interactions are not possible with templates A, T, or C, thus explaining the strong preference of the Rev1 protein for DNA substrates containing a template G [117]. Even more striking is that the side chain of Arg-324, also from the N-digit, forms hydrogen bonds with the O² and N3 atoms of the incoming dCTP and helps position it properly for nucleotide incorporation. The other three possible incoming nucleotides would either have a reduced ability to hydrogen bond or have unfavorable steric clashes with the Arg-324 side chain. Thus, unlike any other DNA polymerase, the Rev1 protein provides the specificity for the incoming nucleotide by using one of its own amino acid side chains as a template to direct nucleotide incorporation [117].

Pre-steady state kinetic studies of the yeast Rev1 protein have examined the kinetic consequences of this unique protein template-directed mechanism [118]. Opposite a non-damaged template G, the selection of the incoming nucleotide is accomplished exclusively at the initial nucleotide binding step and not the subsequent nucleotide incorporation step. An incoming dCTP binds to the Rev1 protein/DNA complex with ~300 fold greater affinity than does dGTP or dTTP (dATP incorporation was not detectable). This is likely due to the intrinsically higher affinity of the Arg-324 side chain for dCTP. Once bound, however, all incoming nucleotides are incorporated at approximately the same rate. The preference for a G residue in the template position of the DNA substrate was achieved at both the initial nucleotide-binding step as well as the subsequent nucleotide-incorporation steps. In other words, when A, T, or C is flipped out of the DNA double helix and into the hydrophobic pocket, the incoming nucleotide is bound weaker and incorporated slower than when a G is present in the hydrophobic pocket.

The kinetic and structural studies described above clearly show that the ability of the Rev1 protein to incorporate a C with high efficiency depends on the flipped out template residue of the DNA fitting properly within the hydrophobic pocket and forming favorable hydrogen bonds with the G loop. Studies of the activity of the Rev1 protein on damaged DNA substrates further support this notion. For example, the Rev1 protein incorporates C residues opposite lesions with modifications at the N² minor groove position of the template G, such as γ -

hydroxy-1,N²-propano-G, N²-methyl-G, N²-ethyl-G, N²-isopropyl-G, and others, occurs with nearly the same efficiency as it does opposite non-damage G [113,114,119]. The structure of the ternary complex of the yeast Rev1 protein bound to an incoming dCTP and a DNA containing 1,N²-propano-G (a stable ring-closed analog of γ -hydroxy-1,N²-propano-G) shows that the Rev1 protein is able to accommodate this bulky lesion (Fig. 6) [119]. The damaged G fits comfortably in the hydrophobic pocket and makes similar hydrogen bonds to the G loop as observed for the non-damaged G. As another example, the Rev1 protein incorporates a C opposite a template abasic site with the same to a ~100-fold lower efficiency than it incorporates a C opposite a non-damaged G [111,112,115,116,118]. This moderate reduction in efficiency likely occurs because, while there are no hydrogen bonds formed between the abasic site and the G loop of the hydrophobic pocket, no unfavorable interactions exist. By contrast, lesion with modifications at either the O⁶ or C8 positions of the template G, such as O⁶-methyl-G, O⁶-benzyl-G, and 8-oxoG, resulted in much less efficient nucleotide incorporation [111,113,118]. This large decrease in the efficiency of nucleotide incorporation is likely due to both the loss of hydrogen bonds between the damaged template residues and the G loop and the presence of steric clashes within the hydrophobic pocket.

4. Concluding remarks

Many important questions remain unanswered regarding the replication of damaged DNA by non-classical DNA polymerases. First, what exactly is the cellular role for some of the non-classical polymerases? Only the role of pol η , which is catalyzing the incorporation of nucleotides opposite template thymine dimers and 8-oxo-G lesions, is very clear. What specific lesions is pol ι responsible for replicating *in vivo*? Does pol κ play a significant role in incorporating nucleotides opposite lesions or does it only function in the extension step during the replication of damaged DNA? Does the Rev1 protein play a significant role in incorporating nucleotides or does it principally function in a structural capacity as a scaffold protein? Do these polymerases play roles in processes other than the replication of damaged DNA? It has been proposed, for example, that Y-family polymerases play a role in somatic hypermutation [120,121]. Second, what other protein factors interact with these non-classical polymerases and how do these interactions affect their mechanism? Most studies of the interacting factors have focused on proliferating cell nuclear antigen (PCNA), the sliding clamp processivity factor for DNA polymerases. All four eukaryotic Y-family polymerases interact with PCNA, and this interaction has been shown to enhance the catalytic activity of pol η , pol κ , and pol ι [122–125]. What other factors interact with non-classical polymerases? Do these interactions stimulate the catalytic function of non-classical polymerases? If so, how do these interactions result in stimulation? Third, how is the polymerase switching event controlled? It has been suggested that the DNA damage-dependent mono-ubiquitination of PCNA by the Rad6-Rad18 complex plays a key role in this process [126–132]. There is, however, little direct evidence to support this hypothesis. Does the mono-ubiquitination of PCNA directly trigger the polymerase switching event? Are other factors necessary for this event? Fourth, how do post-translational modifications of these non-classical polymerases regulate their function? There is evidence that pol η may be regulated by phosphorylation [133] and ubiquitination [134,135]. Do these post-translational modifications impact the mechanism of these polymerases? What signals induce these post-translational modifications?

Despite these remaining unanswered questions, we have come a long way in terms of understanding the key differences between the non-classical and classical polymerases that allow for the replication of damaged DNA templates. It is becoming clear that the mechanisms of these non-classical polymerases are fascinating variations of the mechanism of the classical polymerases. The mechanisms of pol η and pol κ represent rather minor variations, because both enzymes select the incoming dNTP utilizing Watson-Crick base pairs with the template base. These proteins, however, are more tolerant of distortions in the DNA substrate than are

their classical counterparts. The mechanisms of pol ϵ and the Rev1 protein represent rather major variations. Pol ϵ selects the incoming dNTP utilizing Hoogsteen base pairs with the template base, and the Rev1 protein selects the incoming dNTP utilizing hydrogen bonds with an arginine side chain of the protein. The minor and major variations all accomplish the same goal: to allow the polymerases to circumvent problems posed by DNA damage in the template strand. Finally, it should be pointed out that the elucidation of these mechanistic variations has largely been the result of kinetic and structural analyses. These biophysical approaches have provided answers to fundamental questions regarding the function and mechanism of non-classical DNA polymerases that could not have been answered by cellular approaches alone.

Acknowledgments

This work was supported by Award Number R01GM081433 from the National Institute of General Medical Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

We thank Lynne Dieckman, Christine Kondratick, and Marc Wold for valuable discussions.

Abbreviations

8-oxo-G	8-oxo-7,8-dihydro-2'-deoxyguanosine
A	2'-deoxyadenosine
C	2'-deoxycytidine
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidine triphosphate
dGTP	2'-deoxyguanosine triphosphate
dNTP	2' deoxynucleoside triphosphate
dTTP	2'-deoxythymidine triphosphate
G	2'-deoxyguanosine
PAD	polymerase associated domain
pol	polymerase
PP _i	pyrophosphate
T	2'-deoxythymidine

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Fig. 1. Mechanism of DNA synthesis by DNA polymerases. The minimal mechanism of nucleotide incorporation by all DNA polymerases contains at least six steps. Step 1: the polymerase binds the DNA substrate. Step 2: the polymerase-DNA binary complex binds the incoming dNTP substrate. Step 3: the bound nucleotide is incorporated. Step 4: the pyrophosphate (PP_i) product is released. Step 5: the polymerases translocates along the DNA. Step 6: alternatively, the polymerase dissociates from the DNA.

Fig. 2.

The polymerase switching mechanism. Step 1: the classical polymerase (*purple*) dissociates from the stalled replication complex. Step 2: the non-classical polymerase (*blue*) associates with the stalled complex. Step 3: the non-classical polymerase catalyzes nucleotide incorporation opposite the lesion and further extension. Step 4: the non-classical polymerase dissociates from the replication complex. Step 5: the classical polymerase returns to the replication complex.

Fig. 3.

The active site of pol η . The structure of the pol η (*light blue*) determined in the absence of DNA and dNTP substrates with DNA (*orange*) containing a template thymine dimer (*yellow*) and an incoming dATP (*green*) modeled into the active site is shown. This model is based on a previously published model [51]. The oxygen (*red*) and nitrogen (*blue*) atoms on the incoming nucleotide and template residue are shown. The Watson-Crick hydrogen bonds are indicated by dashed lines. (PDB ID: 1JIH, reference [51])

Fig. 4. The active site of pol κ . Pol κ (*light blue*) is shown bound to DNA (*orange*) with A (*yellow*) as the template and dTTP (*green*) as the incoming nucleotide. The oxygen (*red*) and nitrogen (*blue*) atoms on the incoming nucleotide and template residue are shown. The Watson-Crick hydrogen bonds are indicated by dashed lines (PDB ID: 2OH2, reference [80])

Fig. 5.

The active site of pol ι . Pol ι (light blue) is shown bound to DNA (orange) with 1,N⁶-etheno-A template (yellow) as the template and dTTP (green) as the incoming nucleotide. The *anti* or *syn* conformations of the glycosidic bond are indicated. The oxygen (red) and nitrogen (blue) atoms on the incoming nucleotide and template residue are shown. The Hoogsteen hydrogen bonds are indicated by dashed lines (PDB ID: 2DPJ, reference [85])

Fig. 6.

The active site of the Rev1 protein. The Rev1 protein (*light blue*) is shown bound to DNA (*orange*) with 1,N²-propano-G (*yellow*) as the template and dCTP (*green*) as the incoming nucleotide. The side chain of Arg-324 (*purple*) is shown. The oxygen (*red*) and nitrogen (*blue*) atoms on the incoming nucleotide, template residue, and Arg-324 are shown. The hydrogen bonds between the incoming dCTP, the DNA, and the side chain of Arg-324 are indicated by dashed lines (PDB ID: 3BJY, reference [119])