Nucleic Acids and Molecular Biology 30

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Nucleic Acid Polymerases

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Preface

More than any other class of enzymes, nucleic acid polymerases are directly responsible for an overabundance of enzymatic, regulatory, and maintenance activities in the cell. DNA polymerases accurately replicate copies of genomes in all forms of life as well as have specialized roles in DNA repair and immune response. RNA polymerases are most noted for their active roles in controlling gene expression during transcription but can also be utilized in self-replicating ribozymes and viral replication. Although the general sequence homology, structural architecture, and mechanism are conserved, they have evolved to incorporate deoxynucleotides (dNTPs) or ribonucleotides (rNTPs) explicitly. Various nucleic acid polymerases have specificities for RNA or DNA templates, incorporate dNTPs or rNTPs, and can be template dependent or independent. Here, we provide examples on the latest understanding of each class of nucleic acid polymerase, their structural and kinetic mechanisms, and their respective roles in the central dogma of life.

This book provides a catalog and description of the multitude of polymerases (both DNA and RNA) that contribute to genomic replication, maintenance, and gene expression. Evolution has resulted in tremendously efficient enzymes capable of repeated extremely rapid syntheses that have captivated researchers' interests for decades. We are inspired by work that started over 60 years ago and is actively pursued today for a fundamental understanding of life, contributions to human health and disease, and current and future biotechnology applications. Nucleic acid polymerases are fascinating on a number of levels, yet still continue to surprise us with novel modes of action revealed through ongoing and future studies described within this volume.

We wish to thank all the authors for their specific expertise and willingness to participate in this comprehensive review of nucleic acid polymerases. We are also grateful to the many investigators before us (including our research mentors: Stephen Benkovic and Akira Ishihama) who began and continue this important

line of research. We believe this book will be useful for a wide range of researchers in both the early and later stages of their careers. We would be thrilled if this volume becomes the go-to resource for nucleic acid polymerase structure, function, and mechanism for years to come.

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Contents

Chapter 1
Introduction to Nucleic Acid Polymerases: Families, Themes, and Mechanisms

Michael A. Trakselis and Katsuhiko S. Murakami

Keywords Polymerase • Mechanism • Structure • Function • Catalysis

Abbreviations

CPD	Cyclobutane pyrimidine dimers
E. coli	*Escherichia coli*
FDX	Fidaxomicin
FILS	Facial dysmorphism, immunodeficiency, livedo, and short statures
kDa	Kilodaltons
pol	Polymerase
Pol I	*E. coli* DNA polymerase I
RdRp	RNA-dependent RNA polymerase
Rif	Rifampicin
rRNA	Ribosomal RNA
TLS	Translesion synthesis
UV	Ultraviolet light
XPD	*Xeroderma pigmentosum*

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1.1 Introduction/Discovery/Classification

Template-dependent and template-independent nucleotidyl transfer reactions are fundamentally important in the maintenance of the genome as well as for gene expression in all organisms and viruses. These reactions are conserved and involve the condensation of an incoming nucleotide triphosphate at the 3′ hydroxyl of the growing oligonucleotide chain with concomitant release of pyrophosphate. DNA polymerase I (Pol I) isolated from *E. coli* extracts was initially characterized in in vitro reactions well over 50 years ago by the seminal work of Arthur Kornberg's laboratory (Kornberg 1957; Lehman et al. 1958; Bessman et al. 1958). Inspired by this work, the discovery of a DNA-dependent RNA polymerase quickly followed in 1960 from a variety of researchers including Samuel Weiss (Weiss and Gladstone 1959), Jerald Hurwitz (Hurwitz et al. 1960), Audrey Stevens (Stevens 1960), and James Bonner (Huang et al. 1960). These early enzymatic characterizations of DNA-dependent deoxyribonucleotides and ribonucleotide incorporations gave credibility both to Watson and Crick's DNA double helix model (Watson and Crick 1953) and the transcription operon model proposed by François Jacob and Jacques Monod (Jacob and Monod 1961).

Prior to 1990, few DNA polymerase members were known. Pol I, Pol II, and Pol III from bacteria defined the initial A, B, and C families of polymerases (Braithwaite and Ito 1993), respectively. Eukaryotic polymerases adopted a Greek letter nomenclature (Weissbach et al. 1975) and included cellular B-family polymerases pol α, pol β, pol δ, pol ε, and pol ζ, and the mitochondrial A-family polymerase pol γ. Rapid progress in genome sequencing, search algorithms, and further biochemical analysis identified other putative DNA polymerases both in bacteria and eukaryotes prompting the expansion of the Greek letter nomenclature. Families D, X, and Y were created to classify unique polymerase in archaea as well as those with specialized functions in DNA repair (Burgers et al. 2001; Ishino et al. 1998; Ohmori et al. 2001). After inclusion of reverse transcriptase enzymes that are RNA-dependent DNA polymerases including telomerase, the DNA polymerase families now number seven (Table 1.1). Although the number of human DNA polymerases stands at 16 members, a recently characterized human archaeo-eukaryotic (AEP) DNA primase (Prim-Pol) has both RNA and DNA synthesis abilities (L. Blanco, personal communication) suggesting that other uncharacterized enzymes may have additional unidentified roles in DNA synthesis. This chapter introduces and highlights chapters within this series and puts the DNA and RNA polymerase families, structures, and mechanisms in context.

1.1.1 DNA Polymerase Families and Function

Most DNA-dependent DNA polymerases have a single catalytic subunit (Fig. 1.1). These single subunits are generally active on their own but are regulated with

Table 1.1 Model DNA polymerase family members

Family	Viral	Bacterial	Archaeal	Eukaryotic
A	T7 gp5	Pol I		Pol γ (mito)
		Klenow[a]		Pol θ
		Taq Pol		Pol ν
B	T4/RB69 gp43	Pol II	PolB1 (B2 and B3)[b]	Pol α
	phi29 Pol			Pol δ
				Pol ε
				Pol ζ
C		Pol III		
D			Pol D[c]	
X				Pol β
				Pol λ
				Pol μ
				TdT
Y		Pol IV	Pol Y	Pol η
		Pol V		Pol ι
				Pol κ
				Rev1
RT[d]	RT			Telomerase

[a]Klenow is the C-terminal truncation of *E. coli* Pol I
[b]Crenarchaea generally have thee Pol B enzymes, while euryarchaea have one
[c]Pol D is only found in euryarchaea phyla of archaea
[d]Reverse transcriptase (RT) is RNA-dependent DNA polymerase

regard to function through various accessory proteins that direct and restrain catalysis to specific DNA substrates. For the most part, Y-family DNA repair polymerases adopt a slightly more open active site to accommodate base damage and are devoid of any proofreading exonuclease domains (Chap. 4). These structural features are required for replication past a variety of lesions in the template strand during DNA replication to maintain the integrity of the fork. DNA repair polymerases (X and Y family) are actively involved in maintaining our genome under intense DNA-damaging stressors. The ability to prevent mutagenesis is their main cellular role, but changes in expression levels and disruptions of DNA repair pathways are common in promoting cancer and tumorigenesis (Chap. 3). Viral, bacterial, and some archaeal DNA polymerases (A and B families) are primarily single-subunit enzymes. They are held at the replication fork through dynamic interactions with accessory proteins to maintain high local concentrations during active replication (Chap. 6). The DNA replication polymerase enzymatic accuracy (fidelity) is unprecedented and is primarily responsible for maintaining stable genomes of all organisms. In bacteria and eukaryotes, the DNA replication polymerases (B and C families) have evolved to contain additional subunits that are almost always associated with the catalytic subunit as holoenzyme complexes (Chap. 2). The recently discovered D-family replication polymerases from certain archaea are also multisubunit enzymes and are presumably ancestral precursors to their eukaryotic homologs (Chap. 6). The polymerase accessory subunits have a variety of roles that are only just being identified including maintaining structural

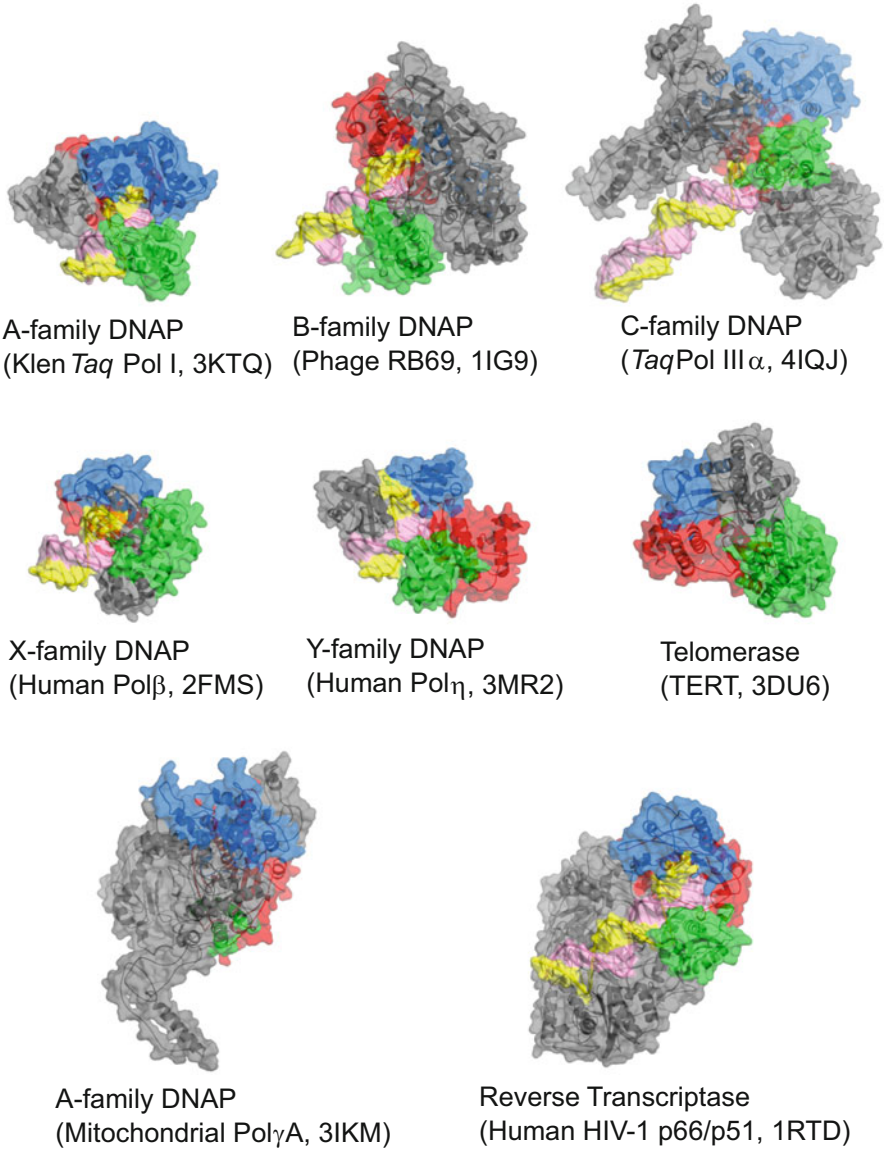

Fig. 1.1 Gallery of DNA polymerases. All polymerases and DNA are shown as cartoon models with partially transparent molecular surfaces. Their names of family, sources, and PDB accession codes are indicated. Protein structures are colored *gray* and key subdomains are colored (thumb, *green*; palm, *red*; fingers, *blue*). Nucleic acids are colored *yellow* for the template DNA and *pink* for the primer DNA. All polymerases are depicted using the same scale in this figure and also in Fig. 1.2 for direct comparison of their sizes

geometries, additional enzymatic activities, and interactions with other polymerase accessory proteins.

Although DNA polymerases are generally template-dependent enzymes and follow Watson and Crick base-pairing rules, there is a subclass of polymerases (primarily X family) that are template independent (Chap. 5). These polymerases are involved in aspects of DNA repair where DNA strands have lost connectivity and require additional nucleotide additions at the ends to facilitate repair. They also have a unique biological role contributing to random incorporations and corresponding diversity of antibodies required for immunological responses. A similar type of DNA extension is also required at the ends of chromosomes to maintain their length during DNA replication, but instead of random nucleotide additions, the enzyme telomerase uses an RNA template strand as a cofactor for sequence-specific DNA repeat additions called telomeres (Chap. 9). This RNA-dependent DNA polymerase, telomerase, is unique to organisms with linear genomes and is also implicated in a variety of human diseases and aging.

1.1.2 RNA Polymerase Families and Function

All cellular organisms including bacteria, archaea, and eukaryotes use multi-subunit DNA-dependent RNA polymerase for transcribing most of the RNAs in cells (Fig. 1.2) (Werner and Grohmann 2011). Bacterial RNA polymerase is the simplest form of this family (composed of the minimum five subunits), whereas archaeal and eukaryotic RNA polymerases possess additional polypeptides to form ~11–17 subunit complexes. Bacteria and archaea use a single type of RNA polymerase for transcribing all genes, whereas eukaryotes have three different enzymes, Pol I, Pol II, and Pol III, and synthesize the large ribosomal RNA (rRNA) precursor, messenger RNA (mRNA), and short untranslated RNAs including 5S rRNA and transfer RNA (tRNA), respectively (Chap. 12). In plant, there are two additional 12-subunit RNA polymerases, Pol IV and Pol V, that play important roles in RNA-mediated gene-silencing pathways (Chap. 13). The archaeal transcription system has been characterized as a hybrid of eukaryotic and bacterial transcription systems; the archaeal basal transcription apparatus is very similar to that of eukaryote, but its transcriptional regulatory factors are similar to those of bacteria (Hirata and Murakami 2009; Jun et al. 2011).

Bacteriophage encodes single-unit RNA polymerase of ~100 kDa molecular weight, which expresses bacteriophage genes on host bacterial cells for generating progeny phage particles (Chap. 10). Although bacteriophage RNA polymerase is about four times smaller than the cellular RNA polymerases (Fig. 1.2), it is able to carry out almost all functions in transcription cycle observed in cellular RNA polymerases. Its primary and three-dimensional structures are similar to A-family polymerase, which also includes mitochondrial RNA polymerase expressing genes from mitochondrial DNA (Fig. 1.2) (Chap. 11).

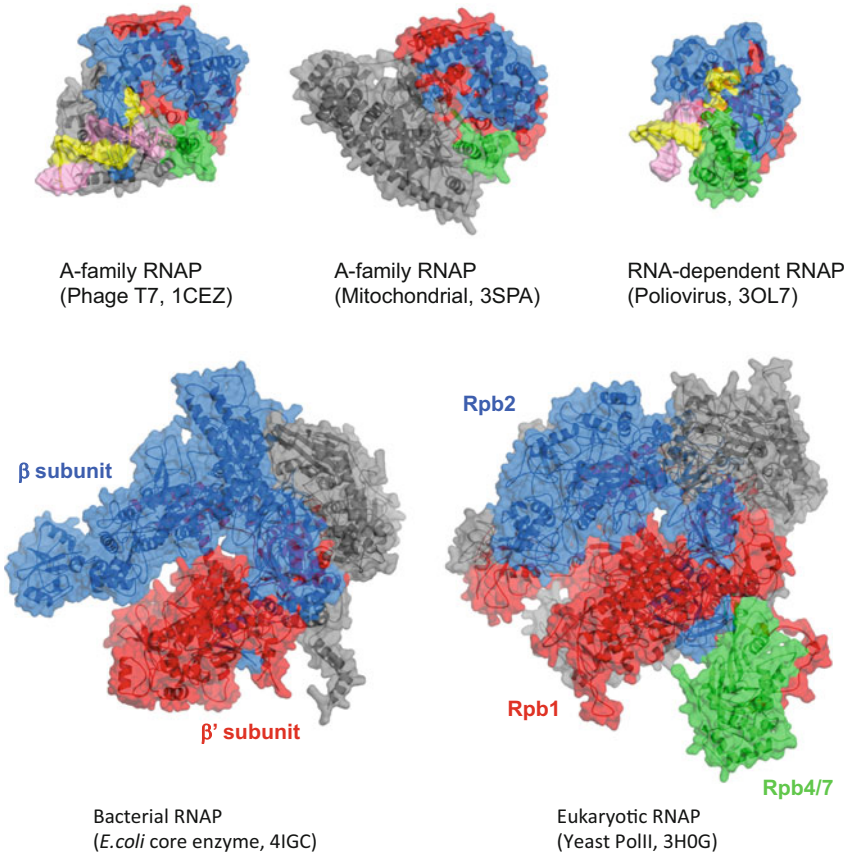

Fig. 1.2 Gallery of RNA polymerases. All polymerases and DNA are shown as cartoon models with partially transparent molecular surfaces. Their names of family, sources, and PDB accession codes are indicated. Protein structures are colored *gray* and key subdomains are colored (thumb, *green*; palm, *red*; fingers, *blue* for the A-family bacteriophage-type RNA polymerase and RdRp; largest subunit, *red*; second largest subunit, *blue*; protruding stalk, *green* for the cellular RNA polymerases). Nucleic acids are colored *yellow* for the template DNA and *pink* for the non-template DNA. All polymerases are depicted using the same scale in this figure and as in Fig. 1.1 for direct comparison of their sizes

RNA viruses including influenza, rhinovirus, hepatitis C, and poliovirus have RNA-dependent RNA polymerases (RdRps) that are responsible for replicating their RNA genomes and expressing their genes (Fig. 1.2). The RdRps are targets for antiviral therapies, but their higher mutation rates due to lack of proofreading endonuclease activity generate resistant variants to compromise antiviral therapies (Chap. 14).

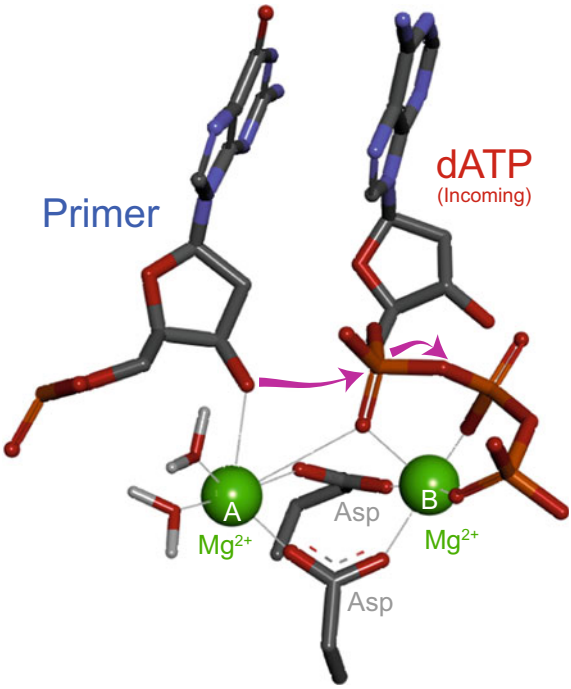

Fig. 1.3 Highlights the two-metal-ion mode of catalysis for DNA and RNA polymerases. Two conserved aspartates coordinate metals A and B in the active site. Metal A activates the 3′-OH for attack on the 5′ α-phosphate of the incoming nucleotide (either dATP or ATP) with release of β-γ pyrophosphate. Metal B neutralizes the negative charge on the phosphates as well as buildup in the transition state

1.2 Conserved Polymerase Structures

The original structure of the C-terminal fragment of *E. coli* Pol I (Klenow fragment) identified the general architecture of DNA polymerases to resemble a right hand with subdomains similar to fingers, thumb, and palm regions (Fig. 1.1) (Ollis et al. 1985). Although sequence homology from different DNA polymerase families has diverged quite significantly, the general organization of all polymerase structures is very similar (Figs. 1.1 and 1.2), suggesting that they may have evolved from a common ancestor. In fact, both the DNA and RNA polymerases catalyze essentially the same chemical reaction with subtle differences ensuring accurate incorporation of their respective nucleotides (Fig. 1.3) (Steitz 1993).

1.2.1 DNA Polymerase Structural and Kinetic Mechanisms

The two most important and conserved residues are aspartates contained within the palm domain that act to coordinate two metal ions (Mg^{2+}) for catalysis (Fig. 1.3). Metal A lowers the bonding potential of the hydrogen at the 3′-OH, activating the 3′-O^- for attack at the α-phosphate of the incoming nucleotide. Metal B aids pyrophosphate leaving and stabilizes structures of the pentacovalent transition

state. The mechanism was originally proposed based on the 3′–5′ removal of nucleotides in the exonuclease site of DNA Pol I (Beese et al. 1993). This two-metal-ion mechanism for phosphoryl transfer is identical for DNA and RNA polymerases and extremely similar to analogous reactions involving RNA-catalyzed reactions including splicing (Steitz and Steitz 1993). It is hypothesized that this mechanism was the basis of catalysis in the RNA world and has maintained its core features with all modern polynucleotide polymerases. Interesting, this basic two-metal-ion mechanism has recently been challenged by the observation of a third metal ion in the active site of pol η that acts to neutralize the negative charge buildup in the transition state and protonates the leaving group pyrophosphate (Nakamura et al. 2012). It will be interesting to see if this transient third metal ion also exists in other polymerases suggesting a common theme and expansion of the traditional two-metal-ion phosphoryl transfer mechanisms. The two other domains (fingers and thumb) have diverged significantly throughout the polymerase families but contain functionally analogous elements. The fingers domain acts to correctly position the incoming nucleotide with the template, while the thumb domain aids in DNA binding and successive nucleotide additions (processivity).

To increase the fidelity (accuracy) of continuous nucleotide incorporation, some DNA polymerases from the A, B, and C families have a separate exonuclease (3′–5′) domain which verifies correct incorporation and removes an incorrectly incorporated base. For bacterial and archaeal family A and B polymerases, the exonuclease activity is included in a separate domain within the contiguous polypeptide sequence. In the *E. coli* Pol III holoenzyme as well as eukaryotic B-family polymerase, the exonuclease activity is contained within a separate polypeptide. The first structure of DNA bound in the exonuclease domain was with the Klenow fragment and suggested a common two-metal-ion catalysis mechanism for removal of nucleotides as well (Beese and Steitz 1991). The exonuclease site was defined as having three conserved carboxylate residues coordinating both metal ions, binding to the DNA, and activating catalysis and removal of an incorrectly incorporate base.

In addition to exonuclease activity, high-fidelity DNA polymerases also maintain accuracy through kinetic checkpoints ensuring accurate base pairing (Fig. 1.4). The general consensus is that the polymerase domain alone accounts for fidelity values of 10^{-5} to 10^{-6} and inclusion of the exonuclease proofreading domain contributes another 10^{-2} for total fidelity values of 10^{-7} to 10^{-8} (1 error in every 100 million or 99.999999 % accurate) (Kunkel 2004). DNA polymerases from other families including X and Y have significantly lower fidelity values (10^{-2} to 10^{-5}) accounted by the more frequent error rates, lesion bypass abilities, and absent exonuclease domains (Chaps. 3–5).

For the majority of A-, B-, as well as some Y-family polymerases, a slow step prior to chemistry (step 3) ensures correct base pairing before phosphodiester bond formation. Based on the fusion of structural and kinetic data, it was originally postulated that an open-to-closed transition in the fingers domain was the slow step in the mechanism. More recently, the open-to-closed transition was measured directly using fluorescence and found to be fast relative to step 3, prompting the

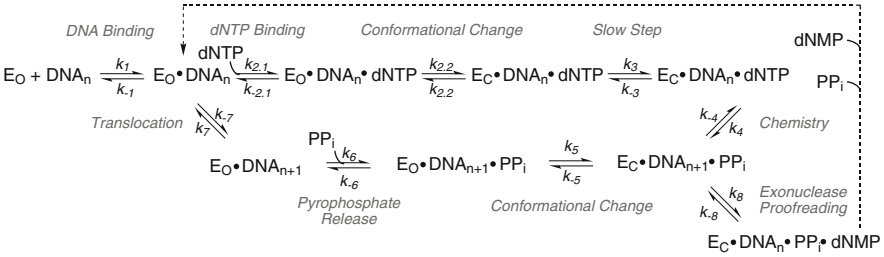

Fig. 1.4 General kinetic mechanism for high-fidelity DNA polymerases. The enzyme undergoes a fast open (E_O)-to-closed (E_C) transition (step 2.2) after binding DNA and nucleotide. Kinetic checkpoints include the slow step prior to chemistry (step 3) as well steps 5 and 8 after chemistry to activate the proofreading function if necessary

inclusion of steps 2a and 2b into the mechanism (Joyce et al. 2008; Johnson 2010). The identity of the slow step 3 is still unknown and may instead be associated with a change in metal ion coordination of either metal B or an incoming metal C in preparation of moving forward through the transition state towards chemistry (Nakamura et al. 2012).

The kinetic checkpoints themselves ensure that correct nucleotides are optimally positioned in the active site over incorrect ones to promote catalysis. Prevention of rNTP binding in DNA polymerase active sites is restricted by a steric gate towards the 2′-OH (step 2.1) (Delucia et al. 2006) as well as reduced rate of fingers closing (step 2.1) limiting their incorporation (Joyce et al. 2008). For Klenow and T7 pol, incorrect dNTP incorporation is prevented by a slower chemistry step 4 than for correct dNTPs defining polymerase fidelity (Dahlberg and Benkovic 1991). Step 5 following phosphoryl transfer is also considered a kinetic slow step and is important for increasing the probability for proofreading (step 8) in the case of a misincorporated base (Kuchta et al. 1988). Although this is not an absolute kinetic mechanism for nucleotide selection in all DNA polymerases (Fig. 1.4), the basic principles explain a number of the mechanistic facets required for maintaining high nucleotide fidelity. Whether or not this complex scheme holds as a general mechanism for all DNA polymerases remains to be determined, but it is likely to be accurate for high-fidelity DNA polymerases in particular.

Fast, successive, and accurate nucleotide additions require that the polymerase remains associated with the template after a translocation step (step 7) for multiple rounds of catalysis or processivity. DNA polymerases by themselves are not highly processive and are not able to incorporate more than 20–50 successive nucleotides in a single binding event. The exception seems to be the B-family DNA polymerase from phi29 which has extremely robust strand displacement activity and processivity of replication of several thousand bases (Blanco et al. 1989; Kamtekar et al. 2006). Phi29 Pol has a specific insertion called the terminal protein region 2 (TPR2) that acts with the palm and thumb subdomains to encircle and close around the DNA template limiting dissociation. Increased processivity has also been seen after oligomerization of some archaeal DNA polymerases effectively

encircling the template (Chap. 6). In both of these examples, the polymerases use a topological linkage of the protein to DNA to remain bound to the template for efficient and successive incorporations. More commonly, interactions of DNA polymerases with toroidal accessory factors (clamp proteins) achieve the same result of increased processivity by coupling the DNA polymerase with the template, limiting dissociation (Trakselis and Benkovic 2001; Bloom 2009). These clamp proteins (PCNA, in particular) have specific interaction domains that bind consensus sequences in DNA polymerases and other genomic maintenance proteins that act to recruit and retain enzymes at the replication fork (Moldovan et al. 2007).

1.2.2 RNA Polymerase Structural Mechanism

For the nucleotidyl transfer reaction by RNA polymerase, a two-metal-ion catalytic mechanism has been proposed, which is common in the DNA polymerase, as the enzyme possesses two divalent catalytic and nucleotide-binding metal cations (Mg^{2+}) chelated by two or three Asp residues at the enzyme active site (Fig. 1.3). Both metal ions are proposed to have octahedral coordination at physiological Mg^{2+} concentrations.

For transcribing RNA using DNA template, DNA-dependent RNA polymerase including cellular RNA polymerases (Chaps. 12 and 13), bacteriophage RNA polymerase (Chap. 10), and mitochondrial RNA polymerase (Chap. 11) unwinds a small region of double-stranded DNA to the single-stranded form and synthesizes RNA as a complementary sequence of the template. The unwound DNA region is called the transcription bubble that contains a DNA-RNA hybrid of ~8 base pairs. For synthesis of RNA, nucleotide substrate and catalytically essential divalent metals in addition to the single-stranded template DNA must be accommodated at the active site. One of four ribonucleotide triphosphates—ATP, GTP, CTP, and UTP—forms a Watson–Crick base pair with a DNA template base, and its α-phosphate group is attached by a 3′-hydroxyl of the growing end of the RNA. As a result, a linear RNA polymer is built in the 5′ to 3′ direction.

The overall shape of cellular RNA polymerases including bacterial, archaeal, and all types of eukaryotic enzymes is crab claw-like with a wide internal channel for double-stranded DNA binding (Fig. 1.2, Chaps. 12 and 13). The enzyme active site is located on the back wall of the channel, where an essential Mg^{2+} ion is chelated by three Asp of the absolutely conserved NADFDGD motif in the largest subunit. Compared to the bacterial RNA polymerase, archaeal and all eukaryotic RNA polymerases possess a characteristic protruding stalk that is formed by a heterodimer, and their relative positioning of the main body and stalk is also highly conserved.

The structure of bacteriophage-type RNA polymerases including mitochondrial enzyme resembles cupped right hand with palm, fingers, and thumb subdomains and a cleft that can accommodate double-stranded DNA (Fig. 1.2). Not only the overall structure of polymerases but also the secondary structures of subdomains in

the bacteriophage-type RNA polymerase are highly conserved in the A-family DNA polymerase. The enzyme active site is located on the palm subdomain, where an essential Mg^{2+} ion is chelated by two Asp of the conserved motifs A and C. The conserved motif B is in the mobile finger subdomain, which changes its position during the nucleotide addition cycle and plays an important role in the nucleotide selection (Chaps. 10 and 11).

The overall shape of RdRps is similar to other nucleic acid polymerases, having "cupped right hand" structure and fingers, thumb, and palm subdomains (Fig. 1.2, Chap. 14). Because of its extension of the fingers, RdRp has more fully enclosed the active site, which may enhance their protein stability and enzyme processivity for their genomic RNA replication function.

1.3 Implications in Disease/Therapy

Although accurate DNA synthesis is a hallmark of high-fidelity DNA replication polymerases, a number of other polymerases have been implicated in various diseases and aging underlying their importance for further study. The best known example involves telomerase. When normal somatic cells replicate in the absence of telomerase, they undergo successive shortening of their telomeric ends, termed the end replication problem (Allsopp and Harley 1995). The shortening of telomeres acts as a clock determining the life of a cell ultimately causing senescence and cell death. However, in cancer cells, telomerase is upregulated preventing telomeric shortening and increasing cellular survival giving rise to immortal cells found in tumors. Although telomerase deficiency is most notable in the genetic disorder, *dyskeratosis congenita*, mutations in telomerase are also associated with anemia, other bone marrow-related diseases, and lung fibrosis. The unifying diagnostic indicator in all cases is short telomeres (Armanios 2009). The mechanism of RNA-mediated DNA telomeric synthesis by telomerase will be discussed in great detail in Chap. 9.

Translesion DNA polymerases are specialized low-fidelity DNA polymerases that can insert bases opposite a lesion, bypassing the damage, while potentially inducing point mutations. It is hypothesized that potential mutagenesis is favored over complete replication arrest and fork collapse. Translesion synthesis (TLS) is regarded as being responsible for the large increase in point mutations found in cancer genomes (Bielas et al. 2006). These Y-family DNA polymerases generally have much less fidelity and more open active sites accommodating a variety of DNA template lesions including oxidations, deaminations, abasic sites, methylations, and a host of environmental mutagens and are described in detail in Chap. 4.

Mutations in the Y-family Pol η account for the inheritable genetic disease, *xeroderma pigmentosum* (XPD) (Masutani et al. 1999). This disease sensitizes cells to UV light, significantly increasing the risk of skin carcinomas. Pol η is known to bypass thymine cyclobutane pyrimidine dimers (CPD) caused by UV cross-linking

of adjacent residues (Johnson et al. 2000). Genetic mutations in Pol η associated with XPD disrupt the contacts with the DNA limiting its activity. Therefore, this translesion DNA polymerase has evolved a specific role in replication over UV-induced damage, and mutations in Pol η are responsible for replication fork collapse, double-strand breaks, and chromosomal breaks. The only other DNA polymerase found to be associated with an inheritable genetic disease is Pol ε where mutations in the large subunit give rise to splicing changes that cause decreased expression and predicted truncated protein products in FILS (facial dysmorphism, immunodeficiency, livedo, and short statures) syndrome patients (Pachlopnik Schmid et al. 2012).

The X-family base excision repair DNA polymerase (Pol β) (Chap. 3) has been found to have sporadic mutations in human tumors (Starcevic et al. 2004). Increased expression of Pol β has also been measured in a number of cancers interfering with normal DNA replication and causing mutations (Albertella et al. 2005; Tan et al. 2005). Several small-molecule inhibitors have been found to increase sensitivity to chemotherapeutic agents by blocking action of Pol β and seem to be a viable avenue for cancer therapy (Goellner et al. 2012). Other X-family DNA polymerases including terminal deoxytransferase (TdT) (Chap. 5) have been implicated in leukemia and carcinomas through altered expression levels. New nucleoside analogs have been shown to be specific towards TdT controlling expression levels and sensitizing cancer cells to conventional treatment.

Therefore, it seems there is an opportunity for targeted X- and Y-family DNA polymerase inhibition by either controlling expression levels or using as adjuvants with DNA-damaging radiation or chemotherapy (Lange et al. 2011). The challenges will be to avoid toxicity issues common with previous inhibitors, selectively target cancer cells, and act specifically on one of the 16 human DNA polymerases. Not an easy task, but with preliminary successes for Pol β and Tdt, the opportunity also exists for other DNA polymerases. The more we can emphasize structural differences in the active sites, identify allosteric regions, or detect novel mechanistic features, the better a position we are in to develop novel therapeutic agents. Success will require understanding the balance of DNA polymerase actions in a variety of cell types and developing screening methods to simultaneously measure effects on multiple DNA polymerases.

RNA polymerase is an essential enzyme in bacteria and virus and, as such, is a proven target for antibiotics and antiviral drugs (Chaps. 11 and 14). Fidaxomicin (FDX) is an inhibitor of bacterial RNA polymerase and is one of the newest antibiotics approved by the US Food and Drug Administration (FDA) for treatment of *Clostridium difficile*-associated diarrhea. The best characterized antibiotic against bacterial RNA polymerase is Rifampicin (Rif), which has been used as the first-line drug for infectious bacteria treatment, including tuberculosis, over four decades. However, a high incidence of Rif-resistant bacterial strains with RNA polymerase mutations is one of our public health challenges. Although many Rif-resistant *Mycobacterium tuberculosis* with RNA polymerase mutants have been derived in laboratory, only three residues with specific amino acid substitution account for ~85 % of *M. tuberculosis* Rif-resistant strains found in clinical isolates.

Therefore, structures of these three Rif-resistant RNAPs can move one step forward the structure-based discovery of improved Rif for tuberculosis treatment.

1.4 Remaining Questions and Future Directions

DNA and RNA polymerases have evolved naturally over millions of years to be highly accurate enzymes for incorporating the four available deoxyribonucleotides for DNA synthesis and ribonucleotides for RNA synthesis to faithfully maintain genomes and to express genes. Recent research efforts have focused on evolving these high-fidelity enzymes to have altered enzymatic properties or nucleotide specificities required for a variety of biotechnology applications (Chap. 7). Some of the goals are to amplify ancient genomes, incorporate alternative genetic alphabets, and replicate chemically and environmentally modified templates. In addition to traditional biotechnology development, these engineered polymerases have the potential to revolutionize synthetic biology by creating safe artificial living systems that incorporate unnatural DNA analogs for the creation of anything from drugs/metabolites to energy.

Although the basic mechanisms of incorporation, proofreading, and fidelity are well characterized for a number of DNA polymerases, there are many remaining questions on how polymerases function within the context of the replisome during normal DNA replication or repair. For example, do the kinetics or fidelities change drastically when accessory proteins are interacting with the polymerase? How does the unwinding rate of the DNA helicase control DNA polymerase kinetics? How is high-fidelity synthesis coordinated with error-prone lesion bypass when multiple polymerases are available? Answers to these questions will require the ability to both assemble and test more components of the replisome simultaneously in vitro and probe the kinetics within the context of an actively replicating cell.

The expression levels of DNA polymerases in various cancer cell types and stem cells are also an exciting avenue for study. Stem cells in particular need to maintain highly stable genomes. In these cells, the distribution of polymerases should favor high-fidelity enzymes and may even include suppressors against X- and Y-family polymerases. On the other hand, cancer cells are active mutators, and it would not be surprising to find inactivating mutations, loss, or rearrangements of DNA polymerases as more individual cancer cell sequencing results are available. In addition, DNA polymerases may be inactivated through alterations in DNA methylation patterns or RNAi changes. Pol θ in particular has been shown to have a significant difference in expression levels in breast tumors over non-tumor cells (Lemee et al. 2010). Therefore, it will also be important to assess any expression level deviations for DNA polymerases in individual cells to understand equilibrium changes that may be occurring at the replication fork and their resulting consequences on genomic stability. If DNA polymerase distributions can be determined first, it is conceivable that targeted DNA polymerase therapies will better sensitize cells to radiation or chemotherapy.

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Chapter 2
Eukaryotic Replicative DNA Polymerases

Erin Walsh and Kristin A. Eckert

Abstract DNA replication is a dynamic process that requires the precise coordination of numerous cellular proteins. At the core of replication in eukaryotic cells are three DNA polymerases, Pol α, Pol δ, and Pol ε, which function cooperatively to ensure efficient and high-fidelity genome replication. These enzymes are members of the B family of DNA polymerases, characterized by conserved amino acid motifs within the polymerase active sites. Pol α is a DNA polymerase of moderate fidelity that lacks 3′→5′ exonuclease activity, while Pols δ and ε are processive, high-fidelity polymerases with functional 3′→5′ exonuclease activities. Each polymerase exists as a holoenzyme complex of a large polymerase catalytic subunit and several smaller subunits. The Pol α holoenzyme possesses primase activity, which is required for de novo synthesis of RNA–DNA primers at replication origins and at each new Okazaki fragment. In one model of eukaryotic DNA replication, Pol ε functions in leading strand DNA synthesis, while Pol δ functions primarily in lagging strand synthesis. This chapter discusses the biochemical properties of eukaryotic replicative polymerases and how biochemical properties shape their functional roles in replication initiation, replication fork elongation, and the checkpoint responses.

Keywords DNA replication fork • S phase checkpoint • DNA polymerase fidelity • primase • proofreading exonuclease • replisome • genome stability

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2.1 Biochemical Properties of Replicative Polymerases

2.1.1 Polymerase α-Primase

2.1.1.1 Overview

A formal nomenclature for eukaryotic DNA polymerases has been adopted, in which cellular DNA polymerases are given Greek letters in order of their historical discovery (Weissbach et al. 1975; Burgers et al. 1990). Accordingly, Pol α-primase was the first mammalian polymerase to be purified and characterized (Yoneda and Bollum 1965). Pol α-primase holoenzyme is a heterotetrameric protein complex in all eukaryotes studied (Table 2.1) (see Muzi-Falconi et al. 2003 for review). The p180 large subunit contains the DNA polymerase active site (Plevani et al. 1985; Wong et al. 1986). The B subunit has no known enzymatic activity, but performs a regulatory function, possibly linking the pol α holoenzyme to components of the replication fork (Collins et al. 1993). DNA polymerases lack the ability to carry out de novo DNA synthesis and, in eukaryotes, require a 3′-OH provided by an RNA primer in order to initiate DNA synthesis. Primase activity is contained within the tightly associated p49/p58 complex (Plevani et al. 1985; Nasheuer and Grosse 1988). The architecture and subunit arrangement of the *Saccharomyces cerevisiae* (*S. c.*) Pol α-primase holoenzyme have been examined by X-ray crystallography and electron microscopy. The B subunit is tethered to the p180 subunit through a structured, flexible linker (Klinge et al. 2009). The four-subunit holoenzyme exists as a dumbbell-shaped particle, with the catalytic primase and polymerase active sites present in distinct lobes of the complex, separated by ~100 Å (Nunez-Ramirez et al. 2011).

2.1.1.2 An Essential Polymerase and Primase

Pol α polymerase activity is essential for chromosomal replication. Genetic analyses of mutants in *S. c.* have demonstrated that both the catalytic and regulatory B subunits are required in vivo for viability, and mutants in either gene exhibit defects in DNA replication and progression through S-phase (Johnson et al. 1985; Foiani et al. 1994; Budd and Campbell 1987). In mammalian cells, Pol α-primase neutralizing antibodies inhibit DNA synthesis (Miller et al. 1985; Kaczmarek et al. 1986). Thermosensitive yeast *POL1* mutants display elevated genetic instability due to defects in replication (Gutiérrez and Wang 2003; Liu et al. 1999). The primase activity is also essential in eukaryotic cells. Disruption of either the *PRI1* or *PRI2* genes is lethal in *S. c.* due to replication defects (Foiani et al. 1989; Lucchini et al. 1987). Characterization of conditional *PRI1* and *PRI2* mutants demonstrates that loss of primase activity causes increased mitotic recombination and spontaneous mutation rates, possibly due to defects in replication and impaired meiosis (Longhese et al. 1993).

Table 2.1 Composition of the major replicative DNA polymerases in eukaryotes

		Designation (gene/protein)		
Subunit	Function	Human	*S. cerevisiae*	*S. pombe*
Pol α holoenzyme				
A	Polymerase	*POLA1*/p180	*POL1*	*pol1*
B	Regulatory	*POLA2*/p70	*POL12*	*pol12*
	Primase regulatory	*PRIM2*/p58	*PRI2*	*pri2*
	Primase catalytic	*PRIM1*/p49	*PRI1*	*pri1*
Pol δ holoenzyme				
A	Polymerase, 3′→5′ exonuclease	*POLD1*/p125	*POL3*	*pol3*
B	Regulatory	*POLD2*/p50	*POL31*	*cdc1*
C	Regulatory	*POLD3*/p68[a]	*POL32*	*cdc27*
D	Regulatory (DNA damage)	*POLD4*/p12	–	*cdm1*
Pol ε holoenzyme				
A	Polymerase, 3′→ 5′ exo	*POLE1*/p261	*POL2*	*cdc20*
B	Regulatory	*POLE2*/p59	*DPB2*	*dpb2*
C	Double-stranded DNA binding	*POLE3*/p17	*DPB3*	*dpb3*
D	Double-stranded DNA binding	*POLE4*/p12	*DPB4*	*dpb4*

[a]Also referred to as the p66 subunit

2.1.1.3 Coordinated Primase and Polymerase Activities

In eukaryotic cells, in vivo studies have demonstrated that RNA primers of Okazaki fragments are attached to short DNA chains (Kitani et al. 1984). The Pol α holoenzyme is a unique replicative protein complex, possessing two coupled catalytic activities (Hu et al. 1984). Primase initiates synthesis of RNA at polypyrimidine tracts within the DNA template, preferentially within a T-rich region, and this occurs at many sites along the DNA template (Yamaguchi et al. 1985; Kitani et al. 1984). The primase catalytic site maps to the p49 subunit (Nasheuer and Grosse 1988), in which a carboxylic triad is essential for its function in generating RNA primers (Copeland and Tan 1995). The *S. c.* Pol α/p49 subunit is sufficient for synthesis of RNA, but highly inefficient without the p58 subunit (Santocanale et al. 1993). Biochemically, p58 is necessary for synthesis of the first dinucleotide of the RNA primer and modulates the rate and stability of subsequent extension steps (Copeland and Wang 1993). GTP is preferentially incorporated into the first dinucleotide, possibly to stabilize the short primer (Sheaff and Kuchta 1993). Although RNA primers generated by purified primase can vary in length from ~2 to 10 nucleotides in the presence of dNTPs (Nasheuer and Grosse 1988), only those $\geq$7 nucleotides are extended by Pol α, and formation of an RNA primer of this length terminates primase activity (Kuchta et al. 1990). The p58 subunit regulates primase processivity and ensures the correct RNA primer length is synthesized (Zerbe and Kuchta 2002). The DNA polymerase activity of Pol α extends RNA primers with ~20–30 dNTPs to generate an RNA–DNA hybrid molecule (Thompson et al. 1995).

Mechanistically, the primase and polymerase activities of the Pol α holoenzyme are tightly coupled to ensure that new primers are not synthesized until the previous primer has been extended by polymerase (Sheaff et al. 1994). Functional coordination of the two activities is dependent upon the presence of dNTPs (Hu et al. 1984). An intramolecular transfer of the RNA primer from the primase active site to the polymerase active site occurs rapidly when dNTPs are present (Sheaff et al. 1994). Structurally, flexible tethering of Pol α holoenzyme lobes containing the primase and polymerase centers increases the efficiency of primer transfer between the two active sites (Nunez-Ramirez et al. 2011). Structural and biochemical studies suggest that the p58 subunit is involved in the switch from primase to polymerase through conformational changes (Arezi et al. 1999; Agarkar et al. 2011). Consistent with its role in synthesizing short RNA–DNA primers, the Pol α holoenzyme lacks the high processivity possessed by the other major replicative holoenzymes (see below), although it does possess the capacity to carry out robust DNA synthesis in vitro. The rate of DNA synthesis by calf thymus Pol α-primase is similar to that of Pols ε and δ (Weiser et al. 1991), and Pol α-primase displays an in vitro processivity of ~20–100 nucleotides (Hohn and Grosse 1987). Pol α-primase interacts with the p70 subunit of replication protein A (RPA), which stimulates both the polymerase activity and processivity of the holoenzyme (Braun et al. 1997), possibly by increasing the affinity for primer termini (Maga et al. 2001).

2.1.1.4 A Moderately Accurate Polymerase

The in vitro fidelity of the mammalian Pol α holoenzyme purified from multiple sources has been determined using several genetic assays (Kunkel and Loeb 1981; Kunkel 1985; Eckert et al. 1997). The majority of errors created by Pol α are single-base substitutions, followed by one-base deletion errors (Kunkel 1985; Eckert et al. 1997). The purified *S. c.* Pol α/p180 catalytic subunit and the *S. c.* Pol α holoenzyme have similar overall fidelities but display statistically significant differences in error rates within specific sequence contexts (Kunkel et al. 1989). The *S. c.* Pol α/p180 subunit exhibits an error rate of 1/9,900 and 1/12,000 per nucleotide synthesized, for single-base substitutions and deletions, respectively (Kunkel et al. 1991). The human Pol α/p180 subunit base substitution error rate can be as low as 1/42,000 nucleotides in low-pH buffer conditions (Eckert and Kunkel 1993). Direct comparison of purified mammalian Pol α-primase, Pol δ, and Pol ε preparations demonstrated that Pol α possesses the lowest fidelity of the three major eukaryotic replicative polymerases (Thomas et al. 1991).

One mechanism by which Pol α maintains its moderate fidelity is the low rate of extending mismatched DNA primers (Perrino and Loeb 1989). Some calf thymus Pol α holoenzyme pause sites correspond to sites of increased mismatched 3′-primer termini (Fry and Loeb 1992), consistent with a low rate of mispair extension. Pol α holoenzyme pausing within microsatellite sequences also is correlated with the rate of misalignment-based errors (Hile and Eckert 2004).

Replication auxiliary factors enhance the DNA fidelity of Pol α-primase. RPA decreases dNTP misincorporation rate by decreasing the affinity of the Pol α holoenzyme to mismatches while increasing the affinity to matched DNA primer termini, suggesting that it may function as a "fidelity clamp" that modulates the Pol α holoenzyme for faithful DNA synthesis (Maga et al. 2001). Terminal misincorporation events at Pol α pause sites are also avoided in the presence of RPA (Suzuki et al. 1994). Pol α-primase lacks 3′→5′ exonuclease activity due to amino acid changes at catalytic residues within the domain, which limits its intrinsic fidelity (Pavlov et al. 2006b). However, *S. c.* Pol δ can perform intermolecular proofreading of errors made by Pol α-primase (Pavlov et al. 2006a). Based on an estimation that ~4–8 × 10^4 RNA–DNA primers are synthesized by Pol α-primase during replication in human cells (Muzi-Falconi et al. 2003), such mechanisms of enhanced fidelity may be essential in maintaining genome stability.

In contrast to its DNA polymerase fidelity, the priming activity exhibits very low fidelity during RNA synthesis. Primase readily misincorporates NTPs during in vitro RNA synthesis, often polymerizing primers containing consecutive mismatches (Sheaff and Kuchta 1994). Importantly, after intramolecular transfer, such mismatched RNA primers are readily extended by the DNA polymerase activity in the presence of dNTP substrates.

2.1.1.5 Posttranslational Regulation

The Pol α holoenzyme exists as an assembled complex throughout the cell cycle (Ferrari et al. 1996), and its activity is regulated by cyclin-dependent kinases in a cell cycle-dependent manner (Voitenleitner et al. 1999). The p180 subunit is a phosphoprotein that becomes hyperphosphorylated in G_2/M phase, while the B subunit is phosphorylated only in G_2/M (Nasheuer et al. 1991). Pol α phosphorylation results in lowered single-stranded DNA binding affinity, lowered DNA synthesis activity, and an inhibition of DNA replication (Nasheuer et al. 1991; Voitenleitner et al. 1999).

2.1.2 DNA Polymerase δ

2.1.2.1 Overview

Pol δ was originally identified as a novel DNA polymerase purified from rabbit bone marrow that possessed a very active 3′→5′ exonuclease activity (Byrnes et al. 1976). Subsequently, *S. c.* POL3 was identified as the yeast homolog of Pol δ (Boulet et al. 1989). In all eukaryotes, Pol δ is a multimeric complex, wherein the largest subunit harbors the DNA polymerase and 3′→5′ exonuclease catalytic domains, and is tightly associated with a regulatory B subunit (Table 2.1). Mammalian Pol δ holoenzyme is a heterotetrameric protein consisting of two additional

C and D subunits, p68 and p12. The fission yeast *Schizosaccharomyces pombe* (*S. p.*) also encodes a heterotetrameric Pol δ, possessing the Cdm1 subunit which shows some sequence similarity to the mammalian p12 subunit. The budding yeast *S. c.* Pol δ is a heterotrimer of the A and B subunits together with a C subunit ortholog, Pol32.

The structure of a truncated form of the *S. c.* Pol δ/p125 subunit in ternary complex with DNA and dNTP substrates has been solved at 2 Å resolution (Swan et al. 2009). The polymerase domain has the characteristic right-hand structure of palm, fingers, and thumb subdomains. The 3′→5′ exonuclease domain is separated from the polymerase domain by 45 Å. Pol δ also possesses a novel N-terminal domain that interacts with 10–20 nucleotides of the ssDNA template, upstream of the polymerase active site. The structure of a truncated *S. c.* Pol δ holoenzyme as determined by small-angle X-ray scattering analysis consists of a globular catalytic core (A subunit) flexibly linked to an elongated tail comprised of the B and C subunits (Jain et al. 2009). The interaction of Pol3 (A subunit) with the Pol31 and Pol32 (B and C subunits) is stabilized by binding of a 4Fe-4S metal cofactor cluster to four conserved cysteine residues in the CysB motif of the Pol3 C-terminal domain (Netz et al. 2012).

2.1.2.2 An Essential Polymerase

Evidence that Pol δ is an essential component of the eukaryotic replication machinery has been derived from several models. Disruption of Pol δ is lethal in *S. p.*, and thermosensitive mutants of pol3 arrest in S-phase of the cell cycle (Francesconi et al. 1993). Deletion of the *S. p.* cdc27 gene (Pol δ/C subunit) results in cell cycle arrest (MacNeill et al. 1996). Although the orthologous *S. c POL*32 gene is not essential, deletion mutants do display abnormal phenotypes (Gerik et al. 1998). *S. c. POL*3 mutants harboring different substitutions at a catalytically essential residue, Leu^{612}, exhibit a wide range of phenotypic deficiencies (Venkatesan et al. 2006). Only 8 of 19 mutants are viable and display varying degrees of genotoxic sensitivity, cell cycle defects, and morphological abnormalities (Venkatesan et al. 2006). Replication studies in *Xenopus* egg extracts demonstrated that immunodepletion of Pol δ leads to a significant decrease in DNA synthesis and the accumulation of unreplicated, single-stranded DNA gaps (Fukui et al. 2004). Knockdown of Pol δ/p125 in HeLa cells causes an accumulation of cells in early, middle, and late S-phase, and cells do not enter mitosis (Bermudez et al. 2011). Homozygous disruption of *Pold1* in mice was shown to cause embryonic lethality between E4.5 and E7.5, due to significant defects in DNA synthesis (Uchimura et al. 2009). Profoundly, homozygous mutations of highly conserved amino acids within motif A of the Pol δ active site (L604G and L604K) are embryonic lethal in mice, and mice heterozygote for the L604K mutant exhibit reduced life spans and accelerated tumorigenesis (Venkatesan et al. 2007). Mouse embryonic fibroblasts heterozygous for these mutant proteins display elevated mutation rates and chromosomal instability relative to wild-type cells (Venkatesan et al. 2007). The severe

replication defects associated with loss of Pol δ in each of these models were vital in uncovering the polymerase's role as a major component of the eukaryotic replication fork.

2.1.2.3 Efficient DNA Synthesis in the Presence of Replication Accessory Factors

Pol δ requires replication accessory proteins to achieve its greatest efficiency. Shortly after the discovery of Pol δ, an auxiliary protein was identified, which co-eluted with Pol δ during purification and significantly enhanced its in vitro DNA synthesis on several DNA templates (Tan et al. 1986). This protein was later identified as proliferating cell nuclear antigen (PCNA) and was shown to greatly enhance calf thymus Pol δ processivity in vitro (Prelich et al. 1987; Bravo et al. 1987). PCNA is now known as the eukaryotic sliding clamp protein, a homotrimer ring-shaped protein that encircles DNA and tethers replication proteins, allowing movement along the DNA template (Moldovan et al. 2007). Replication Factor C (RFC), a heteropentameric complex, is responsible for loading PCNA onto DNA through an ATP-dependent mechanism. In the presence of PCNA, RFC, and RPA, *S. c.* Pol δ processivity is enhanced from 6 nucleotides to >600 nucleotides (Chilkova et al. 2007). Human Pol δ holoenzyme activity increases >50-fold and processivity is stimulated in the presence of PCNA (Xie et al. 2002). However, the human Pol δ holoenzyme differs biochemically from *S. c.* Pol δ holoenzyme, as human Pol δ dissociates more readily from DNA templates, even in the presence of accessory factors (Bermudez et al. 2011).

Recombinant human Pol δ can be purified in several subassemblies, all of which retain DNA synthesis activity (Zhou et al. 2012; Podust et al. 2002). The Pol δ p125/p50 heterodimer (also referred to as the core dimer) is a tightly associated complex with low specific activity. The three subunit complex, Pol δ3 (core + p68 subunit), displays high DNA synthesis activity but is unstable in vitro. The p68 subunit is essential for synthesis of long DNA products by Pol δ. The p12 subunit increases stability of the holoenzyme and increases DNA synthesis activity. All four subunits of human Pol δ individually interact with PCNA, which may allow for flexibility during DNA replication, as many proteins functionally interact with PCNA during Okazaki fragment maturation (Zhang et al. 1999; Wang et al. 2011; Li et al. 2006b). PCNA interacting motifs also were identified within the C-termini of all three subunits of *S. c.* Pol δ, which are needed for efficient DNA synthesis (Acharya et al. 2011). PCNA stimulation of DNA synthesis activity differs quantitatively among the various human Pol δ subassemblies, in the order Pol δ4 > Pol δ3 (core + p68) > Pol δ2 core (Zhou et al. 2012). Kinetically, PCNA reduces the K_m for DNA template binding and increases the V_{max} of the calf thymus Pol δ catalytic core, suggesting that PCNA might stimulate Pol δ processivity by increasing its residence time on the DNA template and the rate of nucleotide incorporation (Ng et al. 1991).

2.1.2.4 An Accurate Polymerase

Replication of the genome requires accurate DNA synthesis in order to avoid the accumulation of deleterious mutations. As demonstrated using in vitro mutation assays, purified Pol δ generally possesses a high DNA synthesis fidelity (see Prindle and Loeb 2012 for review). The *S. c.* Pol δ holoenzyme incorporates less than one-base substitution error out of 80,000 nucleotides synthesized (Fortune et al. 2005). For human Pol δ, less than one-base misinsertion error occurred per 220,000 nucleotides (Schmitt et al. 2009). However, *S. c.* and human Pol δ holoenzymes do not exhibit high fidelity during in vitro synthesis of repetitive microsatellite DNA sequences (Hile et al. 2012; Abdulovic et al. 2011). At the heart of Pol δ's high fidelity is its intrinsic 3′→5′ exonuclease activity (Simon et al. 1991), which enables proofreading upon incorporation of incorrect nucleotides during DNA synthesis (Kunkel et al. 1987). Both human and *S. c.* exonuclease-deficient Pol δ forms exhibit approximately 10-fold higher base substitution error rates than wild-type Pol δ in vitro (Fortune et al. 2005; Schmitt et al. 2009). In vivo, yeast strains carrying inactivating mutations within the *POL3* exonuclease domain exhibit a 100-fold increased spontaneous mutation rate (Morrison and Sugino 1994). Furthermore, mice with homozygous substitutions at highly conserved residues within the proofreading domain of Pol δ exhibit a high incidence of cancer and decreased survival (Goldsby et al. 2002; Albertson et al. 2009).

Intrinsic kinetic properties of Pol δ also are key determinants of its high fidelity. *S. c.* Pol δ strongly favors incorporation of correct versus incorrect nucleotides during synthesis (Dieckman et al. 2010). A highly conserved leucine residue within the Pol δ active site has been extensively studied in yeast, mice, and humans (Leu-612, 604 and 606, respectively) for its role in ensuring high-fidelity DNA synthesis. Amino acid substitutions at this site cause allele-specific phenotypic effects in *S. c.* and mice (Venkatesan et al. 2006, 2007). In vitro, the human Pol δ holoenzyme L606G mutant is highly error prone (Schmitt et al. 2010). Interestingly, the L606K mutant exhibits higher fidelity than wild-type Pol δ but decreased bypass of DNA adducts (Schmitt et al. 2010), suggesting that the high fidelity conferred by this active site residue is balanced by the need for the wild-type enzyme to perform other activities, including translesion synthesis at the replication fork.

Efficient proofreading requires partitioning of DNA substrates containing 3′ terminal mispairs from the polymerase to the exonuclease active sites (Khare and Eckert 2002). An *S. c. POL3* active site mutation that impairs this partitioning results in decreased DNA synthesis fidelity and increased spontaneous mutation rate (McElhinny et al. 2007). Purified *S. c.* Pol δ interacts with base pairs distant from the templating base, which may allow the polymerase to "sense" base mismatches (Swan et al. 2009).

Although PCNA stimulates Pol δ activity, it may decrease its fidelity. In vitro, PCNA increases the rate of nucleotide misincorporation by *S. c.* Pol δ, resulting in a

significant reduction in fidelity (Hashimoto et al. 2003). Similarly, nucleotide misincorporations by calf thymus Pol δ increase ~27-fold in the presence of PCNA, and PCNA may enable Pol δ to extend mismatched base pairs by stabilizing the Pol δ-template-primer complex (Mozzherin et al. 1996). However, addition of both PCNA and RPA had no effect on *S. c.* Pol δ base substitution error rates, and the addition of PCNA and RPA to Pol δ DNA synthesis reactions contributed to a decreased rate of large deletion errors within directly repeated sequences (Fortune et al. 2006). Further studies are needed to fully understand the effect of accessory factors on Pol δ fidelity. Interestingly, the Werner syndrome protein (WRN), a RecQ family helicase and 3′→5′ exonuclease, can enable high-fidelity DNA synthesis by Pol δ through excision of primer-template mismatches prior to polymerase extension (Kamath-Loeb et al. 2012).

2.1.2.5 Posttranslational Regulation

Phosphorylation may represent an important mechanism of Pol δ regulation (see Lee et al. 2012 for review). Mammalian Pol δ protein levels peak at the G1/S border, and the p125 subunit is most actively phosphorylated during S-phase (Zeng et al. 1994). The B subunit (p50) is also phosphorylated in vivo and is an in vitro substrate of the cyclin A-Cdk2 cell cycle-dependent kinase (Li et al. 2006a). The C subunit (p68) can be phosphorylated by cyclin-dependent kinases (CycE-Cdk2, CycACdk1, or CycA-Cdk2) in vitro, and PCNA interferes with this phosphorylation (Ducoux et al. 2001). In addition, mammalian Pol δ/p125, p68, and p12 subunits can be phosphorylated by casein kinase 2 in vitro and subsequently dephosphorylated by protein phosphatase-1 (Gao et al. 2008), suggesting an additional regulatory circuit for regulation. Phosphorylation of Pol δ/p68 coincides with Pol δ association with chromatin at the start of S-phase (Lemmens et al. 2008). The Pol δ/p68 subunit also contains a phosphorylation site for protein kinase A, and phosphomimetic mutation of this residue decreases Pol δ affinity for PCNA and processivity (Rahmeh et al. 2011). Thus, phosphorylation may serve to regulate Pol δ activity by controlling its interaction with DNA and/or auxiliary proteins during replication.

2.1.3 DNA Polymerase ε

2.1.3.1 Overview

Purified Pol ε was first characterized as a larger form of Pol δ that was highly processive, but not stimulated by PCNA in vitro (Syvaoja and Linn 1989). The Pol ε holoenzyme exists as a heterotetrameric protein in all eukaryotes studied (Table 2.1) (Pursell et al. 2008). The large catalytic subunit contains the polymerase and 3′→5′ exonuclease active sites within the N-terminus, and binding domains for smaller

subunits and PCNA within the C-terminus. The C and D subunits form a subcomplex that is important for double-stranded DNA binding (Tsubota et al. 2003). The *S. c.* Pol ε holoenzyme structure has been solved at 20 Å resolution using cryo-electron microscopy and single-particle analyses (Asturias et al. 2006). The three small subunits (B–D) form a discrete extended tail structure, separated from the large catalytic subunit by a flexible hinge. Similar to the *S. c.* Pol δ, a 4Fe-4S cluster is bound to four conserved cysteine residues (the CysB motif) within the C-terminal domain of Pol2 and may be essential for stabilizing the *S. c.* Pol ε holoenzyme (Netz et al. 2012).

2.1.3.2 An Essential Component of the Eukaryotic Replication Machinery

The Pol ε holoenzyme is essential for chromosomal replication. In *S. c.*, *POL2* reading frame disruptions are nonviable due to arrest of DNA replication; however, truncation mutations that maintain catalytic activity are viable with a slow growth phenotype (Morrison et al. 1990). Deletion of the *POL2* N-terminus, which encodes the polymerase catalytic domain, also is viable, although mutants display severe replication defects, including slow fork movement, prolonged S-phase, and shortened telomeres (Kesti et al. 1999; Feng and D'Urso 2001; Dua et al. 1999). Thermosensitive yeast mutants also demonstrate that nonfunctional Pol ε leads to defective chromosomal replication and the accumulation of short DNA fragments (Araki et al. 1992; Budd and Campbell 1993). Immunodepletion of Pol ε from *Xenopus* egg extracts significantly impedes elongation of nascent DNA strands and causes the accumulation of short replication intermediates (Waga et al. 2001). In HeLa cells, transient knockdown of Pol ε causes an accumulation of cells in early S-phase and a decreased rate of replication fork movement (Bermudez et al. 2011). Disruption of Pol ε in mice results in embryonic lethality (Menezes and Sweasy 2012).

2.1.3.3 A Highly Efficient and Processive Polymerase

Pol ε and Pol δ differ in their biochemical interactions with PCNA. PCNA binds the Pol ε p261 (N-terminus), p59, and p12 subunits and increases the rate of nucleotide incorporation by the holoenzyme (Bermudez et al. 2011). In the presence of PCNA, RFC, and RPA, *S. c.* Pol ε processivity is stimulated ~6-fold, less than the ~100-fold stimulation observed for Pol δ (Chilkova et al. 2007). *S. c.* Pol ε has a high affinity for DNA and low affinity for PCNA, while *S. c.* Pol δ displays the opposing affinities for each, suggesting that Pol ε might load onto DNA independently of PCNA, while Pol δ requires preloading of PCNA (Chilkova et al. 2007). However, *S. c.* and human Pol ε are more processive than Pol δ, even in the absence of accessory factors (Dua et al. 2002) (Bermudez et al. 2011). High *S. c.* Pol ε processivity requires a minimal primer duplex stem length of 40 base pairs, and

structural studies suggest that the tail domain formed by the B–D subunits contributes directly to Pol ε processivity by binding double-stranded DNA, precluding the need for PCNA to enhance DNA affinity (Asturias et al. 2006). The processivities of both the *S. c.* Pol ε polymerase and exonuclease activities are reduced in subassemblies lacking the C and D subunits, relative to the holoenzyme form (Aksenova et al. 2010).

2.1.3.4 A Highly Accurate Polymerase

Pol ε is perhaps the most accurate eukaryotic DNA polymerase. Purified calf thymus Pol ε base substitution error rates determined in vitro ranged from 1/30,000 to 1/400,000 mutations per nucleotide synthesized, lower than either Pol δ or Pol α (Thomas et al. 1991). The *S. c.* Pol ε holoenzyme exhibits very low base substitution and single-base deletion error rates, $\leq 2 \times 10^{-5}$ and $\leq 5 \times 10^{-7}$ (Shcherbakova et al. 2003), respectively. Error rates of human Pol ε have not yet been determined because they are close to background rates for in vitro mutation assays (Korona et al. 2011). Although the in vitro fidelity of purified *S. c.* Pol ε lacking the C and D subunits is the same as the Pol ε holoenzyme form, deletion of *DPB3* and *DPB4* results in a slightly increased spontaneous mutation rate in vivo (Aksenova et al. 2010).

The high fidelity of Pol ε is due, in part, to its intrinsic 3′→5′ exonuclease activity. Amino acid substitutions of a conserved motif within the exonuclease active site result in a ~20-fold increase in the *S. c.* spontaneous mutation rate (Morrison et al. 1991). Purified exonuclease-deficient *S. c.* Pol ε exhibits single-base substitution and deletion errors rates that are ~10- and 100-fold higher, respectively, than wild-type Pol ε (Shcherbakova et al. 2003). Kinetically, *S. c.* Pol ε mutants harboring a C1089Y substitution within the polymerase active site exhibit an increased rate of base misincorporation that may result from the inability to perform proper DNA shuffling between the polymerase and exonuclease domains (Shimizu et al. 2002). Human exonuclease-deficient Pol ε is ~5-fold more accurate than wild-type *S. c.* Pol ε for both base substitutions and single-base deletions (Korona et al. 2011). Homozygous loss of Pol ε exonuclease activity in mice results in an elevated spontaneous mutation rate and tumor incidence (Albertson et al. 2009). Specific polymerase active site residues also play a role in determining Pol ε fidelity. For instance, an M644F substitution within the *S. c.* Pol ε active site reduces fidelity due to an increased base misincorporation rate (Pursell et al. 2007a). The Pol ε M644 active site residue may modulate fidelity by maintaining proper geometry of the substrate binding pocket (Pursell et al. 2007a).

2.1.3.5 Posttranslational Modification

The posttranslational regulation of Pol ε has not been extensively studied. However, the *S. c.* B subunit, required for chromosomal replication (Araki et al. 1991), is

phosphorylated during late G1 phase in a cell cycle-dependent manner (Kesti et al. 2004).

2.2 Functions at the Eukaryotic Replication Fork

2.2.1 Replication Initiation

The process of replication initiation takes place as two steps (see Masai et al. 2010 for review). The pre-RC complex (ORC; Cdc6; Cdt1; MCM10 and MCM2-6 helicase complex) is first loaded onto chromosomes at origins of replication. In the second step, pre-RCs are activated to generate replication fork through the kinase activities of Cdc7-Dbf4 and CDK, which allows loading of other proteins required for replication. Loading of the GINS complex, a heterotetrameric complex essential for replication (Takayama et al. 2003), leads to activation of the MCM helicase complex, which enables unwinding of duplex DNA at the replication fork. All three *S. c.* replicative polymerases (α, δ, ε) bind to origins in early S-phase (Hiraga et al. 2005). Pol ε is a component of the preloading complex (Pre-LC), which also contains GINS, Sld2, and Dpb11 (Muramatsu et al. 2010), suggesting that Pol ε may play an active role in replication initiation (Fig. 2.1). Indeed, loss of the Pol ε catalytic subunit in *S. p.* prevents loading of GINS and several other initiation proteins to origins (Handa et al. 2012). GINS function is required for recruitment of Pol ε to chromatin (Pai et al. 2009) and can stimulate Pol ε activity (Bermudez et al. 2011).

Pol α-primase interacts with several protein players in DNA replication initiation (Fig. 2.1). The B subunit of Pol α-primase interacts directly with ORC for its recruitment to initiation sites (Uchiyama and Wang 2004). MCM10 binds to the Pol α/p180 subunit and physically interacts with the Pol α holoenzyme in vitro and in vivo (Fien et al. 2004; Ricke and Bielinsky 2004; Warren et al. 2009). MCM10 maintains steady-state levels of the Pol α/p180 subunit (Ricke and Bielinsky 2004), loads Pol α holoenzyme onto chromatin (Zhu et al. 2007), and facilitates Pol α holoenzyme binding to primed DNA templates (Fien et al. 2004). Additionally, the GINS complex interacts with the primase subunits and stimulates DNA synthesis activity (De Falco et al. 2007).

2.2.2 Leading and Lagging Strand Replication

Upon activation of replication origins, Pol α-primase is responsible for generating RNA–DNA primers that initiate DNA synthesis on the leading and lagging strands of the replication fork. Temporal studies using *Xenopus* extracts demonstrated that Pol α-primase is loaded onto DNA after helicase-mediated unwinding has been

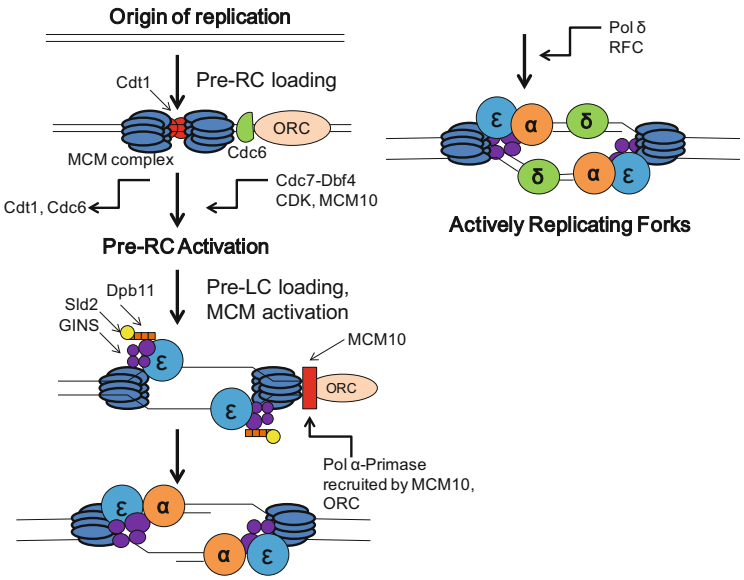

Fig. 2.1 Simplified schematic of replication initiation in eukaryotes. Initiation occurs at origins of replication, at which a pre-RC complex, consisting of the MCM complex, Cdc6, and Cdt1, is first loaded and then activated by the Cdc7-Dbf4 and CDK kinases. This leads to the recruitment of the pre-LC complex, consisting of GINS, Sld2, Dpb11, and Pol ε. Pol α-primase is recruited through interactions with MCM10 and also directly interacts with GINS. Mechanisms leading to Pol δ recruitment are currently unknown. Upon recruitment of all three replicative Pols, an active, bidirectional replication fork is generated, in which Pol ε is modeled as the leading strand Pol, and Pol δ is modeled as the lagging strand Pol. For simplicity, some replication-initiating factors and protein–protein interactions are not displayed. The reader is referred to Masai et al. 2010 and Araki 2010 for further details

initiated (Walter and Newport 2000). Switching from Pol α to Pol δ or Pol ε involves RFC. In vitro, RFC inhibits Pol α activity once a sufficient RNA–DNA primer is generated, by decreasing its affinity for the DNA template, thus coordinating a switch from Pol α to Pol δ (Mossi et al. 2000; Maga et al. 2000).

Several studies have suggested that Pols δ and ε have distinct responsibilities at the replication fork (Kunkel and Burgers 2008). In HeLa cells, Pol ε is more active in early S-phase while Pol δ activity increases in late S-phase (Rytkonen et al. 2006). Early biochemical evidence that Pol δ functions as the lagging strand Pol came from studies demonstrating its role in Okazaki fragment maturation, in which Pol δ functions together with FEN1, a 5′ flap endonuclease; DNA ligase 1; and PCNA (Burgers 2009). As replication takes place on the lagging strand, Pol δ encounters RNA primers within downstream Okazaki fragments every ~100–200 nucleotides, which must be removed in order to prevent deleterious duplication mutations (Burgers 2009). Yeast genetic analyses and in vitro studies demonstrated that Pol δ carries out strand displacement of one to two nucleotides within the RNA primer, followed by idling through its 3′→5′ exonuclease activity, which allows for FEN1 to remove the resulting ribonucleotide flap. This process occurs in several

cycles until all ribonucleotides are removed and a DNA–DNA nick is generated, which is sealed by DNA ligase 1 (Garg et al. 2004; Jin et al. 2003; Burgers 2009). In contrast, Pol ε does not undergo idling at downstream primers or interact with FEN1 to generate ligatable nicks (Garg et al. 2004), evidence that is not consistent with a role in lagging strand replication.

Early studies of exonuclease-deficient *S. c.* Pol δ and Pol ε strains provided genetic evidence that the two polymerases act on opposite DNA strands during replication (Morrison and Sugino 1994; Shcherbakova and Pavlov 1996; Karthikeyan et al. 2000). More recently, yeast DNA polymerases δ and ε variants that have distinctive error signatures in vitro have been used to infer the strand preference for DNA synthesis (e.g., leading versus lagging) during in vivo DNA replication. Such genetic studies of Pol ε and Pol δ mutants are consistent with roles as the leading and lagging strand polymerase, respectively. The asymmetric *S. c.* Pol ε mutant (M644G) exhibits a high rate of T-dTMP mismatches relative to complementary A-dAMP mismatches in vitro and an elevated rate of T-dTMP mismatches in an origin orientation-dependent manner in vivo, consistent with its role in replication on the leading strand (Pursell et al. 2007b). Conversely, the *pol3-L612M* is an asymmetric Pol δ mutator that increases the rate of dT-dGMP mismatches but has a low rate for generating the complementary dA-dCMP mismatch in vitro (McElhinny et al. 2007), and genetic analysis of the *pol3-L612M* mutant showed that its signature errors occurred in vivo primarily on the lagging strand (Nick McElhinny et al. 2008). Deep DNA sequencing and analyses of L612M mutant genomes revealed the Pol error signature genome wide, suggesting that Pol δ is responsible for lagging strand replication across the *S. c.* genome (Larrea et al. 2010). Although such evidence supports the model in which Pol δ functions as the lagging strand Pol, alternative models have been proposed in which Pol δ may also contribute to replication on the leading strand (as reviewed in Kunkel and Burgers 2008). Importantly, it remains to be determined whether this model applies to replication in mammalian cells and to the fork after replication restart.

2.2.3 *Replicative Polymerase Functions in Checkpoint Responses*

Replicative polymerases are integral components of pathways monitoring progression of the mitotic cell cycle (Fig. 2.2). Pol α-primase is a central player in checkpoint signaling that ensures DNA replication is complete prior to progression into mitosis. Checkpoint activation of the Chk1 kinase in *Xenopus* extracts requires RNA synthesis by primase, but not DNA synthesis (Michael et al. 2000), and yeast mutants defective for primase activity are unable to activate the Chk1 and Rad53 kinases (Griffiths et al. 2001; Marini et al. 1997). The Cds1 protein kinase interacts directly with Pol α-primase, possibly to signal downstream cell cycle checkpoint

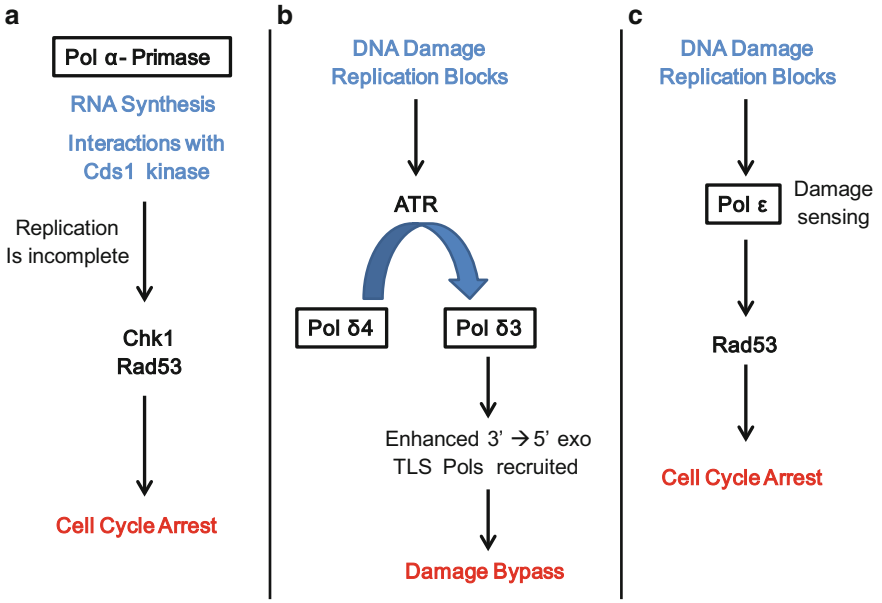

Fig. 2.2 Eukaryotic DNA polymerases in cellular checkpoint responses. (**a**) During S-phase of the cell cycle, Pol α-primase activity results in RNA synthesis, which serves as a signal that replication is incomplete and prevents cell cycle progression through activation of Chk1 and Rad53. Interactions between Pol α-primase and the Cds1 kinase also act as an upstream signal for Chk1 and Rad53 activation. (**b**) DNA damage and replication blocks act as a signal for ATR activation in cells, which leads to degradation of the p12 subunit of Pol δ4 and the formation of Pol δ3. The three-subunit form of Pol δ is proposed to play a role in damage bypass. (**c**) DNA damage and replication blocks act an upstream signal for Pol ε, which then triggers signaling for activation of Rad53 and cell cycle arrest under these conditions

responses (Murakami and Okayama 1995). Thus, the presence of RNA primers synthesized by primase during S-phase acts as a signal that replication is not yet complete in order to prevent cell cycle progression.

Over the past several years, a three subunit form of Pol δ, devoid of the p12 subunit, has been extensively studied for its role in regulating the DNA damage response in human cells (reviewed in Lee et al. 2012). Upon treatment of cells with various DNA damage-inducing agents, including UV, methyl methanesulfonate, hydroxyurea, and aphidicolin, the p12 subunit undergoes ubiquitylation-dependent degradation to form Pol δ3 (core + p68 subunit). Under conditions of low UV doses, this is dependent on activation of ATR, a major checkpoint response pathway in eukaryotic cells (Zhang et al. 2007). Interestingly, Pol δ3 displays increased exonuclease partitioning and decreased potential for bypass of various DNA lesions (Lee et al. 2012). These findings led to a model in which Pol δ3 is responsible for slowing replication progression at sites of DNA damage, which might allow for switching to a translesion synthesis polymerase (Lee et al. 2012).

Pol ε also is an essential component of cell cycle checkpoint responses. Pol ε functions upstream of Rad53 in the DNA damage response pathway (Navas et al. 1996). The C-terminus of *S. c.* POL2 has an essential role in initiating the S-phase checkpoint response, but is not involved in the G1 or G2/M checkpoints (Navas et al. 1995; Dua et al. 1998). In response to replication stress, Pol ε is required for one of two independent pathways leading to checkpoint activation (Puddu et al. 2011).

2.3 Perspective

Intensive basic research over the past 4 decades, using biochemical and genetic approaches and model systems from yeast to humans, has demonstrated that DNA polymerases cooperate to ensure efficient and accurate eukaryote genome replication. This chapter has provided a glimpse into how the biochemical properties of Pols α, δ, and ε shape their functional roles at the replication fork. The multisubunit holoenzyme structure of each polymerase provides a mechanism for regulating polymerase activities, allowing proper cell cycle progression and cellular responses to replication roadblocks, such as DNA damage. Studies of budding yeast have provided strong evidence that a fourth polymerase holoenzyme complex is present at the eukaryotic replication fork, namely, Pol ζ, whose biochemical activities are critical for translesion DNA replication (see Pavlov and Shcherbakova 2010 for review). Intriguingly, very recent papers have shown that the B and C subunits of Pol δ are shared with Pol ζ, suggesting that the catalytic domains of replicative polymerases can be exchanged within a holoenzyme complex (Baranovskiy et al. 2012; Johnson et al. 2012). This new evidence highlights the very dynamic nature of DNA polymerases at the replication fork to efficiently process all aspects of genomic maintenance as they arise during replication. Clearly, elucidating exactly how polymerases are regulated during DNA replication in eukaryotic cells is an emerging area of research but one that is already providing new and unexpected twists to the current paradigm.

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Chapter 3
DNA Repair Polymerases

Robert W. Sobol

Abstract The nuclear and mitochondrial genomes are under constant attack from endogenous (metabolic) and exogenous genotoxins. The resulting genomic insults include damaged bases and nucleotides, deoxyribo- and ribonucleotide misincorporation, intra-strand and interstrand DNA cross-links, and single-strand and double-strand DNA breaks. As expected, efficient recognition and removal of these genotoxic lesions is critical to begin the repair process and restore genome integrity. With the exception of direct reversal mechanisms, repair of both the nuclear and mitochondrial genomes requires DNA synthesis to replace the nucleotides or DNA strands removed during the repair process. Whereas some DNA repair pathways co-opt replicative DNA polymerases to synthesize the DNA in the "repair patch," other DNA repair pathways have dedicated DNA polymerase enzymes. This chapter will detail the DNA polymerases central to the major mammalian DNA repair pathways and, where applicable, highlight the unique roles these DNA polymerases may play in protecting normal cells from mutagenic or genotoxic agents and in providing resistance to genotoxic chemotherapeutic treatments.

Keywords DNA repair • DNA polymerase • Mutation • Genome stability

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Abbreviations

5′dRP	5′-deoxyribose phosphate
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine
AP	Apurinic/apyrimidinic
APE1	Apurinic/apyrimidinic endonuclease
BER	Base excision repair
dsDNA	Double-stranded DNA
FEN1	Structure-specific flap endonuclease 1
HR	Homologous recombination
KO	Knockout
LigI	DNA ligase I
LigIII	DNA ligase III
MEF	Mouse embryonic fibroblast
MGMT	O^6-methylguanine-DNA methyltransferase
MMR	Mismatch repair
NER	Nucleotide excision repair
NHEJ	Nonhomologous end joining
PARP1	Poly(ADP-ribose)polymerase-1
PARP2	Poly(ADP-ribose)polymerase-2
PCNA	Proliferating cell nuclear antigen
Polκ	DNA polymerase kappa
Polλ	DNA polymerase lambda
Polβ	DNA polymerase beta
Polα	DNA polymerase alpha
Polγ	DNA polymerase gamma
Polδ	DNA polymerase delta
Polη	DNA polymerase eta
Polθ	DNA polymerase theta
Polι	DNA polymerase iota
Polκ	DNA polymerase kappa
Polμ	DNA polymerase mu
RFC	Replication factor C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SSBs	Single-strand breaks
ssDNA	Single-stranded DNA
TdT	Terminal deoxynucleotidyltransferase
UV	Ultraviolet
WRN	Werner syndrome protein helicase

3.1 Introduction

Human cells repair thousands of DNA lesions per day to prevent the accumulation of DNA mutations or genome aberrations that can impact cellular survival and genomic integrity (Lindahl 1993). These lesions manifest as base (e.g., deamination of cytosine to uracil) or nucleotide modifications (e.g., thymine–thymine dimers), deoxyribo- and ribonucleotide misincorporation (mismatches), intra-strand or inter-strand DNA cross-links, and single-strand or double-strand DNA breaks. These numerous and diverse types of DNA lesions derive from both endogenous and exogenous sources. Base damage can be induced by a variety of reactive oxygen species (ROS), reactive nitrogen species (RNS), and sources of DNA alkylation (Svilar et al. 2011). Such molecules stem from endogenous sources via cellular metabolism and exogenous sources mediated by environmental exposure. Additional modifications include simple and complex DNA adducts (Marnett et al. 2003; Marnett 2000; Knutson et al. 2007, 2009; Otteneder et al. 2006), ultraviolet light-induced pyrimidine dimers (Setlow and Setlow 1962), replication errors that give rise to mutations, deletions, or insertions (Kunkel and Erie 2005), as well as gross modifications such as DNA cross-links (Deans and West 2011) and DNA double-strand breaks (DSBs) from environmental, genetic, and endogenous sources (Friedberg et al. 2006).

To facilitate the repair of these lesions, cells have multiple DNA damage response and DNA repair mechanisms that signal the presence of lesions and promote DNA repair (Jackson and Bartek 2009; Friedberg et al. 2006; Wood et al. 2001, 2005; Hoeijmakers 2001). A general overview of mammalian DNA repair pathways and the lesions each repairs is depicted in Fig. 3.1. With the exception of direct reversal (DR) repair processes, each pathway utilizes one or more DNA polymerases as an integral part of the overall DNA repair pathway. The major DR pathway proteins include O^6-methylguanine DNA methyltransferase (MGMT) and the α-ketoglutarate-dependent dioxygenase enzymes: AlkB homologues 1, 2, and 3 (ALKBH1, ALKHB2, ALKBH3). These proteins directly reverse the damage to the DNA base without the requirement of new DNA synthesis (Fu et al. 2012; Yi and He 2013), each with unique lesion specificity. Further detail on this mode of direct reversal DNA repair can be found elsewhere (Fu et al. 2012; Yi and He 2013).

The remaining DNA repair pathways depicted in Fig. 3.1 utilize DNA polymerases to replace the excised lesion-containing nucleotides. Base lesions and DNA SSBs are primarily repaired by the base excision repair (BER) pathway (Almeida and Sobol 2005, 2007; Svilar et al. 2011). As shown and as will be discussed, BER utilizes specific DNA polymerases depending on the initiating lesion, the subcellular location (nuclear vs. mitochondria), and the BER sub-pathway. Similarly, nonhomologous end joining (NHEJ), a pathway for repair of DNA DSBs, also utilizes specialized DNA polymerases (Lieber 2008; Lange et al. 2011; Ramsden 2011; Ramsden and Asagoshi 2012). Another class of specialized DNA polymerases, translesion DNA polymerases, are discussed in

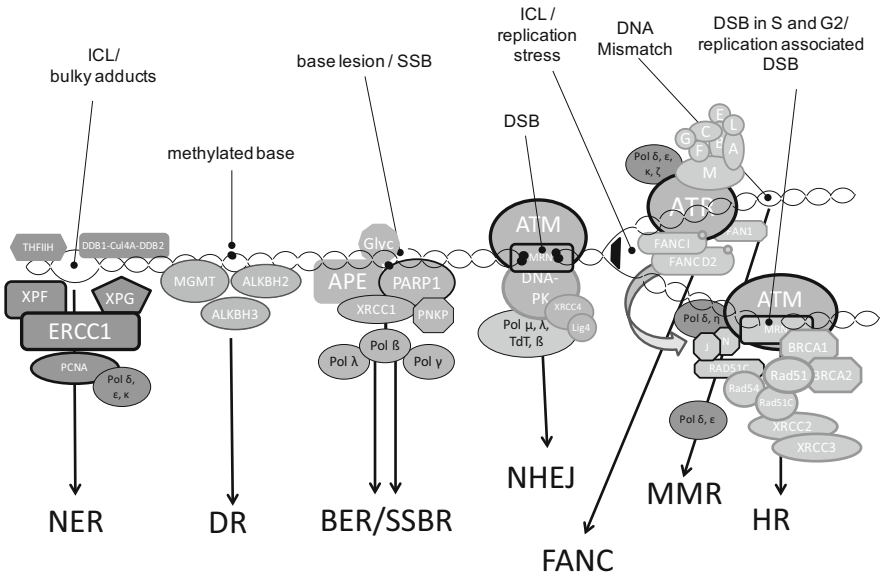

Fig. 3.1 Schematic representation of the mammalian DNA repair pathways. This figure depicts the mammalian DNA repair pathways, the major proteins within each pathway and highlights (*black lettering*) the DNA polymerases involved in each pathway. Adapted from Vens and Sobol (2013)

Chap. 4. The nucleotide excision repair (NER) pathway is a multi-protein, highly complex DNA repair pathway that plays an important role in the repair of DNA lesions induced by many genotoxins and facilitates the removal of bulky DNA adducts that grossly distort the DNA double helix and those that cause a block to transcription (Hoeijmakers 2001; de Laat et al. 1999; Wood 1996; Shuck et al. 2008). As depicted in Fig. 3.1, NER utilizes primarily replicative DNA polymerases but also uses DNA polymerase kappa (Polκ) separate from its role in lesion bypass or translesion DNA synthesis (Ogi et al. 2010). The remaining pathways for the repair of DNA mismatches (MMR), DNA DSBs via homologous recombination (HR), or DNA intra-strand cross-links via the FANC pathway either co-opt replicative DNA polymerases or use specialized polymerases to synthesize DNA after lesion removal or to replicate DNA from the homologous template. The following sections will provide an overview of these DNA repair pathways, emphasizing the role of the DNA polymerases specific to each pathway. Where appropriate, each section will also include relevant discussion on the alterations in these DNA polymerases in cancer since defects in these DNA repair pathways can promote tumorigenesis and are common in human cancers (Hanahan and Weinberg 2011; Harper and Elledge 2007; Curtin 2012; O'Driscoll 2012; Hoeijmakers 2009).

3.2 DNA Polymerases in Base Excision Repair

The proteins of the base excision repair (BER) pathway participate in the repair of dozens of base modifications that result from alkylating agents, reactive nitrogen species, and reactive oxygen species (oxidative DNA damage), among others (Svilar et al. 2011; Almeida and Sobol 2005, 2007). Such damage can arise from numerous exogenous and endogenous sources, resulting in a multitude of detrimental cellular effects, including mutations, genome rearrangements, altered gene expression, and the onset of cell death or senescence (Hoeijmakers 2001; Baute and Depicker 2008; Hegde et al. 2011). The BER pathway model shown in Fig. 3.2 is initiated by a DNA glycosylase such as MYH, a unique glycosylase with specificity for a normal base (adenine) when paired opposite the ROS modified form of deoxyguanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Svilar et al. 2011; David et al. 2007). The remaining ten DNA glycosylases are specific for many types of base lesions, as reviewed elsewhere (Almeida and Sobol 2007; Svilar et al. 2011; Fu et al. 2012). Once the base lesion is removed, the product, an abasic or apurinic/apyrimidinic (AP) site, is a substrate for an endonuclease specific for AP sites, the AP endonucleases APE1 or APE2 (although the majority activity results from APE1) (Almeida and Sobol 2007). There is general consensus that the resulting DNA single-strand break after APE1 (or APE2) cleavage forms a nucleation site for scaffold proteins such as PARP1 and XRCC1 followed by recruitment of the proteins needed to complete repair (not shown in this figure) (Almeida and Sobol 2007). Either DNA polymerase β (Polβ) or DNA polymerase lambda (Polλ) can be recruited to conduct end-trimming and DNA synthesis. Polβ is considered the major end-trimming (5′dRP lyase activity) and DNA polymerase enzyme in BER although, as will be detailed below, Polλ plays a significant role in oxidative damage repair. Alternate DNA polymerases have also been suggested to participate in BER, depending on the base lesion and the subcellular location (nuclear vs. mitochondrial), as will be discussed below. The short-patch BER pathway (Fig. 3.2, left panel) likely contributes 90 % of the repair mediated by BER, but if the 5′ end of the gap is blocked such that end-trimming (5′dRP lyase activity) is attenuated, both Polβ and DNA polymerase δ (Polδ) or DNA polymerase ε (Polε) can extend the repair patch to 2–12 bases, completing a form of BER known as long-patch BER (Fig. 3.2, right panel). Finally, the repair gap is sealed or ligated by either DNA ligase III (LigIII) or DNA ligase I (LigI). Recently, it was suggested that LigI functions as the primary BER DNA ligase in the nucleus with LigIII playing a predominant role in the mitochondria (Gao et al. 2011; Simsek et al. 2011).

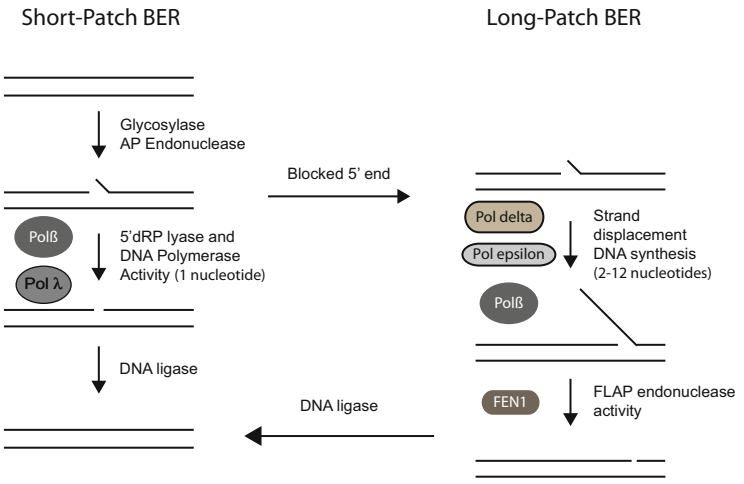

Fig. 3.2 Schematic for short-patch and long-patch BER. Simplified diagram depicting the two sub-pathways for BER: short-patch and long-patch. In short-patch BER, the cleaved AP site can be further processed by the 5′dRP lyase activity of Polβ or Polλ, followed by DNA synthesis and ligation. However, if the 5′ end of the downstream DNA is blocked and cannot be processed, strand-displacement DNA synthesis can proceed. Processing requires FEN1 to remove the 2–12 base flap, followed by DNA ligation

3.2.1 DNA Polβ as the Primary BER Polymerase

DNA polymerase β (Polβ) is a member of the X-family of DNA polymerases (Burgers et al. 2001; Bebenek and Kunkel 2004) and is an essential BER protein, considered the major or primary BER DNA polymerase. At 335 amino acids (39 kDa), Polβ is the smallest of the human DNA polymerases (Beard and Wilson 2006; Lange et al. 2011; Sobol et al. 1996). Polβ has two active sites. The 5′dRP lyase activity is restricted to the 8 kDa N-terminal domain and requires the active site residue K72, whereas the nucleotidyl transferase or DNA polymerase activity resides within the C-terminal domain and requires the aspartate triad D190, D192, and D256, as depicted in Fig. 3.3a. Structurally, the enzyme contains four domains (8K, Fingers, Palm, and Thumb), with the 8K and Fingers domain comprising the dRP lyase activity and the Palm and Thumb domains comprising the majority of the nucleotidyl transferase activity. As depicted in the diagram and structural representation (Fig. 3.3b), the single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) binding domains reside mostly in the N-terminus, with the enzyme inducing a bend in the DNA upon binding and nucleotide incorporation (Batra et al. 2006).

Since its initial discovery (Weissbach 1977; Weissbach et al. 1975a, b), Polβ was found to be unique in its enzymatic properties (Ono et al. 1979; Tanabe et al. 1979; Yoshida et al. 1979) as compared to the other newly characterized mammalian DNA polymerases alpha (Polα), gamma (Polγ), and delta (Polδ) (Byrnes

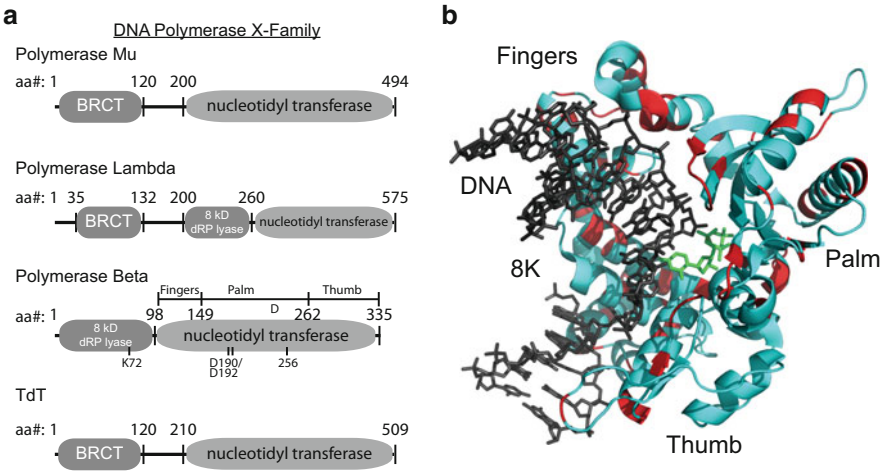

Fig. 3.3 Structural and functional details for DNA Polβ. (**a**) Cartoon depiction of DNA polymerases μ, λ, β, and TdT. For each, the amino acid length is indicated, as well as the domains for protein binding (BRCT), lyase activity (8 kDa lyase), and DNA polymerase activity (nucleotidyl transferase). The amino acid residues spanning each domain are also indicated. A linear depiction of the amino acid residues (1–335) of Polβ is shown, indicating the structural sub-domains as determined by crystallographic analysis (8K, fingers, palm, and thumb), the functional domains (dRP lyase and nucleotidyl transferase), and the essential active site residues (K72 and D190/192/256). (**b**) Ternary structure of DNA Polβ with DNA substrate and incoming nucleotide—structure (pdb2fms) depicting DNA Polymerase β (Polβ) with a gapped DNA substrate and dUMPNPP with magnesium in the catalytic site (Batra et al. 2006). The fingers, palm, and thumb domains of Polβ are indicated. The 8K domain is at the back of the structure facing away from the plane of the image and is shown behind the DNA in this orientation. Amino acids altered by germline or somatic mutations are colored *red* (Sobol 2012b; Donigan et al. 2012)

et al. 1976; Weissbach 1977). Of the four eukaryotic DNA polymerases identified by 1977, Polβ was considered to be "the" DNA repair polymerase (Hubscher et al. 1979; Siedlecki et al. 1980; Waser et al. 1979; Wawra and Dolejs 1979). These early studies defined a role for Polβ in repair using isolated nuclei or nuclear extracts, monitoring the incorporation of radioactive nucleosides following DNA damage (Hubscher et al. 1979; Siedlecki et al. 1980; Waser et al. 1979; Wawra and Dolejs 1979; Mosbaugh and Linn 1983). Although it was subsequently shown that Polα can also carry out gap-filling DNA synthesis in a base excision repair (BER) reaction (Mosbaugh and Linn 1984), the evidence continued to mount in support of Polβ acting as "the" DNA repair polymerase in the nucleus. Studies continued to identify a role for Polβ in the repair of damage induced by many different DNA damaging agents, including bleomycin (Seki and Oda 1986; DiGiuseppe and Dresler 1989), cigarette smoke (Cui et al. 2012), arsenic (Lai et al. 2011), UV-radiation (Orlando et al. 1988), benzo[a]pyrene (Ishiguro et al. 1987), methylmethane sulfonate (Park et al. 1991), ionizing radiation (Price 1993), G-T mis-pairs (Wiebauer and Jiricny 1990), and uracil (Dianov et al. 1992; Nealon et al. 1996; Singhal et al. 1995; Singhal and Wilson 1993). Interestingly, a truncated

version of Polβ expressed in MEFs mediates a dependence of the cells on homologous recombination (HR) and sensitizes cells to radiation (Neijenhuis et al. 2009, 2010). Several groups reported complete BER in vitro with Polβ and additional purified proteins (Singhal et al. 1995; Nealon et al. 1996; Kubota et al. 1996). Like many DNA repair proteins, Polβ has been reported to be modified by and/or regulated by posttranslational modification, including phosphorylation (Tokui et al. 1991; Guo et al. 2008; Phosphosite 2010), acetylation (Hasan et al. 2002), methylation (El-Andaloussi et al. 2006, 2007), as well ubiquitinylation (Parsons et al. 2008, 2009; Sobol 2008). It is not yet clear how these modifications impact overall BER or the role of Polβ in BER as in many cases these observations have been limited to in vitro studies (Sobol 2008; Goellner et al. 2012).

Although it was demonstrated in heterologous systems (*Escherichia coli* and *Saccharomyces cerevisiae*) that Polβ can conduct DNA replication and repair in vivo (Blank et al. 1994; Ohnishi et al. 1990), it was not until a mouse gene knockout (KO) (Gu et al. 1994) was made that the specificity of the repair conducted by Polβ was defined (Sobol et al. 1996).

Characterization of the Polβ KO mouse (Gu et al. 1994; Sugo et al. 2000) and mouse embryonic fibroblasts (MEFs) deficient in Polβ (Sobol et al. 1996) clearly demonstrated a requirement for Polβ in repair of alkylation and oxidative DNA damage (Sobol et al. 1996; Horton et al. 2002) and provided a valuable resource to explore additional functions of Polβ (Esposito et al. 2000; Gonda et al. 2001), to evaluate the impact of Polβ on mutagenesis (Niimi et al. 2005; Cabelof et al. 2003; Sobol et al. 2002; Bennett et al. 2001; Poltoratsky et al. 2005) and mechanisms of genotoxin-induced cell death (Ochs et al. 1999, 2002; Horton et al. 2003, 2005; Le Page et al. 2003; Sobol et al. 2003; Trivedi et al. 2005; Cabelof et al. 2004; Tomicic et al. 2001), to investigate alternate or compensatory repair pathways in the absence of Polβ (Biade et al. 1998; Fortini et al. 1998, 1999; Stucki et al. 1998; Dianov et al. 1999; Braithwaite et al. 2005b), to address structure–function relationships or protein partners of Polβ in vivo (Kedar et al. 2002; Niimi et al. 2005; Sobol et al. 2000) and most recently to evaluate changes of gene expression in response to Polβ depletion (Li et al. 2012a). The most definitive and reproducible endpoint that has been used to evaluate Polβ participation in repair in vivo is survival following DNA damage such as exposure to alkylating agents (Sobol et al. 1996, 2000). Unfortunately, the Polβ mouse knockout is lethal just after birth (neonatal lethality) (Sugo et al. 2000; Gu et al. 1994), complicating efforts to evaluate the role of Polβ in an animal model. Surprisingly, it is the 5′dRP lyase function of Polβ (Matsumoto and Kim 1995) that appears to be essential and sufficient for alkylating agent resistance (Sobol et al. 2000). In the absence of Polβ (in MEFs), cells are unable to efficiently repair the highly toxic 5′dRP moiety and therefore are hypersensitive to different types of alkylating agents such as methylmethane sulfonate, *N*-methyl-*N*-nitrosourea, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Sobol et al. 1996, 2000, 2002, 2003; Trivedi et al. 2005), the thymidine analog 5-hydroxymethyl-2′-deoxyuridine (Horton et al. 2003), as well as the therapeutic agent temozolomide (Trivedi et al. 2005; Horton et al. 2003) and radiation (Neijenhuis et al. 2005, 2009, 2010; Vens and Begg 2010). In human cells, Polβ

is suggested to play a role in the cellular response to cisplatin (Kothandapani et al. 2011) but plays a significant role in the repair of the DNA lesions induced by the clinical alkylating agent temozolomide (Trivedi et al. 2008; Tang et al. 2010, 2011; Goellner et al. 2011; Stachelek et al. 2010), prompting a robust series of investigations to identify specific Polβ inhibitors (Goellner et al. 2012; Wilson et al. 2010; Barakat et al. 2012; Jaiswal et al. 2009).

In mice, an intact 5′dRP lyase domain appears to rescue the neonatal lethality of the Polβ mouse knockout yet does not appear to be sufficient to rescue all of the defects associated with the loss of Polβ in the mouse (Senejani et al. 2012). By expression of a mutant of Polβ deficient in polymerase activity (Y265C) in a Polβ KO background, it was demonstrated that the mice (homozygous for the Y265C mutant Polβ) were born at the expected Mendelian ratios, but loss of the polymerase function in vivo led to the accumulation of repair intermediates and less than 40 % survived 24 h after birth, remaining smaller than the WT littermates even 3 weeks after birth (Senejani et al. 2012).

Dozens of somatic or germline mutations of Polβ have been identified and characterized (Sobol 2012b; Nemec et al. 2012; Donigan et al. 2012), prompting the suggestion that Polβ may be mutated in as much as 30 % of human tumors (Starcevic et al. 2004; Sweasy et al. 2006; An et al. 2011). As depicted in Fig. 3.3b, these mutations are not limited to a single domain or active site and are found throughout the Polβ open reading frame (note the red-shaded regions in the cartoon depicting locations of somatic and germline mutations in the structure of Polβ). In many cases, these mutations show little or no effect but several mutations have significant impact on DNA polymerase activity or 5′dRP lyase activity. For example, the E295K mutant, first identified in gastric cancer (Iwanaga et al. 1999), is defective in nucleotidyl transferase activity, and the resulting protein is defective in BER, inducing cellular transformation when over-expressed (Lang et al. 2007; Li et al. 2012b). Conversely, the L22P cancer mutant is reported to be defective only in 5′dRP lyase activity (Dalal et al. 2008). It has been suggested that tumor-specific defects in BER such as a defect in Polβ may be exploited for selective therapeutic options (Neijenhuis et al. 2010), and so it remains to be determined if the presence of these Polβ mutants can be exploited clinically.

3.2.2 Emerging Role of Polλ in BER of Oxidative Damage

DNA polymerase lambda (Polλ) was first isolated and characterized as a beta-like (Polβ-like) polymerase (Aoufouchi et al. 2000; Garcia-Diaz et al. 2000; Nagasawa et al. 2000). Overall, Polλ is a 575 amino acid enzyme that participates in both BER and nonhomologous end joining (NHEJ). Unique to Polλ is an N-terminal BRCT domain that is essential for its role in NHEJ (see Sect. 3.2 below). Similar to Polβ, Polλ is an X-family polymerase with multiple domains, including both the nucleotidyl transferase activity domain and an 8K domain that contributes the 5′dRP lyase activity important for complete BER (Garcia-Diaz et al. 2001)

(Fig. 3.3a). Although its primary role is likely in NHEJ, the presence of the 5′dRP lyase activity (Garcia-Diaz et al. 2001) has prompted continued investigation into the role of Polλ in BER, even suggesting that in some cases, both Polβ and Polλ compete for repair of the same lesions but may have nonredundant roles in vivo depending on cellular state (Garcia-Diaz et al. 2002). However, it is clear that whether it is a backup or competing player in BER, Polλ has a significant role in BER (Lebedeva et al. 2005; Braithwaite et al. 2005a, b).

Both Polβ and Polλ participate in BER in DT40 cells (Tano et al. 2007) and have redundant as well as independent BER roles in MEFs (Braithwaite et al. 2010). Further, Polλ-deficient cells are sensitive to radiation (Vermeulen et al. 2007a), similar to that found by the same group for Polβ (Vermeulen et al. 2007b). However, the most prominent role for Polλ in BER appears to be in MYH-initiated BER, as depicted in the short-patch BER model shown in Fig. 3.2a. This unique BER process requires the removal of the normal adenine base when opposite the ROS lesion 8-oxodG and insertion of a cytidine opposite 8-oxodG to allow a second round of BER initiated by OGG1 (Sobol 2012a; David et al. 2007). Interestingly, both Polβ and Polλ can fill the gap opposite the 8-oxodG lesion (Brown et al. 2007) although Polλ may be more error prone (Brown et al. 2011). A role for Polλ in MYH-mediated repair has been shown in cells and with purified proteins (van Loon and Hubscher 2009).

Multiple structural studies have been completed for Polλ, specific for the lyase domain (DeRose et al. 2003) or the catalytic core (Garcia-Diaz et al. 2004), providing significant insight with regard to structure and function (Garcia-Diaz et al. 2005). The enzyme is phosphorylated by cdk2 (Frouin et al. 2005; Wimmer et al. 2008) and its stability is regulated by ubiquitylation (Markkanen et al. 2011). Further, the involvement of Polλ in BER of oxidative lesions is regulated by both cdk2-mediated phosphorylation and MULE-mediated ubiquitylation (Markkanen et al. 2012). Most interestingly, there is functional cross talk between these two PTMs in that phosphorylation of Thr553 on Polλ prevents ubiquitylation and proteasome-mediated degradation (Wimmer et al. 2008). More recently, it is suggested that long-patch DNA repair synthesis mediated by Polλ is enhanced by binding to the Werner syndrome protein helicase (WRN) (Kanagaraj et al. 2012).

Only one cancer mutant of Polλ has been characterized, but it appears to have a defect in NHEJ as opposed to BER (Terrados et al. 2009). Polλ inhibitors have also been developed and are suggested to have tenfold greater specificity to Polλ as compared to Polβ (Strittmatter et al. 2011). It remains to be determined if these novel tools can advance our understanding on the role of Polλ in BER.

3.2.3 Alternate or Backup Polymerases in BER

The most relevant or obvious backup DNA polymerases that function in BER (besides Polλ) are those that have been found to participate in the long-patch sub-pathway of BER (Fig. 3.2b). Long-patch BER is initiated similarly to

short-patch BER to produce a nicked DNA intermediate but appears to have different DNA polymerase requirements. Repair completion requires a 3′OH moiety for proper nucleotidyl transfer and chain elongation. In cases where the 5′ moiety within the gap is refractory to Polβ lyase activity (Gary et al. 1999), Polδ, Polε, or Polβ, coupled with proliferating cell nuclear antigen (PCNA) and a variety of other proteins including the flap structure-specific endonuclease 1 (FEN1), poly (ADP-ribose)polymerase 1 (PARP1), and LigI, synthesizes DNA to fill the gap, resulting in a displaced DNA flap of 2–12 bases in length (Fortini et al. 1998; Stucki et al. 1998; Gary et al. 1999; Parlanti et al. 2002; Pascucci et al. 1999; Podlutsky et al. 2001; Matsumoto et al. 1999). DNA synthesis and strand displacement by Polβ is stimulated by the combined presence of FEN1 and PARP1 (Prasad et al. 2000, 2001) or RPA (DeMott et al. 1998). WRN is also observed to stimulate strand-displacement activities of Polβ (Harrigan et al. 2003) to facilitate long-patch BER (Harrigan et al. 2006). FEN1 then catalyzes the removal of the ensuing DNA flap, leaving a nick that has been transferred 2–12 nucleotides downstream of the original damage site. Finally, the intact DNA strand is restored by ligation mediated by LigI (Fig. 3.2b).

Recent studies with purified proteins or in cells (DT40 KO cells, MEF KO cells, or human cells following RNA interference) have implicated additional DNA polymerases that may participate in nuclear BER. DNA polymerase iota (Polι) is a Y-family polymerase and encodes a 5′dRP lyase activity (Bebenek et al. 2001) located in the 40-kDa domain spanning residues M79 to M445 (Prasad et al. 2003). Although Polι is shown to protect cells from oxidative stress suggesting a more prominent role in BER of oxidative damage (Petta et al. 2008), Polι appears to play little or no role in the repair of alkylation damage (Poltoratsky et al. 2008; Sobol 2007). Efforts are continuing to uncover the most significant biological role for Polι (Vidal and Woodgate 2009).

DNA polymerase theta (Polθ), an A-family polymerase, has also been suggested to be involved in BER (Ukai et al. 2006). As with the other BER DNA polymerases, Polθ contains a 5′dRP lyase domain (Prasad et al. 2009) and Polθ KO cells are sensitive to oxidative damage (Goff et al. 2009; Yousefzadeh and Wood 2013), all supportive for a role for Polθ in BER, as well as a role in the response to radiation (Higgins et al. 2010b). Interestingly, Polθ is known to be upregulated in breast cancers and, when over-expressed, correlates with poor prognosis (Higgins et al. 2010a; Lemee et al. 2010; Begg 2010).

3.2.4 Mitochondrial BER

BER has a well-defined role in repair of the mitochondrial genome (Bogenhagen et al. 2001), although recently it has been suggested that other repair pathways function in mitochondria (Kazak et al. 2012). Several nuclear BER enzymes also encode mitochondrial isoforms, i.e., UNG1 (Slupphaug et al. 1993). Additional mitochondrial BER enzymes have been summarized elsewhere (Svilar et al. 2011;

Liu and Demple 2010). The sole polymerase in mitochondria is DNA polymerase γ (Polγ), an A-family DNA polymerase essential for mitochondrial replication (Liu and Demple 2010) and implicated in mitochondrial BER (Stuart et al. 2005; Bogenhagen et al. 2001). The enzyme is comprised of the catalytic subunit (Polγ) and an accessory subunit (POLG2 or POLGB), reported to enhance the BER capacity of Polγ (Pinz and Bogenhagen 2006). As with the other BER DNA polymerases mentioned above, Polγ encodes a 5′dRP lyase activity domain (Longley et al. 1998), supporting its role in the short-patch sub-pathway of BER. Similar to that observed for nuclear BER, Polγ also supports a long-patch BER sub-pathway in mitochondria via both a FEN1-dependent (Liu et al. 2008) and a FEN1-independent (Szczesny et al. 2008) reaction, the latter possibly involving either DNA2 (Zheng et al. 2008) or EXOG (Tann et al. 2011). Interestingly, over 40 disease mutations have been identified in the gene for Polγ (POLG), many of which lead to mitochondrial disorders (Longley et al. 2005). It has yet to be determined if these mutations impact the role of Polγ in BER.

3.3 Unique and Specialized DNA Polymerases in Nonhomologous End Joining

The majority of DNA DSBs are repaired in mammalian cells by the nonhomologous end joining (NHEJ) pathway (Fig. 3.4a) (Lieber 2008; Downs et al. 2007). Primarily, the requisite DNA synthesis associated with NHEJ-mediated repair is via X-family DNA polymerases (Fig. 3.3a). As will be described below, repair of DSBs in the G_2-phase of the cell cycle or during the latter part of the S-phase of the cell cycle is primarily handled by the homologous recombination (HR) pathway (Fig. 3.4b). It is suggested that in G_2-phase and late S-phase, there are numerous factors that contribute to DSB repair pathway choice between HR and NHEJ (Brandsma and Gent 2012). However, in G_0-phase, G_1-phase and in the early part of S-phase, DSBs are repaired primarily by the NHEJ pathway. As depicted in Fig. 3.4a, the DNA ends are recognized by the KU heterodimer (KU70/KU80), a large DNA-binding protein with significant binding affinity to DNA ends (Doherty and Jackson 2001). Bound and activated KU undergoes a conformational change, increasing its affinity (hence recruitment) to the other critical factors required for NHEJ (Lieber 2008), including the nuclease complex Artemis/DNA-PKcs (Gell and Jackson 1999; Rivera-Calzada et al. 2007), the DNA polymerases (primarily Polμ and Polλ) (Ramsden 2011; Ramsden and Asagoshi 2012), and the ligase complex XLF/XRCC4/LigIV (Costantini et al. 2007; Gell and Jackson 1999). This large protein complex subsequently processes the broken, modified DNA ends, resulting in relegation/joining to repair the DSB. The overall process has been extensively reviewed elsewhere (Lieber 2008; Brandsma and Gent 2012; Murray et al. 2012; Boboila et al. 2012; Chapman et al. 2012; Kass and Jasin 2010; Malu et al. 2012; Pawelczak et al. 2011). Note that the recruitment of the

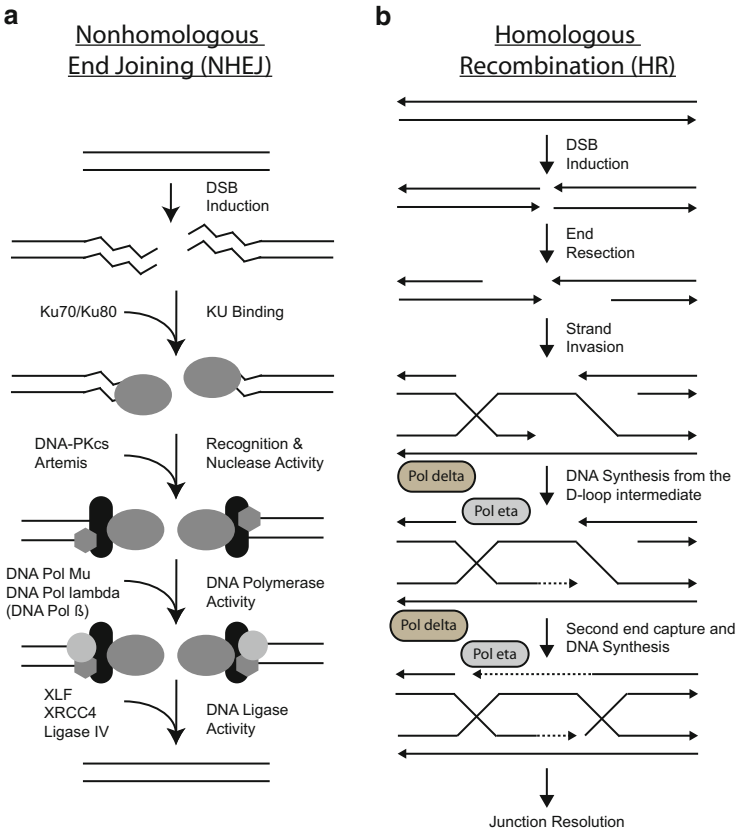

Fig. 3.4 DNA polymerases involved in double-strand break repair. (**a**) Schematic depicting the mechanism of NHEJ functioning in all phases of the cell cycle, showing KU binding to the broken ends of a DNA DSB, followed by nuclease activity to trim the ends, DNA polymerase activity for end processing, and DNA ligase activity to seal the DSB. (**b**) Classical scheme for HR-mediated repair of a DSB, showing DNA synthesis by either Polδ or Polη extending from the D-loop intermediate and followed by second-end capture and coordinated DNA synthesis of the opposite strand

essential polymerases for NHEJ primarily occurs via the BRCT domain of the polymerases (Fig. 3.3a) (DeRose et al. 2007; Matsumoto et al. 2012; Mueller et al. 2008; Gell and Jackson 1999). Details for each of the polymerases involved in mammalian NHEJ are described below.

3.3.1 DNA Polymerase Mu

DNA polymerase μ (Polμ) is an X-family DNA polymerase (Nick McElhinny and Ramsden 2003) with homology to TdT (Dominguez et al. 2000; Ruiz et al. 2001) containing both a BRCT domain in the N-terminus and the nucleotidyl transferase activity in the C-terminus (Fig. 3.3a). A role for Polμ in NHEJ was suggested following the discovery that Polμ interacts with KU and LigIV (Mahajan et al. 2002; Paull 2005). There are known NHEJ-related functional differences between Polμ and the other X-family polymerases (Bertocci et al. 2006). For example, mice deficient for Polμ are defective for immunoglobulin kappa chain rearrangement (Bertocci et al. 2003) but not Ig gene hypermutation (Bertocci et al. 2002). Further, over-expression of Polμ can impact the rate of somatic hypermutation (Ruiz et al. 2004). In addition, Polμ-deficient mice have a defect in hematopoiesis (Lucas et al. 2009). Structural studies have contributed to an in-depth understanding for the role of Polμ in NHEJ, highlighting slight but important differences with other X-family DNA polymerase members that might explain some of the substrate specificity for Polμ as compared to Polλ and Polβ (Moon et al. 2007).

Analysis of Polμ KO MEFs clearly establishes a role for Polμ in DSB repair of a variety of NHEJ substrates (Chayot et al. 2010, 2012; Capp et al. 2007). Similar to that seen for Polλ (see below), gap-filling activities in the NHEJ process mediated by Polμ are dependent on XLF (Akopiants et al. 2009), likely via interaction with the BRCT domain of Polμ (Mueller et al. 2008; DeRose et al. 2007). Polμ is also known to conduct translesion DNA synthesis, as will be discussed elsewhere in this series (Chap. 4). Defects in Polμ with regard to NHEJ can give rise to an increase in genomic abnormalities (e.g., chromosome aberrations) and should be associated with an increase in cancer.

3.3.2 DNA Polymerase Lambda

As described in Sect. 3.1.2, Polλ has a high degree of similarity to Polβ (Garcia-Diaz et al. 2000) and has a significant role in BER, particularly for oxidative damage (Lebedeva et al. 2005; Braithwaite et al. 2005a, b; Markkanen et al. 2012; Kanagaraj et al. 2012). The enzymatic properties of Polλ also suggested a role for this polymerase in NHEJ (Fan and Wu 2004; Lee et al. 2004; Bebenek et al. 2003). As was described above and as shown in Fig. 3.3a, Polλ has an N-terminal BRCT domain that promotes its role in NHEJ (Mueller et al. 2008). Polλ interacts with the XRCC4/LigIV complex via residue R57 in the BRCT domain (Mueller et al. 2008). Polλ-mediated gap filling during NHEJ also requires XLF, a core protein in the NHEJ pathway (Ahnesorg et al. 2006; Buck et al. 2006; Cavero et al. 2007; Revy et al. 2006). By characterizing whole-cell extracts from XLF-deficient human cells, it was determined that XLF is essential for gap filling

by both Polλ and Polμ (Akopiants et al. 2009). It is suggested that XLF may align the DNA ends in the repair reaction, in concert with XRCC4 (Andres et al. 2012), DNA ligase IV (Ropars et al. 2011), KU (Yano et al. 2008, 2011), and APLF (Grundy et al. 2013). Proper polymerase fidelity is also required for Polλ with regard to NHEJ-mediated DNA synthesis, as was discovered by characterizing the single-nucleotide polymorphism (SNP) mutant at codon 438 (R438W) (Terrados et al. 2009). This point mutant of Polλ retains nucleotidyl transferase activity and 5′dRP lyase activity but has a reduction in base substitution fidelity (Terrados et al. 2009). Interestingly, this mutant negatively impacts the role of Polλ in NHEJ, leading to an increase in chromosomal aberrations (Terrados et al. 2009).

3.3.3 TdT

Terminal deoxynucleotidyltransferase (TdT) participates in a very restricted capacity in NHEJ. Expression of TdT is limited to cells productive for V(D)J recombination (Benedict et al. 2000), suggesting that a role for TdT is limited to NHEJ during V(D)J recombination. This unique X-family polymerase catalyzes the addition of nucleotides by a template-independent mechanism, for example, at the junction of rearranged Ig heavy chain and T-cell receptor gene segments during B-cell and T-cell maturation. This activity, even with purified protein, is consistent with a role in NHEJ (Ma et al. 2004). Recently, it has also been shown that TdT can carry out non-template-mediated nucleotide addition at a DSB junction but only in the presence of KU80 and XRCC4 (Boubakour-Azzouz et al. 2012). TdT binds to the essential NHEJ protein KU (Mahajan et al. 1999) as well as the DSB repair protein hPso4 (Mahajan and Mitchell 2003), and its role in V(D)J recombination is suppressed by binding to PCNA (Ibe et al. 2001). As with other X-family polymerases involved in NHEJ, the N-terminal BRCT domain of TdT (Mueller et al. 2008) does have a positive effect on nucleotide addition activity (Repasky et al. 2004). Although TdT shares significant sequence homology with the other X-family polymerase members (Fig. 3.3a), there does not appear to be any significant overlapping function of TdT with Polλ or Polμ (Bertocci et al. 2006). Further activities of TdT are discussed in Chap. 5.

3.3.4 DNA Polymerase Beta

DNA polymerase β (Polβ) is genetically similar to TdT (Anderson et al. 1987) and exhibits strong similarity to Polμ (Ruiz et al. 2001) as well as structural (DeRose et al. 2003) and functional (Ramadan et al. 2003) similarity to Polλ (Fig. 3.3a). In this light, Polβ has long been suggested to have a role, albeit minor, in NHEJ. Although there is evidence of a genetic interaction between Polβ and the NHEJ protein DNA-PKs (Niimi et al. 2005), this by itself does not implicate Polβ in

NHEJ. Mice with a reconstituted lymphoid system using Polβ KO fetal liver cells showed normal patterns of somatic hypermutation, suggesting little role for Polβ in this process (Esposito et al. 2000). Further, the lack of a BRCT domain in Polβ (Fig. 3.3a) would negatively impact its role in NHEJ since this protein–protein interaction domain (Woods et al. 2012) in Polλ, Polμ, and TdT is important for interacting with NHEJ protein partners (Mueller et al. 2008). However, it is possible that Polβ may play a limited role in microhomology-mediated end joining (MMEJ) (Crespan et al. 2012), a sub-pathway of NHEJ that is independent from KU and DNA ligase 4/XRCC4 (McVey and Lee 2008).

3.4 DNA Polymerases Critical to Homologous Recombination Repair of DNA Double-Strand Breaks

The homologous recombination (HR) pathway participates in several critical biological processes, including DNA repair, the rescue of stalled/collapsed DNA replication forks, meiotic chromosome segregation, and telomere maintenance (Sung and Klein 2006; Friedberg et al. 2006; Hoeijmakers 2001). As with NER and MMR, much of the effort in recent years to characterize the proteins involved in HR has focused on the early steps in this pathway including lesion (DSB) recognition, HR protein regulation, DSB repair pathway choice (HR vs. NHEJ), strand exchange processes, as well as the proteins involved in the resolution of holiday junctions (Barzel and Kupiec 2008; Bordeianu et al. 2011; Krejci et al. 2012; Sung and Klein 2006; Symington and Gautier 2011). A classical schematic for the HR pathway is depicted in Fig. 3.4b. Upon recognition of the DSB, the ends are processed through an end resection step, allowing strand invasion of the homologous strand of the sister chromatid, providing the template for HR-directed DNA synthesis from the D-loop and subsequently after second-end capture. Defining the DNA polymerase in this process was first shown in yeast where it was demonstrated that Polδ is preferentially recruited to complete DNA synthesis for HR (Maloisel et al. 2008). There are in fact numerous genetic examples demonstrating that yeast Polδ is involved in HR (Giot et al. 1997; Lydeard et al. 2007; Maloisel et al. 2004, 2008; Wang et al. 2004; Fabre et al. 1991). More recently, using purified proteins, it was shown that yeast Polδ, together with PCNA, is essential for DNA synthesis from the D-loop during HR (Li et al. 2009). In a more recent study, both yeast Polδ and yeast polymerase eta (Polη) contributed equally to DNA synthesis to extend the D-loop (Sebesta et al. 2011).

However, in chicken DT40 cells it was demonstrated that Polη participates in both HR and TLS (Kawamoto et al. 2005). Simultaneously, using purified human proteins and cell lysates, it was shown that human Polη promoted DNA synthesis from the D-loop intermediate (Fig. 3.4b) but this DNA synthesis step could not be conducted by human Polδ or by human polymerase iota (Polι) (McIlwraith et al. 2005). Human Polη, but not human Polδ or human Polι, was also able to

mediate the capture and annealing of the second end of the resected DSB, in concert with RAD52. This was subsequently followed by DNA synthesis from the captured "second" DNA end (McIlwraith and West 2008) (Fig. 3.4b). Of course, some aspects of HR may require a TLS step. This will be discussed elsewhere in this series (Chap. 4).

3.5 DNA Polymerases as Essential Components in Nucleotide Excision Repair

The nucleotide excision repair (NER) pathway plays an important role in the repair of DNA lesions (Kuper and Kisker 2012) induced by many genotoxins and chemotherapeutics including DNA cross-linking agents such as chloroethylating agents, cisplatin, carboplatin, and lesions induced by a host of environmental agents including cigarette smoke (Friedberg et al. 2006) and ultraviolet (UV) light (Wood 1996; de Vries et al. 1995; Yeh et al. 2012). Put simply, NER facilitates the removal of bulky DNA adducts that grossly distort the DNA double helix and those that cause a block to transcription. Overall, the pathway consists of two complementary sub-pathways that have some overlap. These two sub-pathways are referred to as global genome repair (GGR–NER) and transcription-coupled repair (TCR–NER) and facilitate lesion recognition/confirmation and the assembly of the pre-incision complex. Molecular details on the proteins involved in NER can be found in several excellent reviews (Hoeijmakers 2001; de Laat et al. 1999; Wood 1996; Shuck et al. 2008; Hanawalt and Spivak 2008; Gillet and Scharer 2006). The two sub-pathways are distinct regarding the lesion recognition step but converge and utilize the same proteins to remove the ~22–28 base oligonucleotide containing the lesion. Until recently, the molecular details of the later steps in the pathway, the DNA synthesis steps, were not fully characterized (Kunkel and Van Houten 2006). Although much has yet to be worked out, recent studies have provided compelling evidence that the DNA synthesis step of NER involves three DNA polymerases (Fig. 3.5), the replicative DNA polymerases delta (Polδ) and epsilon (Polε) as well as the Y-family DNA polymerase kappa (Polκ) (Kunkel and Van Houten 2006; Ogi et al. 2010; Ogi and Lehmann 2006).

3.5.1 Replicative Polymerases Delta and Epsilon in NER

DNA polymerases δ (Polδ) and ε (Polε) are both B-family DNA polymerases with primary roles in DNA replication (Kunkel and Burgers 2008). The involvement of replicative DNA polymerases in DNA synthesis in human NER evolved from earlier studies with human cell extracts that implicated a requirement for the replication cofactor PCNA (Shivji et al. 1992). Subsequent studies demonstrated

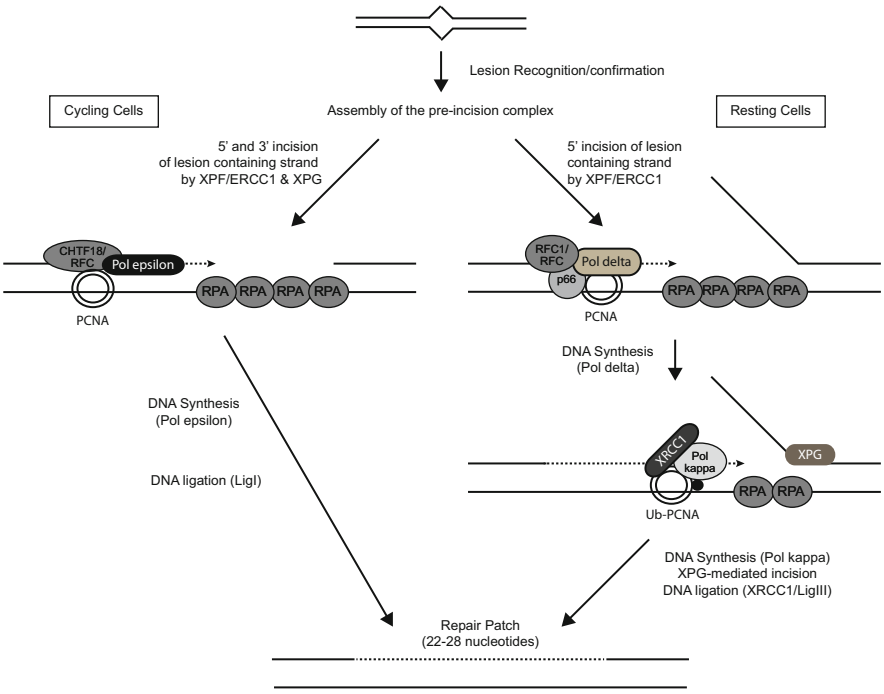

Fig. 3.5 DNA synthesis in NER by Polδ, Polε, and Polκ. Schematic depicting a role for Polδ, Polε, and Polκ in the DNA synthesis step of NER. The diagram shows the NER DNA polymerases in cycling and resting cells (Polδ, Polε, and Polκ) as well as the clamp loaders required for each polymerase (Ogi et al. 2010)

that DNA synthesis during NER was not affected by neutralizing antibodies to polymerase α (Polα) but was blocked by aphidicolin, suggesting a possible role for Polδ and/or Polε (Coverley et al. 1992). Subsequent elegant studies with purified human proteins clearly established the requirement for Polδ and/or Polε in DNA synthesis during NER (Shivji et al. 1995; Aboussekhra et al. 1995), ultimately defining a core set of proteins required for the repair of a cisplatin DNA adduct (Araujo et al. 2000). Replication factor C (RFC) was observed to be required for recruitment of Polδ (Overmeer et al. 2010).

As suggested above, the latest models suggest that Polδ, Polε, and Polκ (see below) are all involved in DNA synthesis during NER (Fig. 3.5) (Lehmann 2011; Ogi and Lehmann 2006; Ogi et al. 2010). Although it is not yet fully resolved as to the conditions or parameters that dictate polymerase choice in NER, several clues have emerged from biochemical studies (Fig. 3.5). Polδ is recruited by RFC1/p66 and loaded onto PCNA. The recruitment of Polκ does not require RFC1 but in fact is mediated by XRCC1 and is loaded onto ubiquitylated PCNA (see below). Conversely, Polε appears to be the polymerase of choice in cycling (dividing) cells in which CHTF18-RFC recruits Polε to load onto PCNA. Recruitment of Polε appears to favor dividing cells with high dNTPs and after dual incision by

XPF/ERCC1 and XPG, whereas Polδ requires RFC and PCNA for recruitment and likely favors nondividing cells (Lehmann 2011).

3.5.2 *A Role for DNA Polymerase Kappa in NER Unrelated to Translesion Synthesis*

DNA polymerase kappa (Polκ) is a Y-family DNA polymerase with a high error rate typical for this family of polymerases (Ohashi et al. 2000). Like other Y-family polymerases, Polκ can participate in DNA synthesis past bulky DNA lesions (translesion DNA synthesis, TLS) (Chap. 4) (Lange et al. 2011; Ziv et al. 2009) and would not be expected to participate in the DNA synthesis step of NER (Kunkel and Van Houten 2006). The low processivity and fidelity of Y-family polymerases (McCulloch and Kunkel 2008) (e.g., synthesis and incorporation of only one to five nucleotides before dissociation from the primer-template) would likely preclude Polκ from participation in NER to fill the gap of 22–28 nucleotides (Friedberg et al. 2006). However, in vitro studies have demonstrated that Polκ polymerizes up to 25 nucleotides before dissociation (Ohashi et al. 2000), supporting a possible role for Polκ in NER gap-filling DNA synthesis.

The first clue that Polκ may participate in NER gap-filling DNA synthesis was the demonstration that Polκ localized to repair foci with PCNA in a pattern that was unlike the other Y-family TLS polymerases eta (Polη) and iota (Polι) (Ogi et al. 2005). In a surprising finding using Polκ-KO MEFs, it was demonstrated that loss of Polκ reduced the level of NER following UV damage. Repair was not completely absent but was significantly reduced and clearly established a novel role for Polκ in NER (Ogi and Lehmann 2006). The same group followed this with a more detailed report implicating Polδ, Polε, and Polκ in NER (Ogi et al. 2010). As described above for Polδ and Polε and in Fig. 3.5, Polκ (in a complex with XRCC1) is recruited to complete DNA repair synthesis by ubiquitylated PCNA following repair DNA synthesis initiated by Polδ (Fig. 3.5, right side). Once repair is completed, the polymerase dissociates (Polκ) and XPG facilitates the 3′ incision to release the flap. This latter step is consistent with the observation that the 5′ incision by XPF/ERCC1 precedes the 3′ incision by XPG and that repair synthesis can proceed in the absence of XPG catalytic activity (Staresincic et al. 2009). Based on this recent model and available biochemical analysis (Fig. 3.5), recruitment and involvement of Polκ in NER requires XRCC1 and ubiquitylated PCNA for recruitment and likely favors low dNTPs and synthesis after 5′ incision by XPF/ERCC1 (Lehmann 2011).

3.6 The Mismatch Repair Pathway as a Replicative Polymerase Fidelity Factor

The DNA mismatch repair (MMR) pathway is involved in numerous processes involving DNA metabolism including repair of damage due to environmental or chemotherapeutic exposures, meiotic recombination, DNA damage signaling, and the correction or repair of numerous base–base mismatches and insertion/deletion loops (Fu et al. 2012; Li 2008; Wyatt and Pittman 2006; Modrich 2006; Jiricny 2006). The latter role of the MMR pathway functions to significantly improve DNA replication fidelity, as much as 1,000-fold, repairing errors made by Polα (Liberti et al. 2013; Nick McElhinny et al. 2010; Niimi et al. 2004), Polδ (Nick McElhinny et al. 2010; Lujan et al. 2012), and Polε (Lujan et al. 2012). Loss of MMR therefore promotes a mutator/genome instability phenotype that can predispose to an increase in mutations and cancer in eukaryotic model systems and humans (Arana and Kunkel 2010; Hubscher 2009; Kunkel 2009; Preston et al. 2010; Reha-Krantz 2010; Albertson et al. 2009).

3.6.1 A Reconstituted Human Mismatch Repair Pathway Utilizes DNA Polymerase Delta

A functional human MMR system has been reconstituted using recombinant proteins and artificial (plasmid) substrates (Modrich 2006). As depicted in the significantly simplified model shown in Fig. 3.6, mismatch recognition is primarily mediated by the heterodimers MUTSα (comprised of the proteins MSH2/MSH6 or MSH2/MSH3) and MUTLα (comprised of the proteins MLH1/PMS1) (Friedberg et al. 2006). Further details on mismatch recognition and MMR can be found in numerous reviews (Fu et al. 2012; Li 2008; Wyatt and Pittman 2006; Modrich 2006; Jiricny 2006). In an elegant series of biochemical studies, a completely reconstituted system was developed that was capable of supporting directional MMR ($3' \geq 5'$ or $5' \geq 3'$) that exploits a previously undiscovered latent endonuclease activity of MUTLα that is both ATP and mismatch dependent (Kadyrov et al. 2006). Together with EXO1, this in vitro system yields the proper substrate for DNA polymerase loading onto PCNA to allow DNA synthesis of the repair patch for MMR, estimated at 1,000 bases (Thomas et al. 1991) but can range from 200 to >2,000 base pairs, depending on the location of the mismatch and the cellular state (Modrich 2006). In this system, purified Polδ was utilized and found to be fully capable of supporting MMR DNA synthesis (Fig. 3.6).

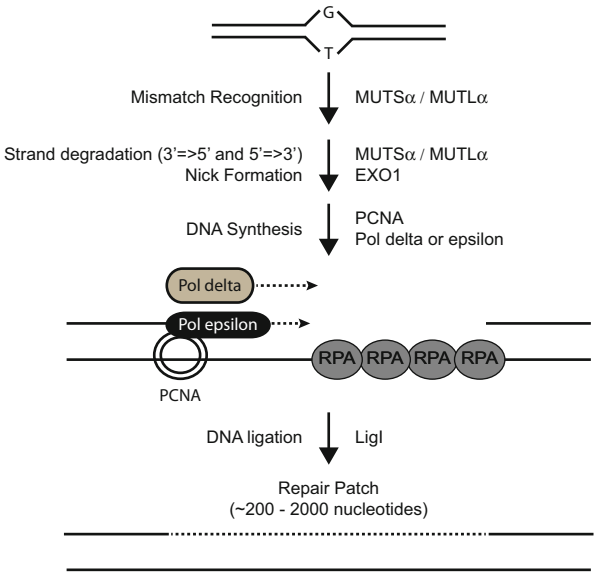

Fig. 3.6 DNA synthesis in human MMR. Schematic depicting a role for Polδ and Polε in the DNA synthesis step of MMR, resulting in a DNA repair patch ranging from 200 to 2,000 bases

3.6.2 Replicative Polymerases Delta and Epsilon in Eukaryotic MMR

Functional (in vivo) studies of eukaryotic MMR and the DNA polymerase requirements for MMR have been limited to *S. cerevisiae* or mouse model systems. As might be expected from the reconstituted system, both replicative polymerases (Polδ and Polε) likely play a role in MMR DNA synthesis. In this model system it is suggested that Polδ, together with Polα, uses the lagging strand as the template for DNA replication whereas Polε uses the leading strand as template (Larrea et al. 2010; Nick McElhinny et al. 2008). It is not yet established if the polymerase used in MMR is also strand specific. Although leading strand (Polε) and lagging strand (Polδ and Polα) fidelity differs, evidence is clear that MMR balances fidelity across both DNA strands (Lujan et al. 2012).

3.7 DNA Polymerase Involved in DNA Cross-link Repair

Characterizing the mechanism or mechanisms that mediate the repair of DNA interstrand cross-links (ICLs) has been a significantly challenging task, complicating the identification of DNA polymerases that may be involved in the repair process. Models have been proposed that depend on replication (Raschle et al. 2008) as well as those that are replication independent (Williams et al. 2012) and involve NER-related transcription-coupled repair or global genome repair and both models (replication dependent and replication independent) involve DNA

translesion synthesis (Enoiu et al. 2012). It is generally accepted that complete repair of an ICL involves proteins from several pathways, including the FANC proteins for ICL recognition and signaling (Kim and D'Andrea 2012), HR and NER proteins for lesion processing (Hinz 2010; Wood 2010), as well as TLS polymerases to synthesize DNA across from the unhooked lesion (Enoiu et al. 2012; Ho et al. 2011; Ho and Scharer 2010; Klug et al. 2012; McHugh and Sarkar 2006; Nojima et al. 2005; Sharma and Canman 2012; Shen et al. 2006). One plausible model for the repair of ICLs is shown in Fig. 3.7. In this model, repair can be achieved by a replication-dependent (right panel) or replication-independent (left panel) process. In the left panel, the replication-independent process utilizes NER proteins to "unhook" the cross-link followed by a translesion DNA polymerase to synthesize DNA across the "lesion." This is followed by a second round of NER-mediated repair and DNA synthesis. The NER proteins involved in ICL repair may vary with the lesion. It was recently reported that cisplatin lesions are repaired in a replication-independent fashion utilizing TCR–NER proteins (Enoiu et al. 2012) whereas MMC and psoralen cross-links are suggested to utilize GGR–NER proteins for ICL repair (Hlavin et al. 2010; Wang et al. 2001; Muniandy et al. 2009). In some cases, BER proteins appear to play a role in ICL repair (Kothandapani et al. 2011; Kothandapani and Patrick 2013). The replication-dependent process utilizes FANC proteins to recognize the ICL and mediate unhooking and induce ICL-associated DSBs, in preparation for HR-mediated repair. Both processes rely heavily on translesion DNA polymerases to synthesize DNA opposite the "unhooked" DNA cross-link (Lange et al. 2011; Sharma and Canman 2012). A separate chapter in this series will discuss translesion DNA polymerases (Chap. 4).

3.8 Summary and Concluding Remarks

There are as many as 15 human DNA polymerases to facilitate DNA replication, DNA repair, and DNA lesion bypass (tolerance) in the nucleus and mitochondrial genomes (Burgers et al. 2001; Bebenek and Kunkel 2004). These are characterized by family or class based upon phylogenetic relationships, as described by Burgers et al. (2001). In most cases, the role of some human DNA polymerases in specific DNA repair pathways is expected, based either on data from *E. coli* or *S. cerevisiae* or on biochemical parameters. The high-processivity, high-fidelity replicative DNA polymerases (Polδ and Polε) are a likely option for synthesis of the longer repair patches needed for NER, MMR, and even long-patch BER, whereas the biochemical parameters of the X-family polymerases suggest they are well suited for DNA synthesis required for the short-patch or minimal DNA synthesis observed in BER and NHEJ. Yet, as the field advances, surprises still abound. For example, the Y-family human DNA polymerase Polκ participates in NER and human Polη but not Polδ or Polι is involved in HR-mediated DNA synthesis from the D-loop

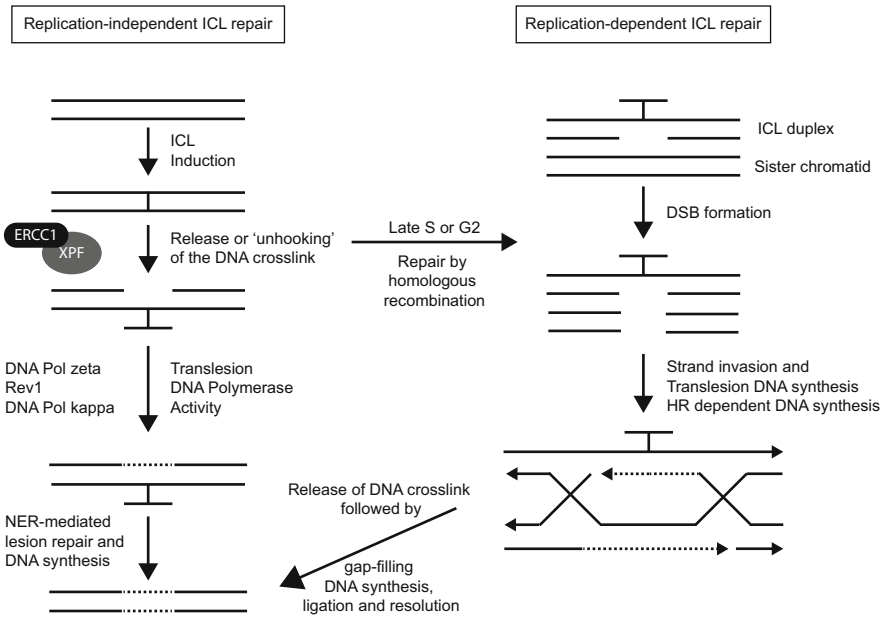

Fig. 3.7 Proposed mechanisms for repair of DNA cross-links. Schematic depicting a current model for the repair of DNA cross-links for cells in the G0/G1 phase of the cell cycle (*left*). Here, repair is mediated by a replication-independent mechanism. Following release or "unhooking" of the lesion (ICL), translesion DNA polymerases can fill the gap across from the lesion, followed by a second lesion removal step and DNA synthesis as in classical NER. On the *right* is a scheme for replication-dependent ICL repair. Here, the repair of DNA cross-links for cells in the late S-phase or G2 phase of the cell cycle would have the availability of the HR pathway to encode the information opposite the unhooked cross-link followed by a second round of DNA synthesis once the lesion (an unhooked cross-link) is removed

intermediate nor involved in second-end capture and the subsequent DNA synthesis step.

Considerable effort is still required to identify and characterize the DNA polymerases involved in many aspects of DNA repair and DNA metabolism. Mutations or defects in DNA polymerases affect response to DNA damaging agents (Sobol et al. 1996, 2000; Trivedi et al. 2005), antibody diversity (Seki et al. 2005), organism survival (Friedberg and Meira 2006), and overall genome maintenance (Prindle and Loeb 2012). The interrelationship between DNA synthesis fidelity and DNA repair is most evident by the cancer predisposition observed when replicative DNA polymerases are mutated in their proofreading domain (Palles et al. 2013). As more details emerge regarding the role of each DNA polymerase in DNA repair, it is expected that we will begin to understand the need for so many different DNA polymerases to maintain genome integrity as well as the multiple roles they may play in the diverse yet interrelated pathways for DNA repair.

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Chapter 4
Eukaryotic Y-Family Polymerases: A Biochemical and Structural Perspective

John M. Pryor, Lynne M. Dieckman, Elizabeth M. Boehm, and M. Todd Washington

Abstract Classical DNA polymerases, which replicate DNA rapidly and with high fidelity, stall upon encountering DNA damage. Thus nonclassical polymerases, which have evolved to accommodate DNA damage, are necessary to overcome these replication blocks. These nonclassical polymerases mainly belong to the Y-family and replicate DNA slower and with lower fidelity than their classical counterparts. Y-family polymerases employ surprising strategies to incorporate nucleotides opposite DNA damage. These include the use of larger and less constrained active sites, the use of Hoogsteen base pairing, and the use of amino acid side chains as templates. Y-family polymerases also engage in protein–protein interactions that are important for their recruitment to stalled replication forks and the coordination of their activities on the DNA. These polymerases function within a dynamic network of protein–protein interactions that are mediated by intrinsically disordered regions of these enzymes. This review focuses on the biochemical and structural studies of the Y-family polymerases, which have provided clear insights into their function.

Keywords DNA replication • DNA repair • DNA polymerase • Protein–protein interactions • PCNA • Rev1 • Translesion synthesis • Intrinsically disordered proteins

Abbreviations

BRCT	BRCA1 C-terminal
CTD	C-terminal domain
NMR	Nuclear magnetic resonance

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PAD	Polymerase-associated domain
PCNA	Proliferating cell nuclear antigen
PIP	PCNA-interacting protein
Pol	Polymerase
RIR	Rev1-interacting region
SAXS	Small-angle X-ray scattering
TT	Thymine–thymine
UBM	Ubiquitin-binding motif
UBZ	Ubiquitin-binding zinc finger
UV	Ultraviolet
XPV	Xeroderma pigmentosum variant form

4.1 Introduction

Classical DNA polymerases, despite their remarkable rate of DNA synthesis and their high fidelity, are unable to efficiently incorporate nucleotides opposite DNA damage. The structural distortions in the DNA caused by the damage are generally not tolerated by their highly constrained active sites. Thus, when one of these enzymes encounters a DNA lesion, it is blocked and the replication fork stalls. Translesion synthesis (TLS) is one strategy for overcoming such replication blocks. In TLS, one or more nonclassical DNA polymerases are recruited to the stalled replication fork. These nonclassical polymerases, which have evolved to accommodate DNA damage, replace the stalled classical polymerase and catalyze DNA synthesis past the lesion. The most studied of these nonclassical polymerases are the Y-family polymerases (Burgers et al. 2001; Ohmori et al. 2001). In prokaryotes, these include the SOS-inducible DNA polymerase IV and DNA polymerase V. In eukaryotes, these include DNA polymerase eta (Pol η), DNA polymerase iota (Pol ι), DNA polymerase kappa (Pol κ), and Rev1. This chapter focuses on the biochemical activities and structures of the eukaryotic Y-family polymerases.

Although most Y-family polymerases can inefficiently incorporate nucleotides opposite a wide range of DNA lesions, each polymerase appears to be specific for efficiently incorporating nucleotides opposite a small number of lesions or families of closely related lesions. These are referred to as the cognate lesions for a given polymerase (Friedberg et al. 2002; Lehmann et al. 2007; Waters et al. 2009). The clearest example of this concept is Pol η and the ultraviolet (UV) radiation-induced *cis–syn* thymine–thymine (TT) dimer (Johnson et al. 1999b). Another cognate lesion of Pol η is 8-oxoguanine (Haracska et al. 2000). The cognate lesions of Pol ι are some minor groove purine adducts and some exocyclic purine adducts (Washington et al. 2004c; Wolfle et al. 2005; Pence et al. 2009; Nair et al. 2006). The cognate lesions of Pol κ are a few minor groove guanine adducts (Choi et al. 2006). The cognate lesions of Rev1 are abasic sites (Pryor and Washington 2011), several minor groove guanine adducts, and some exocyclic guanine adducts

(Washington et al. 2004b; Nair et al. 2008; Choi and Guengerich 2008; Zhang et al. 2002b).

DNA lesions present two kinetic barriers to nucleotide incorporation by DNA polymerases. The first occurs during the incorporation step, when the lesion is in the nascent base pair itself. The second occurs during the subsequent nucleotide-incorporation step (i.e., the extension step), when the lesion is in the primer-terminal base pair. In some cases, Y-family polymerases have evolved to overcome both of these barriers. Pol η, for example, readily inserts nucleotides opposite TT dimers and extends beyond the lesion (Johnson et al. 1999b). In many cases, however, Y-family polymerases have evolved to overcome only one of these two barriers. Thus to bypass these lesions, multiple Y-family polymerases have to work in tandem with one polymerase incorporating a nucleotide opposite the lesion and another catalyzing subsequent extension (Prakash and Prakash 2002; Livneh et al. 2010). For example, Pol ι can efficiently incorporate a nucleotide opposite the acrolein-derived γ-hydroxy-1,N^2-propanoguanine, and Pol κ can efficiency extend beyond this lesion (Washington et al. 2004c). It should be noted that in most cases, this extension step is catalyzed by DNA polymerase zeta (Pol ξ), another nonclassical polymerase. This enzyme is a B-family polymerase, and many excellent review articles describing the biological function and the biochemical properties of Pol ξ are available (Prakash and Prakash 2002; Lawrence 2002, 2004; Waters et al. 2009; Livneh et al. 2010).

4.2 Overview of the Structures of Y-Family Polymerases

The overall structures of eukaryotic Y-family polymerases are similar to each other. These enzymes have a conserved catalytic core region comprised of approximately 400–500 amino acid residues that constitute a polymerase domain and a polymerase-associated domain (PAD). X-ray crystal structures of the catalytic core regions of all four human Y-family polymerases have been determined (Ummat et al. 2012; Nair et al. 2004; Lone et al. 2007; Swan et al. 2009). These structures show that the polymerase domains all contain fingers, thumb, and palm sub-domains analogous to those found in classical DNA polymerases. The polymerase domain binds to the primer-template DNA and an incoming nucleotide and catalyzes the incorporation reaction. The structures and mechanisms of the catalytic core regions of these polymerases are discussed in more detail below (see Sect. 4.3).

In all four enzymes, the catalytic core region is followed by a C-terminal region comprised of 300–400 amino acid residues. An analysis of the C-terminal regions of these polymerases shows that they are mostly intrinsically disordered (Fig. 4.1) (Ohmori et al. 2009). It has been estimated that as many as a third of eukaryotic proteins and a half of mammalian proteins are partially or fully disordered (Fink 2005; Dunker et al. 2008). In general, the disordered regions of proteins are often involved in interactions with multiple protein partners (Fink 2005; Dunker et al. 2008; Cortese et al. 2008). This clearly is the case with the C-terminal

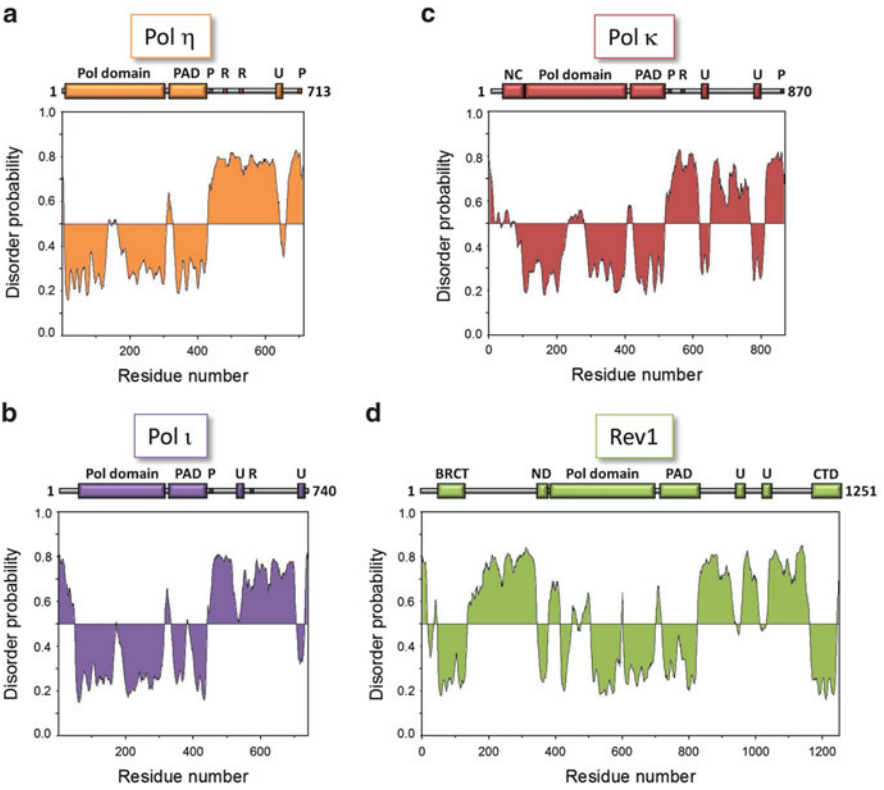

Fig. 4.1 The structured and unstructured regions of Y-family polymerases. The graphs of disorder probability for (**a**) Pol η, (**b**) Pol ι, (**c**) Pol κ, and (**d**) Rev1 were obtained using the meta-approach for predicting disordered regions of proteins (Ishida and Kinoshita 2008). In the diagrams of each polymerase, the structured regions are shown as *thick rectangles*, and the disordered regions are shown as *thin rectangles*. The polymerase (Pol) domain and PAD of each protein are indicated. The N-clasp (NC) of Pol κ as well as the N-digit (ND), the BRCT domain, and the CTD of Rev1 are indicated. PCNA-binding, ubiquitin-binding, and Rev1-binding motifs are indicated by P, U, and R, respectively

disordered regions of the Y-family polymerases. The C-terminal regions of Pol η, Pol ι, and Pol κ all contain small structured and unstructured motifs involved in binding several proteins including the key replication accessory factor proliferating cell nuclear antigen (PCNA), ubiquitin, and Rev1. The C-terminal region of Rev1 contains two small ubiquitin-binding motifs as well as a small, structured C-terminal domain (CTD) that binds Pol η, Pol ι, and Pol κ. In addition, Rev1 is the only eukaryotic Y-family polymerase possessing an N-terminal region comprised of approximately 350 amino acid residues that is also mostly disordered. This region contains a small, structured BRCA1 C-terminal (BRCT) domain. The structure and function of the motifs and small domains in the disordered regions of Y-family polymerases are discussed in more detail below (see Sects. 4.4 and 4.5).

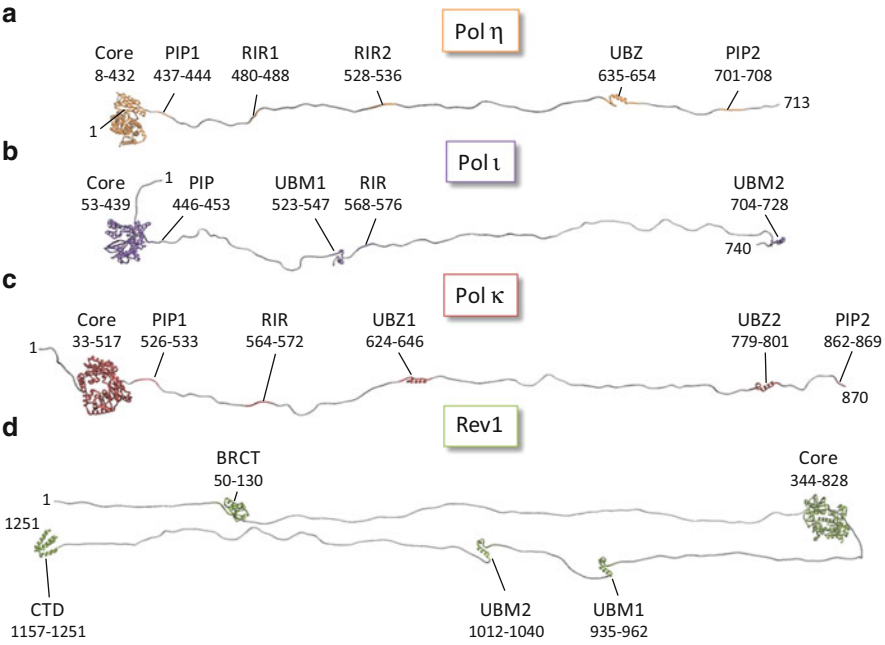

Fig. 4.2 Structural models of the full-length Y-family polymerases. The models of full-length (**a**) Pol η, (**b**) Pol ι, (**c**) Pol κ, and (**d**) Rev1 were built using Coot (Emsley and Cowtan 2004) starting with the X-ray crystal structures of the catalytic core regions of these polymerases (PDB ID: 3TQ1, 1T3N, 2OH2, and 3GQC, respectively) (Ummat et al. 2012; Nair et al. 2004; Lone et al. 2007; Swan et al. 2009) and the NMR structures of the UBZ of Pol η (PDB ID: 2I5O) (Bomar et al. 2007), the UBM of Pol ι (PDB ID: 2KHU) (Bomar et al. 2010), the Rev1 CTD (PDB ID: 2LSY) (Pozhidaeva et al. 2012), and the Rev1 BRCT domain (PDB ID: 2EBW). The UBZ of Pol κ was modeled based on the UBZ of Pol η, and the UBM of Rev1 was modeled based on the UBM of Pol ι. The disordered regions were then built as random coils. The various PIP motifs, UBZs, UBMs, and RIR motifs are indicated

We have built structural models of the full-length versions of all four human Y-family polymerases (Fig. 4.2). We started with the X-ray crystal structures of the catalytic core regions of these polymerases (Ummat et al. 2012; Nair et al. 2004; Lone et al. 2007; Swan et al. 2009) as well as the NMR structure of the ubiquitin-binding zinc finger (UBZ) of Pol η (Bomar et al. 2007), the ubiquitin-binding motif (UBM) of Pol ι (Bomar et al. 2010), the Rev1 CTD (Pozhidaeva et al. 2012), and the Rev1 BRCT domain (Riken Structural Genomics/Proteomics Initiative). We then built the disordered regions as random coils. Given the accuracy of disorder predictions, these extremely long regions are unlikely to contain any folded domains other than the few small domains (the ubiquitin-binding elements, the Rev1 BRCT domain, and the Rev1 CTD) that have already been identified. These disordered regions, however, may still contain some small secondary structural elements that are not shown in the models. Nevertheless, these models provide an

excellent global view of the size and extended nature of the disordered regions of these polymerases relative to the structured catalytic core regions.

4.3 The Catalytic Activity of Y-Family Polymerases

Classical polymerases have evolved to synthesize DNA with high fidelity. Consequently, the active sites of classical polymerases are tightly constrained so that only the correct Watson–Crick base pairs can fit. The structural distortions in the DNA caused by lesions generally do not fit properly in the active sites of classical polymerases, and as a result, classical polymerases cannot efficiently incorporate nucleotides opposite DNA lesions. By contrast, nonclassical polymerases such as the Y-family polymerases have evolved to efficiently incorporate nucleotides opposite DNA damage using a variety of strategies. However, their active sites are not as tightly constrained, and as a result, they synthesize DNA with considerably lower fidelity. Because of their intrinsic low fidelity, replication by Y-family polymerases is highly error prone. Thus to reduce the likelihood of errors, these enzymes all synthesize DNA with low processivity. In this section, we discuss the biochemical activities and structures of the catalytic core regions of the four eukaryotic Y-family polymerases.

4.3.1 DNA Polymerase η

Pol η, the best studied of the Y-family polymerases, is found in all eukaryotes. In yeast, the lack of Pol η leads to an increase in UV-induced mutagenesis (McDonald et al. 1997; Roush et al. 1998). In humans, the lack of Pol η results in the genetic disorder xeroderma pigmentosum variant form (XPV), which is characterized by an extreme sensitivity to sunlight and a predisposition to skin cancer (Johnson et al. 1999a; Masutani et al. 1999). Steady-state and pre-steady-state kinetic studies showed that Pol η synthesizes DNA with low fidelity with error frequencies ranging from 10^{-2} to 10^{-3} (Washington et al. 1999, 2001; Matsuda et al. 2000; Johnson et al. 2000c). This error frequency is approximately 1,000-fold greater than those of classical DNA polymerases. While Pol η can incorporate nucleotides opposite all four non-damaged template residues, it synthesizes DNA with low processivity only incorporating about four or five nucleotides per DNA-binding event (Washington et al. 1999).

The cognate lesions of Pol η include the *cis–syn* TT dimer, which is caused by exposure to UV radiation, and 8-oxoguanine, which is a common form of oxidative base damage. In the case of the TT dimer, purified Pol η incorporates adenines opposite both bases of the dimer with the same kinetics as it incorporates opposite non-damaged thymine (Washington et al. 2000, 2003; Johnson et al. 2000c). In the case of 8-oxoguanine, it incorporates cytosine opposite the lesion with the same

kinetics as it incorporates opposite non-damaged guanine (Haracska et al. 2000; Carlson and Washington 2005). Moreover, it incorporates cytosine 20–60-fold more efficiently than it incorporates adenine. By contrast, classical polymerases often preferentially incorporate adenine opposite 8-oxoguanine because the 8-oxoguanine–adenine base pair is less distorting to the DNA geometry than is the 8-oxoguanine–cytosine base pair.

Insight into the structural basis of the ability of Pol η to replicate through TT dimers was provided by X-ray crystal structures of the catalytic core region of Pol η bound to DNA and incoming nucleotide substrates (Silverstein et al. 2010; Ummat et al. 2012; Biertuempfel et al. 2010). The DNA is contacted by the finger, thumb, and palm sub-domains of the polymerase domain and by the PAD (Fig. 4.3a). The active site of Pol η is larger than those of classical polymerases due in part to a truncation of the fingers sub-domain. This larger active site allows Pol η to accommodate the two cross-linked bases in its active site without steric clashes. The structure of Pol η bound to DNA containing a TT dimer shows that the incoming dATP forms Watson–Crick base pairs with the damaged thymines of the dimer in exactly the same way that it forms base pairs with non-damaged thymines (Fig. 4.3a) (Silverstein et al. 2010). In addition, Pol η also makes several direct contacts with the template strand, and these contacts ensure that the DNA bound in the active site is in the same stable, nearly B-form conformation whether it is damaged or non-damaged. Together, these properties allow Pol η to catalyze nucleotide incorporation opposite the TT dimer with nearly identical kinetics and fidelity as it does opposite non-damaged DNA.

4.3.2 DNA Polymerase ι

Pol ι is found in many higher eukaryotes including insects and mammals; it is not found in yeast and nematodes. The biological role of Pol ι is not well understood, and the lack of Pol ι is not associated with any disease. However, mice lacking Pol ι are at a higher risk for urethane-induced lung cancers (Lee and Matsushita 2005), and human fibroblasts lacking Pol ι were shown to be sensitive to oxidative stress (Petta et al. 2008). Steady-state and pre-steady-state kinetic studies showed that the fidelity and efficiency of nucleotide incorporation by Pol ι varies greatly depending on the template base (Johnson et al. 2000b; Tissier et al. 2000; Zhang et al. 2000; Washington et al. 2004a). Opposite a template adenine, Pol ι carries out nucleotide incorporation with a moderate-to-high efficiency and fidelity with error frequencies ranging from 10^{-4} to 10^{-5}. Opposite both a template guanine and cytosine, the efficiency of incorporation is reduced about tenfold and the fidelity is significantly lower with error frequencies ranging from 10^{-1} to 10^{-2}. Opposite a template thymine, incorporation is very inefficient and the fidelity is extraordinarily low with error frequencies ranging from 10^{-1} to 10^{1}. In fact, Pol ι incorporates the incorrect guanine opposite a template thymine about tenfold more efficiently than it incorporates the correct adenine.

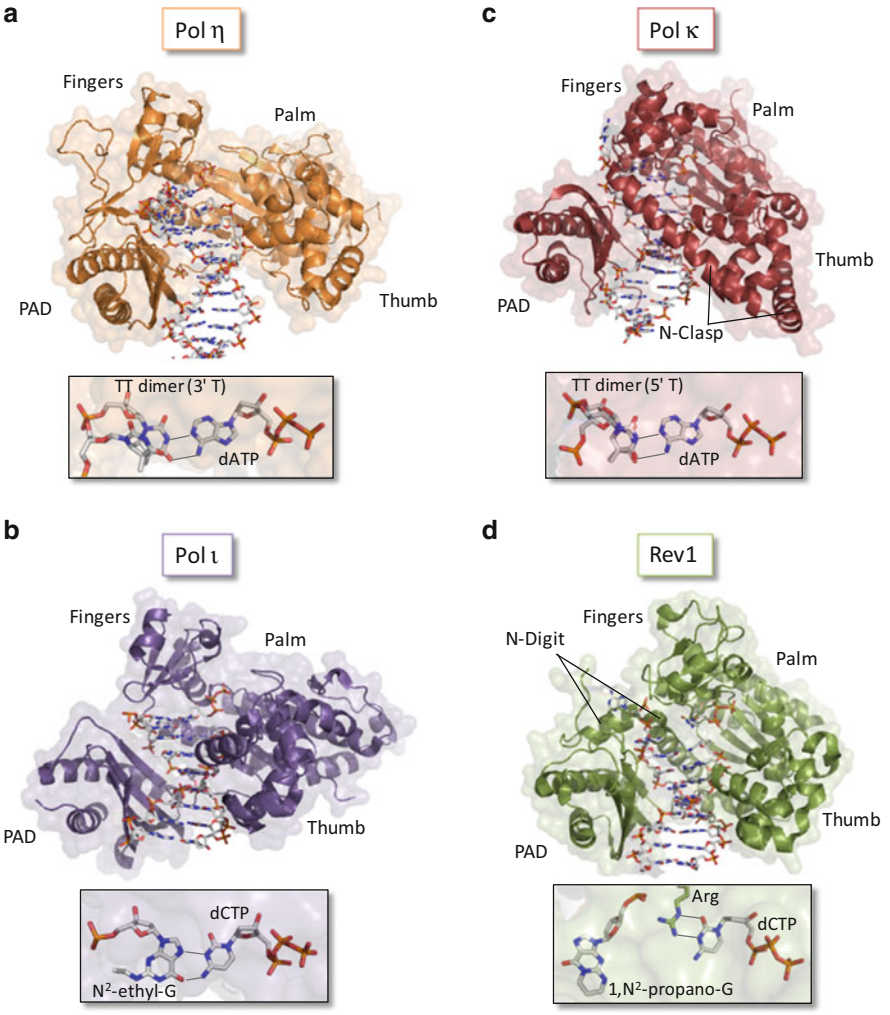

Fig. 4.3 Structure of the catalytic core regions and active sites of Y-family polymerases. Ribbon diagrams of the catalytic core regions of (**a**) Pol η, (**b**) Pol ι, (**c**) Pol κ, and (**d**) Rev1 are shown bound to DNA and an incoming nucleotide. In the case of Pol η, the template base is the 3′ thymine of a TT dimer (PDB ID: 3MFI) (Silverstein et al. 2010). In the case of Pol ι, the template base is N^2-ethylguanine (PDB ID: 3EPI) (Pence et al. 2009). In the case of Pol κ, the template base is the 5′ thymine of a TT dimer (PDB ID: 3PZP) (Vasquez-Del Carpio et al. 2011). In the case of Rev1, the template base is γ-hydroxy-1,N^2-propanoguanine (PDB ID: 3BJY) (Nair et al. 2008). The fingers, palm, and thumb sub-domains of the polymerase domain and the PAD are indicated. The N-clasp and the N-digit of Pol κ and Rev1, respectively, are indicated. The *insets* below the ribbon diagrams show the nascent base pair in the polymerase active site

Kinetic studies of purified Pol ι suggest that minor groove purine adducts and some exocyclic purine adducts are cognate lesions for this polymerase. For example, Pol ι incorporates cytosine opposite N^2-ethylguanine with the same efficiency with which it incorporates opposite a non-damaged guanine (Pence et al. 2009). This is remarkable, because minor groove adducts like this are a strong block to most polymerases because of steric clashes in their active sites. Even more striking is the ability of Pol ι to efficiently incorporate the correct nucleotide opposite lesions such as γ-hydroxy-1,N^2-propanoguanine (Washington et al. 2004c; Wolfle et al. 2005), which has an exocyclic ring connecting N1 and N^2 of the guanine, and 1,N^6-ethenoadenine (Nair et al. 2006), which has an exocyclic ring connecting N1 and N^6 of the adenine. In both of these lesions, the exocyclic ring disrupts their Watson–Crick base-pairing edge preventing them from forming Watson–Crick base pairs.

Insight into the structural basis of the ability of Pol ι to incorporate nucleotides opposite both minor groove purine adducts as well as exocyclic purine adducts was provided by X-ray crystal structures of the catalytic core region of Pol ι bound to DNA and incoming nucleotide substrates (Nair et al. 2004, 2005a, 2006; Pence et al. 2009). When Pol ι forms a ternary complex with the incoming nucleotide and DNA, the template backbone is held in a position that reduces the distance between the two C1′ atoms of the nascent base pair by approximately 2 Å relative to other DNA polymerases. As a result of this, when the template is a purine, Pol ι rotates the template base from the normal *anti* configuration around the N-glycosidic bond to the *syn* configuration in order to accommodate this reduced distance. This forces the incoming nucleotide to bind the template base using Hoogsteen base pairs, not Watson–Crick base pairs (Nair et al. 2004). In the case of the minor groove purine adducts, such as N^2-ethylguanine, Hoogsteen base pairing allows for efficient incorporation because the rotation of the damaged bases from the *anti* to the *syn* configuration places these adducts in the major groove where there are no steric clashes (Fig. 4.3b) (Pence et al. 2009). In the case of the exocyclic purine adducts, the rotation of the damaged base to the *syn* configuration allows for efficient incorporation because these exocyclic rings are easily accommodated in the active site and do not interfere with Hoogsteen base pairing.

4.3.3 DNA Polymerase κ

Pol κ is found in many higher eukaryotes including mammals, but it is not found in yeast and insects. The biological role of Pol κ is also poorly understood. Mouse cells lacking Pol κ have an enhanced spontaneous mutation rate (Stancel et al. 2009) and are sensitive to benzo(*a*)pyrene diol epoxide (Ogi et al. 2002). Unlike other Y-family polymerases, Pol κ is capable of synthesizing DNA with moderate fidelity with error frequencies ranging from 10^{-3} to 10^{-4} (Ohashi et al. 2000; Johnson et al. 2000a). Moreover, Pol κ synthesizes DNA with a higher processivity than other Y-family polymerases; it can incorporate up to

25 nucleotides per DNA-binding event (Ohashi et al. 2000). Thus among the Y-family polymerases, Pol κ is most similar to classical DNA polymerases.

Kinetic studies of Pol κ have led to two proposed roles for this enzyme in TLS. The first is that Pol κ catalyzes nucleotide incorporation opposite cognate lesions as do the other Y-family polymerases. The possible cognate lesions for Pol κ include several minor groove guanine adducts. Kinetic studies have shown that Pol κ incorporates nucleotides opposite N^2-methylguanine, N^2-ethylguanine, N^2-isobutylguanine, N^2-benzylguanine, and N^2-naphthylguanine with nearly the same efficiency as opposite non-damaged guanine (Choi et al. 2006). The second proposed role is that Pol κ functions during the extension step of TLS when two Y-family polymerases work in tandem to bypass a lesion. This comes from the observation that Pol κ efficiently extends from aberrant primer-terminal pairs containing mismatches (Washington et al. 2002). Pol κ also efficiently extends from primer-terminal pairs containing O^6-methylguanine and 8-oxoguanine (Haracska et al. 2002a), N^2-benzo(*a*)pyrenyl guanine adducts (Zhang et al. 2002a), and the TT dimer (Washington et al. 2002). In this latter case, Pol κ extends from the nucleotide already inserted opposite the first base of the TT dimer (the 3′ thymine) by directly incorporating an adenine opposite the second base of the dimer (the 5′ thymine). Given the strong biochemical support for both of these proposed roles, it is likely that Pol κ functions in some contexts to insert nucleotides opposite DNA lesions and in other contexts to extend from nucleotides inserted opposite lesions by other polymerases.

X-ray crystal structures of the catalytic core region of Pol κ in ternary complexes with DNA and incoming nucleotides have shown that like Pol η, it utilizes Watson–Crick base pairing to form the nascent base pair. Its active site, however, is more constrained than those of other Y-family polymerases (Uijon et al. 2004; Lone et al. 2007), which is presumably why this enzyme is capable of higher fidelity DNA synthesis. Pol κ possesses a structural feature called an N-clasp comprised of about 75 amino acid residues that immediately precedes the polymerase domain (Fig. 4.3c). The N-clasp allows Pol κ to completely encircle the DNA substrate when it binds, and deletion of the N-clasp significantly reduces DNA binding by Pol κ (Lone et al. 2007). The N-clasp may provide additional stability to the polymerase–DNA complex and in so doing may contribute to the ability of Pol κ to extend from aberrant primer-terminal pairs. An X-ray crystal structure of Pol κ shows this enzyme in the act of extending from an aberrant base pair, in this case a TT dimer (Fig. 4.3c) (Vasquez-Del Carpio et al. 2011). While the highly restrictive active site does not allow Pol κ to incorporate a nucleotide opposite the first base of this lesion (the 3′ thymine), it does readily accommodate the TT dimer in the active site when extending from this aberrant base pair (i.e., when incorporating opposite the second base of the lesion, the 5′ thymine).

4.3.4 *Rev1*

Rev1, which is found in all eukaryotes, has highly unusual substrate specificity. It preferentially incorporates cytosine opposite every template base, albeit with different efficiencies (Haracska et al. 2002b; Masuda et al. 2002; Masuda and Kamiya 2002). Opposite a template guanine, Rev1 incorporates cytosine with high efficiency. Although Rev1 is capable of misincorporating other incoming nucleotides, it does so at relatively low frequencies ranging from 10^{-3} to 10^{-4}. Thus opposite a template guanine, Rev1 has a moderate fidelity. Opposite the other three non-damaged templates, Rev1 still preferentially incorporates cytosine, although the efficiency of incorporation is reduced by as much as 500-fold. Because of this unique substrate specificity, Rev1 has not been officially designated as a DNA polymerase and assigned a Greek letter. Nevertheless, it has the same general structure and chemical mechanism as other DNA polymerases. It can even processively synthesize DNA if the template contains a series of guanines (Haracska et al. 2002b). Thus Rev1 is a DNA polymerase in everything but name.

Kinetic studies of purified Rev1 suggest that abasic sites as well as several guanine adducts are cognate lesions for this polymerase. Pre-steady-state kinetic studies have shown that Rev1 is capable of highly efficient incorporation opposite an abasic site (Pryor and Washington 2011). Similarly, Rev1 incorporates nucleotides opposite minor groove N^2-guanine adducts including N^2-methylguanine, N^2-ethylguanine, N^2-isobutylguanine, N^2-benzylguanine, N^2-naphthylguanine, N^2-anthracenylguanine, and N^2-benzo(*a*)pyrenyl guanine with nearly the same efficiency as opposite non-damaged guanine (Choi and Guengerich 2008). Incorporation opposite similar major groove guanine adducts is much less efficient, except for O^2-benzylguanine, opposite which Rev1 incorporates nucleotides efficiently. In addition, efficient incorporation by Rev1 was also observed opposite the exocyclic γ-hydroxy-1,N^2-propanoguanine adduct (Washington et al. 2004b). Rev1 also incorporates opposite 8-oxoguanine but does so with low efficiency (Haracska et al. 2002b; Pryor and Washington 2011).

Insight into the structural basis of this unique preference for cytosine incorporation was provided by X-ray crystal structures of Rev1 in a ternary complex with an incoming dCTP and a DNA substrate containing a template guanine (Nair et al. 2005b; Swan et al. 2009). Surprisingly, this structure showed that the template guanine and the incoming dCTP are not base-paired. Instead, the template base is flipped out of the DNA double helix and into a binding pocket in the enzyme comprised in part from amino acid residues in the PAD. Situated between the polymerase domain and the PAD is the N-digit, a structural element comprised of about 50 amino acid residues (Fig. 4.3d). The N-digit contains a highly conserved leucine and a highly conserved arginine that are both critical for catalysis. The leucine is responsible for flipping out the template base into its binding pocket, and the arginine is responsible for hydrogen bonding with the incoming dCTP. Thus Rev1 utilizes an amino acid side chain as a template to direct the incorporation of cytosine irrespective of the identity of the template base,

provided that the flipped out template base fits nicely within its binding pocket. An X-ray crystal structure of Rev1 bound to a DNA substrate containing an exocyclic γ-hydroxy-1,N^2-propanoguanine shows that this lesion is readily accommodated by the template base-binding pocket (Fig. 4.3d) (Nair et al. 2008). Another structure of Rev1 bound to DNA containing an abasic site shows that this lesion does not distort the template base-binding pocket because the pocket is occupied by a series of ordered water molecules (Nair et al. 2011).

4.4 Interactions of Y-Family Polymerases with PCNA

The Y-family polymerases are recruited to stalled replication forks and regulated in part by their interactions with the key replication accessory factor PCNA. PCNA is a ring-shaped homo-trimer that encircles the DNA and greatly enhances the processivity of classical DNA polymerases (Krishna et al. 1994). It also interacts with a wide range of other proteins and in so doing recruits them to sites of DNA replication (Maga and Hubscher 2003; Moldovan et al. 2007; Naryzhny 2008; Zhuang and Ai 2010; Dieckman et al. 2012). When cells are exposed to DNA damaging agents, PCNA is ubiquitylated on lysine-164 by the Rad6–Rad18 ubiquitin-conjugating complex (Hoege et al. 2002; Stelter and Ulrich 2003; Kannouche et al. 2004), and ubiquitin-modified PCNA recruits Y-family polymerases to replication forks. In the structure of ubiquitin-modified PCNA, the ubiquitin moiety sits on the back face of the PCNA ring (Freudenthal et al. 2010). In this section, we will discuss the interactions of Y-family polymerases with unmodified and ubiquitin-modified PCNA.

4.4.1 Interactions with Unmodified PCNA

Pol η, Pol ι, and Pol κ all possess one or more PCNA-interacting protein (PIP) motifs in their disordered C-terminal regions (Figs. 4.1 and 4.2). These motifs contain eight amino acid residues and are found in a large number of proteins that interact with PCNA (Tsurimoto 1999; Hingorani and O'Donnell 2000; Maga and Hubscher 2003). The fourth residue of the motif is hydrophobic (usually a leucine, an isoleucine, or a methionine) and the seventh and eighth residues are aromatic (usually phenylalanine or tyrosine). The X-ray crystal structures of PCNA bound to a variety of PIP motifs have been determined, and these motifs all bind on the front face of the PCNA ring near the inter-domain connector loop. The conserved hydrophobic and aromatic residues bind in a pocket at the interface of the two domains of PCNA. Structures of PCNA bound to the PIP motifs of Pol η and Pol ι have been determined (Hishiki et al. 2009). While the Pol η PIP motif binds to PCNA by forming the same 3_{10} helix that other PIP motifs form (Fig. 4.4a, b), the Pol ι PIP binds to PCNA by forming a novel β-bend structure. The significance of

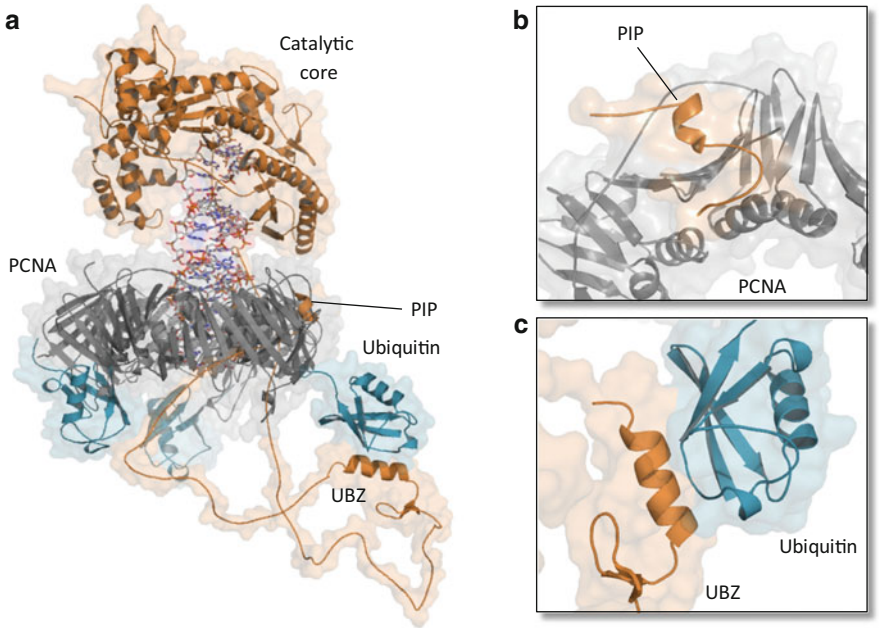

Fig. 4.4 Structural model of full-length Pol η bound to ubiquitin-modified PCNA. (**a**) The model of Pol η bound to ubiquitin-modified PCNA was built using Coot (Emsley and Cowtan 2004) starting with the X-ray crystal structures of the catalytic core region of Pol η (PDB ID: 3TQ1) (Ummat et al. 2012), ubiquitin-modified PCNA (PDB ID: 3L10) (Freudenthal et al. 2010), and PCNA bound to the Pol η-PIP motif (PDB ID: 2ZVK) (Hishiki et al. 2009) and with the NMR structure of the Pol η UBZ (PDB ID: 2I5O) (Bomar et al. 2007). (**b**) A close-up of the Pol η PIP motif bound to the PCNA portion of ubiquitin-modified PCNA is shown. (**c**) A close-up of the Pol η UBZ bound to the ubiquitin portion of ubiquitin-modified PCNA is shown

this unusual PIP conformation is unclear. It should be noted that a structure of PCNA bound to the Pol κ PIP has also been determined (Hishiki et al. 2009), but in this case, additional amino acid residues not found in Pol κ were added to the PIP construct to allow PCNA binding. The native Pol κ PIP does not seem to bind PCNA, so this particular structure is of limited value.

Purified Pol η, Pol ι, and Pol κ physically interact with unmodified PCNA (Haracska et al. 2001a–c, 2002c). Unlike the interactions between PCNA and classical polymerases, the interactions between PCNA and the Y-family polymerases do not substantially increase the processivity of DNA synthesis. Nevertheless, steady-state kinetics shows that interacting with PCNA significantly increases the catalytic efficiency of nucleotide incorporation by all three of these enzymes on both non-damaged and damaged templates. For example, in the case of Pol η, the increase in efficiency of incorporation opposite non-damaged DNA ranges from three- to tenfold and the increase in efficiency on a template abasic site, a non-cognate lesion, ranges from 3-fold to as much as 300-fold depending on experimental conditions (Haracska et al. 2001a, c; Freudenthal et al. 2008). These

physical and functional interactions with PCNA are dependent on intact PIP motifs. Moreover, in human cells, intact PIP motifs are required for both Pol η and Pol ι to localize to nuclear foci containing PCNA following DNA damage (Bienko et al. 2005; Acharya et al. 2008; Vidal et al. 2004). In the case of Pol η, there are two PIP motifs, named PIP1 and PIP2. Disruptions of the individual PIP motifs have only a moderate affect on localization to nuclear foci and Pol η-dependent TLS suggesting that the two PIP motifs are able to functionally substitute for one another. Simultaneous disruption of both PIP motifs, however, completely eliminates localization and Pol η-dependent TLS in vivo (Acharya et al. 2008).

Like the other Y-family polymerases, Rev1 physically interacts with PCNA (Guo et al. 2006; Wood et al. 2007), and this interaction stimulates the catalytic activity of Rev1 (Wood et al. 2007). Unlike these other polymerases, however, Rev1 does not contain a canonical PIP motif, and there has been some debate about the regions of Rev1 that are required to interact with PCNA. It has been reported that the localization of Rev1 to nuclear foci containing PCNA requires either the N-terminal half of Rev1 (residues 1–730) or the C-terminal half (residues 730–1251) (Tissier et al. 2004). Another report, however, showed that localization requires the C-terminal region of Rev1 (residues 826–1251), but not the N-terminal region (Murakumo et al. 2006). It has also been reported that the N-terminal BRCT domain of Rev1 is required for localization to foci in non-damaged cells but is not required in UV-treated cells (Guo et al. 2006). This too is controversial as another study failed to detect a direct interaction between PCNA and the Rev1 BRCT domain (de Groote et al. 2011). Moreover, the stimulation of Rev1's catalytic activity by PCNA does not require an intact BRCT domain (Wood et al. 2007). Thus questions remain regarding the structural basis of the PCNA–Rev1 interaction.

4.4.2 Interactions with Ubiquitin-Modified PCNA

All four Y-family polymerases possess one or more small ubiquitin-binding domains in their disordered C-terminal regions (Figs. 4.1 and 4.2). In the case of Pol η and Pol κ, these small domains are UBZs, which contain about 20 amino acid residues and form a short, two-stranded antiparallel β-sheet followed by an α-helix (Fig. 4.4a, c) (Bomar et al. 2007). Two conserved cysteine residues and two conserved histidine residues coordinate a zinc ion, which likely provides structural stability to this small domain. In the case of Pol ι and Rev1, these small domains are UBMs, which contain about 30 amino acid residues and form a helix-turn-helix motif (Bomar et al. 2010; Burschowsky et al. 2011). NMR titrations have shown that the UBZs and the UBMs interact in slightly different ways with the canonical protein–protein interaction surface of ubiquitin, which is made up of a conserved hydrophobic patch containing leucine-8, isoleucine-44, and valine-70. Neither the conformation of the ubiquitin nor the conformation of the ubiquitin-binding domains seems to change upon complex formation.

In vitro pull-downs using purified Rev1 have shown that this polymerase interacts with ubiquitin-modified PCNA with qualitatively higher affinity than it interacts with unmodified PCNA (Wood et al. 2007). We have quantitatively analyzed the interaction of the disordered C-terminal region of Pol η, which contains the PIP and UBZ motifs, with unmodified PCNA and ubiquitin-modified PCNA, and we find that the attachment of ubiquitin to PCNA enhances the binding affinity of Pol η by approximately 20-fold (unpublished data). In human cells, Pol η specifically interacts with ubiquitin-modified PCNA, but not unmodified PCNA. Immunoprecipitation of PCNA from normal cells pulled down only unmodified PCNA, whereas immunoprecipitation of PCNA from UV-irradiated cells pulled down ubiquitin-modified PCNA and Pol η. Moreover, localization of Pol η to nuclear foci and Pol η-dependent TLS require that the UBZ be intact (Bienko et al. 2005). Localization of Pol ι to foci requires that both UBMs be intact (Bienko et al. 2005; Bomar et al. 2010). Thus ubiquitin-binding domains are important for localization to nuclear foci.

The complex of Y-family polymerases and ubiquitin-modified PCNA is likely flexible. First, the PIP and ubiquitin-binding domains are located within large regions of the Y-family polymerases that are intrinsically disordered. Second, experimental evidence obtained using small-angle X-ray scattering (SAXS) and computational studies using Brownian dynamics simulations show that the ubiquitin moieties of ubiquitin-modified PCNA are dynamic (Tsutakawa et al. 2011). Nevertheless, while the ubiquitin moieties (still attached to lysine-164 of PCNA) are capable of moving around, they have preferred positions on the back face and the side of the PCNA ring. This is important as nearly all PCNA-binding proteins interact with the front face of PCNA. This suggests that Y-family polymerases can bind to the back or side of the PCNA ring without affecting ongoing activity of other proteins bound to the front face of PCNA. Thus the Y-family polymerases can be held in reserve on the back or side of PCNA until their activities are required. Then because of the flexible nature of this complex, they can move to the front face of PCNA and engage the primer-terminus of the DNA substrate. A model of Pol η bound to the DNA substrate on the front face of ubiquitin-modified PCNA is shown in Fig. 4.4a.

4.5 Other Interactions of Y-Family Polymerases

Y-family polymerases function within a dynamic network of protein–protein interactions. The interactions in this network, which are mediated by intrinsically disordered regions of the polymerases, govern their recruitment and regulation. In this network, ubiquitin-modified PCNA, Rev1, and (to a lesser extent) Pol η function as hub proteins, which interact with multiple binding partners (Fig. 4.5a). We discuss the interactions of Y-family polymerases with ubiquitin-modified PCNA above (see Sect. 4.4). In this section, we discuss the other interactions of Pol η and Rev1. We also discuss speculations that Pol η functions as a "first responder" during TLS and that Rev1 functions as a scaffold to recruit other nonclassical polymerases.

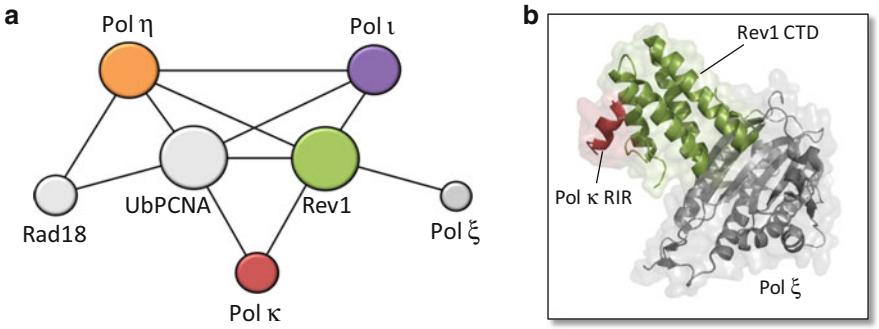

Fig. 4.5 The network of protein–protein interactions of Y-family polymerases. (**a**) The network diagram shows the known protein–protein interactions among Pol η, Pol ι, Pol κ, Rev1, ubiquitin-modified PCNA (Ub-PCNA), Rad18, and the B-family Pol ξ. The size of the *circles* is proportional to the number of contacts in the network. Ubiquitin-modified PCNA and Rev1 are hub proteins each contacting five other proteins in the network. (**b**) A structure of the Rev1 CTD bounds simultaneously to both the Pol κ RIR motif and the Rev7 non-catalytic subunit of Pol ξ (PDB ID: 4FJO) (Wojtaszek et al. 2012a)

4.5.1 Interactions of DNA Polymerase η

Pol η directly interacts with Rad18 (Watanabe et al. 2004), the E3 ubiquitin ligase that, along with Rad6 (the E2 ubiquitin-conjugating enzyme), catalyzes the ubiquitylation of PCNA in the presence of DNA damage. Rad18 binds to the intrinsically disordered C-terminal region of Pol η, and Rad18 and Pol η co-localize to nuclear foci following DNA damage (Watanabe et al. 2004). Moreover, cells expressing a mutant form of Rad18 that is unable to bind Pol η are defective in forming Pol η-containing nuclear foci and are sensitive to UV radiation (suggesting a defect in Pol η-dependent TLS). Moreover, Rad18 is phosphorylated in its Pol η-binding site in response to DNA damage, and this modification enhances the association of Pol η and Rad18 (Day et al. 2010; Barkley et al. 2012). These studies have led to a model in which Rad18 chaperones Pol η to PCNA. Then Rad18 (along with Rad6) catalyzes the ubiquitylation of PCNA and subsequently hands off Pol η to the ubiquitin-modified PCNA. Such a model would ensure that Pol η is the first Y-family polymerase to arrive at the stalled replication fork. Once there, Pol η could either catalyze nucleotide incorporation opposite the lesion or recruit other Y-family polymerases to the fork. While the idea that Pol η functions as a "first responder" during TLS is speculative, it does make sense given that the replication of TT dimers and 8-oxoguanines by Pol η actually reduces the mutagenic potential of these lesions.

Pol η also directly interacts with both Rev1 and Pol ι. Rev1 binds to a short motif containing approximately 10 amino acids located in the disordered C-terminal region of Pol η. This motif on Pol η is called the Rev1-interacting region (RIR), and it binds to the Rev1 CTD (Ohashi et al. 2004, 2009). The RIR motif of Pol η forms an α-helix upon binding to the CTD of Rev1 (see Sect 4.5.2 below)

(Pozhidaeva et al. 2012). Cells producing Pol η mutant proteins that are unable to interact with Rev1 showed defects in forming Rev1-containing foci (Akagi et al. 2009). Pol ι also interacts with Pol η, and the intrinsically unstructured C-terminal regions of both polymerases are involved in this interaction (Kannouche et al. 2002). In cells lacking Pol η, there is a significant reduction in the number of Pol ι-containing nuclear foci. Taken together, these studies suggest that interactions with Pol η are important for recruiting Rev1 and Pol ι to stalled replication forks.

4.5.2 Interactions of Rev1

In addition to its role as a polymerase, Rev1 also plays an essential non-catalytic role in TLS. Yeast cells producing a mutant form of Rev1 with an amino acid substitution in its BRCT domain have very low frequencies of UV-induced mutagenesis suggesting that error-prone TLS is not occurring in these cells (Lemontt 1971). This defect in TLS cannot be attributed to a decrease in the catalytic activity of Rev1 for two reasons. First, the mutant protein with a substitution in the BRCT domain retains a substantial amount of enzymatic activity (Nelson et al. 2000). Second, cells producing a catalytically inactive Rev1 mutant protein have nearly normal frequencies of UV-induced mutagenesis suggesting that error-prone TLS is occurring normally in these cells (Haracska et al. 2001d; Zhou et al. 2010; Otsuka et al. 2005). These results have led to the suggestion that Rev1 functions as a scaffold protein that mediates protein–protein interactions that are required for error-prone TLS.

The CTD of Rev1 has been shown to be essential for its non-catalytic role in TLS. This small domain is comprised of approximately 100 amino acid residues that bind Pol η, Pol ι, and Pol κ (Tissier et al. 2004; Ohashi et al. 2004; Guo et al. 2003). This domain interacts with short RIR motifs found in Pol η, Pol ι, and Pol κ that contain two conserved phenylalanine residues (Ohashi et al. 2009). Recent structures of the RIR motifs of Pol η and Pol κ bound to the Rev1 CTD showed that this domain forms a four-helix bundle, and when the RIR from another polymerase binds, it forms an α-helix that contacts two of the helices in the CTD (Wojtaszek et al. 2012a, b; Pozhidaeva et al. 2012). Expression of a Pol κ mutant protein that had substitutions in its RIR that blocked interactions with Rev1 was not able to rescue the decreased viability of a Pol κ-deficient cell line after induction of DNA damage (Ohashi et al. 2009). This suggests that the interaction with Rev1 is necessary for the function of Pol κ in vivo.

Rev1 also interacts with the non-catalytic Rev7 subunit of Pol ζ (Murakumo et al. 2001), a nonclassical B-family polymerase that functions in the extension step during the bypass of a wide range of DNA lesions. Interactions with Rev1 are necessary for the proper localization of Pol ζ to nuclear foci and for the enhancement of its catalytic activity in yeast (Acharya et al. 2006). Recent structural studies have shown that the Rev7 subunit of Pol ζ is able to interact with the Rev1 CTD via a linker region between two CTD α-helices (Wojtaszek et al. 2012a; Kikuchi

et al. 2012). Moreover, an X-ray crystal structure was determined for the complex containing the Rev7 subunit of Pol ζ, the Rev1 CTD, and the Pol κ RIR, demonstrating that the Rev1 CTD can bind multiple DNA polymerases simultaneously (Fig. 4.5b) (Wojtaszek et al. 2012a). Together, these data suggest that Rev1 plays a critical role in recruiting other TLS polymerases including Pol ξ and perhaps even Pol κ and Pol ι to stalled replication forks.

4.6 Concluding Remarks

The biochemical and structural studies reviewed here have provided tremendous insight into how Y-family polymerases differ from their classical counterparts and can efficiently incorporate nucleotides opposite DNA lesions. They have revealed some surprising strategies employed by Y-family polymerases to accommodate these lesions in their active sites. These strategies include having larger and less constrained active sites, forming Hoogsteen base pairs, and using amino acid side chains as templates. Unfortunately, far less is known about how Y-family polymerases are recruited to stalled replication forks and how their activities are coordinated on the DNA. Biochemical studies of the protein–protein interactions of these polymerases have begun to provide some insight into the recruitment and regulation of Y-family polymerases. It is becoming clear that these enzymes function within a dynamic network of protein–protein interactions in which ubiquitin-modified PCNA and Rev1 play critical roles. Moreover, these interactions are mediated largely via the intrinsically disordered regions of these polymerases. Exciting new breakthroughs will almost certainly emerge from further biochemical and structural studies of this network of protein–protein interactions.

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Chapter 5
DNA Polymerases That Perform Template-Independent DNA Synthesis

Anthony J. Berdis

Abstract DNA polymerases typically catalyze the incorporation of mononucleotides into a growing primer using a DNA or RNA template to properly guide each incorporation event. However, several members of the X-family of DNA polymerases are capable of replicating DNA in the complete absence of a templating strand. This form of template-independent DNA synthesis typically occurs during the repair of double-strand DNA breaks. In addition, there is one exceptional polymerase, denoted as terminal deoxynucleotidyl transferase, that exclusively replicates single-strand DNA rather than duplex DNA. This chapter describes the biological roles for the ability of these DNA polymerases to perform template-independent DNA synthesis during nonhomologous end joining. The biochemical mechanisms for how members of the X-family of DNA polymerases perform this type of replication are compared with other "conventional" DNA polymerases that perform template-dependent synthesis. Enzymatic steps encompassing the binding of DNA and dNTP substrates, the involvement of conformational changes that precede chemistry, and kinetic steps associated with product release are described. The influence of other cellular proteins on the activity of these DNA polymerases during nonhomologous end joining is discussed. Finally, the roles of these specialized DNA polymerases in pathological conditions such as cancer are described with a special emphasis on several new nucleoside analogs that function as therapeutic agents against these DNA polymerases.

Keywords DNA polymerases • Template-independent DNA synthesis • Immunology • Cancer • Chemotherapy

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Abbreviations

3-Eth-5-NIdR	3-Ethynyl-5-nitoindolyl-deoxyribose
3-Eth-5-NITP	3-Ethynyl-5-nitoindolyl-deoxyribose triphosphate
5-NITP	5-Nitroindoyl-deoxyribose triphosphate
AID	Activation-induced deaminase
BRCT	BRCA1 c-terminal domain
DNA-PKcs	Serine/threonine protein kinase that is stimulated by free DNA ends
HR	Homologous recombination
Ig	Immunoglobulin
NHEJ	Nonhomologous DNA end joining
PCNA	Proliferating cell nuclear antigen
PK	Protein kinase
Pol λ	DNA polymerase lambda
Pol μ	DNA polymerase mu
PP_i	Inorganic pyrophosphate
RAG-1	Recombination-activating gene 1
RAG-2	Recombination-activating gene 2
RSS	Recombination signal sequences
SHM	Somatic hypermutation
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
V(D)J	Variable diversity and joining
XLF	XRCC4-like factor (also called Cernunnos)

5.1 Introduction

DNA polymerases extend nucleic acid primers using a DNA template (or RNA during reverse transcription) to guide each nucleotide incorporation event (Fig. 5.1a). However, using a template is not a universal requirement for all DNA polymerases as there are several polymerases that can perform this process in the absence of templating information (Garcia-Diaz et al. 2005; Paull 2005; Bollum 1960). These include specialized DNA polymerases such as polymerase lambda (pol λ) and polymerase mu (pol μ) which can incorporate nucleotides at the blunt end of duplex DNA (Fig. 5.1b). In addition, there is another unique polymerase denoted as *t*erminal *d*eoxynucleotidyl *t*ransferase (TdT) that has the unusual ability to perform polymerization in a completely template-independent manner using only single-strand DNA as the substrate (Fig. 5.1c). At face value, performing DNA synthesis in the absence of any templating information appears to be a highly risky endeavor for the cell because of the implied risk of introducing genetic mutations into chromosomal DNA. Indeed, this is typically true for cells that respond to various DNA damaging agents. However, template-independent DNA

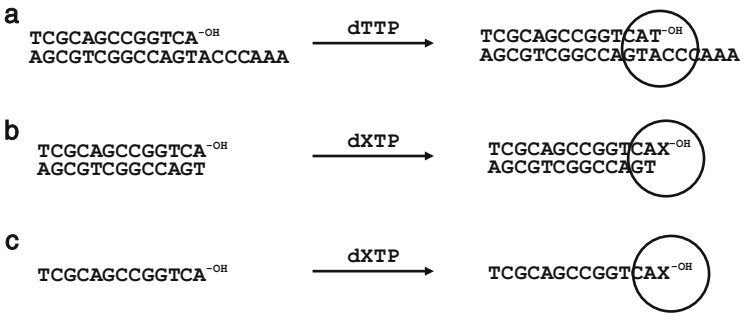

Fig. 5.1 Models for template-dependent and template-independent DNA synthesis. (**a**) Conventional DNA polymerases involved in replicating genomic DNA require double-stranded DNA as a substrate in which the 5′ → 3′ strand is used as a primer and the complementary strand 3′ → 5′ is used as a template. (**b**) Members of the X-family of DNA polymerases including pol λ and pol μ can perform template-independent DNA synthesis using blunt-end DNA as the substrate. (**c**) Terminal deoxynucleotidyl transferase is unique in its ability to catalyze phosphoryl transfer in the absence of a template that cannot be accommodated in its active site

synthesis catalyzed by TdT, for example, is very important for generating random mutations in specific regions of DNA during V(D)J recombination (Kepler et al. 1996; Kunkel et al. 1986). By subtly randomizing genetic material, TdT plays a key role in the maturation of the vertebrate immune system (Komori et al. 1993; Bertocci et al. 2006a; Kallenbach et al. 1990; Landau et al. 1987a). In fact, randomly incorporating nucleotides during V(D)J recombination serves as a viable way to increase antigen receptor diversity to produce approximately 10^{14} different immunoglobulins and 10^{18} unique T-cell antigen receptors that can neutralize potential antigens (Sadofsky 2001; Janeway 1999).

This chapter describes the cellular roles for template-independent DNA synthesis catalyzed by three distinct DNA polymerases including pol λ, pol μ, and TdT. Particular emphasis is placed on TdT as this enzyme plays an important role during V(D)J recombination. The molecular mechanisms responsible for the unique activities of these specialized DNA polymerases are also described. These discussions include the use of the reported structure of TdT which provides important insights into several properties of the polymerase such as recognition of nucleic acid and nucleotide substrates as well as the utilization of various metal ion cofactors. Finally, the biomedical importance of these specialized DNA polymerases are discussed in the context of pathological conditions including leukemia and Merkel cell carcinoma.

5.2 Why Is Template-Independent DNA Synthesis Necessary?

Double-strand breaks (DSBs) arise in dividing cells about ten times per cell per day (reviewed in Ohnishi et al. 2009). DSBs are formed by several mutually exclusive mechanisms including replication across nicked DNA, damage from free radicals caused by oxidants or ionizing radiation, and by the inadvertent action of enzymes involved in DNA metabolism. The two major pathways involved in repairing DSBs are homologous recombination (HR) and nonhomologous end joining (NHEJ) (Kass and Jasin 2010). Of the two pathways, HR is considered to be more faithful since it requires that there be sequence identity in regions that undergo recombination. However, the repair capacity of HR is limited as it occurs only during S-phase of the cell cycle and typically requires that there be hundreds of base pairs of homology in order to be fully efficient. The more commonly used pathway is NHEJ which can repair a DSB at any time during the cell cycle. In addition, when two DSBs occur on different chromosomes, their rejoining is almost always performed by NHEJ. This ability reflects the fact that NHEJ does not require significant amounts of sequence homology, although a few nucleotides of terminal microhomology are often utilized by the NHEJ enzymes. The proteins involved in NHEJ include Ku, DNA-PKcs, Artemis, pol μ, pol λ, XLF (aka Cernunnos), XRCC4, and DNA ligase IV.

5.2.1 The X-Family of DNA Polymerases

Mammals possess four DNA polymerase X-family members that include pol β, pol μ, pol λ, and TdT (Yamtich and Sweasy 2010) and Chap. 2. Pol β is a single polypeptide with an apparent molecular weight of 39 kDa that primarily functions during base excision repair. Pol β possesses both template-dependent polymerase activity and deoxyribose phosphate (dRP) lyase activity (Matsumoto and Kim 1995). Pol λ is most closely related to pol β as both possess dRP lyase activity (Aoufouchi et al. 2000; Nagasawa et al. 2000). In addition, pol λ can substitute for pol β in single-nucleotide base excision repair reactions, at least under in vitro conditions (Braithwaite et al. 2005). Pol μ participates in the resynthesis of missing nucleotides during NHEJ repair of DNA breaks (Ruiz et al. 2001). While pol μ can replicate normal DNA, it also has the ability to incorporate nucleotides opposite damaged DNA. With respect to primary amino acid sequence, pol μ and TdT are approximately 40 % identical to each other and approximately 20 % identical to other Pol X-family members including pol β (Domínguez et al. 2000; Yang 2003). On the other hand, TdT is a very specialized DNA polymerase that adds random nucleotides to DNA ends during V(D)J recombination. TdT activity is generally confined to B- and T-cells and is used to generate diversity during an immune response. During V(D)J recombination, TdT and pol μ participate during heavy

chain rearrangements, while pol λ is believed to function only during light chain rearrangements of antibodies.

5.2.2 *Polymerase Activity During Nonhomologous End Joining*

The relatively high degree of similarity between TdT and pol μ suggests that both polymerases might interact with other core end-joining proteins when processing DSBs (Nick McElhinny et al. 2005). However, their contrasting activities would produce opposite effects on genomic fidelity. For example, the function of TdT is to produce diversity in genomic material during V(D)J recombination, while the template-dependent activity of pol μ should maintain genomic fidelity by allowing accurate gap-filling DNA synthesis during DSB repair. Although pol μ is efficient during alignment-based gap fill-in synthesis in vitro (García-Díaz et al. 2000a; Lee et al. 2004), the polymerase is also prone to frameshift synthesis (Zhang et al. 2001) and is unable to perform strand displacement DNA synthesis (Lee et al. 2004). Ex vivo data also suggests that pol μ can participate in general DSB repair. This is evident as cells exposed to DNA damaging agents show higher levels of pol μ expression and co-localization with γH2AX, a biochemical marker associated with DSB formation (Lee et al. 2004). This is in contrast with the activity of pol β, which does not associate with end-joining factors nor performs alignment-based gap fill-in synthesis (García-Díaz et al. 2000a; Lee et al. 2004).

Pol λ, another Pol X-family member, is similar to TdT and pol μ as all three have an amino-terminal BRCT domain (conserved C-terminal domain in BRCA1) and a carboxy-terminal catalytic domain (Fig. 5.2). The BRCT domain is an important structural feature as it mediates protein/protein and protein/DNA interactions during DNA repair pathways and cell cycle checkpoint regulation. However, the role of this domain in coordinating polymerase activity during DNA repair is still poorly understood. For example, while pol λ can substitute for pol β during in vitro base excision repair reactions (García-Díaz et al. 2001), it is not clear if pol λ performs this function in vivo. Despite this uncertainty, the dRP lyase activity of pol λ has been proposed to participate in end-joining reactions as a way to deal with "abortive" DSB intermediates that form during base excision repair of radiation damage (Bebenek et al. 2003). Mechanistically, pol λ is similar to pol μ as both are prone to frameshift DNA synthesis (Bebenek et al. 2003) and neither performs strand displacement DNA synthesis (Capp et al. 2006).

Depletion of pol λ can block the end joining of substrates that require gap-filling synthesis in cell extracts (Bertocci et al. 2002). However, a deficiency in pol λ activity does not influence the efficiency of end-joining during V(D)J recombination (Maga et al. 2005). In contrast, the efficiency of V(D)J recombination is affected by deficiencies in pol μ activity. Collectively, these results suggest that the cellular role of pol μ is limited to V(D)J recombination while pol λ functions

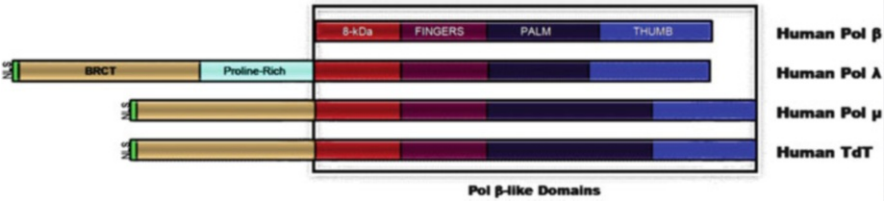

Fig. 5.2 Schematic representations of the different domains found in the four X-family DNA polymerases. Each domain is labeled and colored for clarity. *NLS* represents the nuclear localization signal motif, and *BRCT* indicates the BRCA1 carboxy terminus domain

almost exclusively during general DSB repair. The exact details of how various X-family members interact with end-joining factors to coordinate their activities remain unclear. Current models suggest that TdT, pol μ, and pol λ are recruited to DSBs by interactions of their BRCT domains with ligase IV. Competition amongst these polymerases has only been observed between pol μ and TdT (Nick McElhinny et al. 2005). The details of these studies are described later as they occur only during V(D)J recombination.

Another important consideration for regulating polymerase activity during DSB repair is the availability of dNTP substrates. Since dNTPs are at their highest concentration during the S-phase of the cell cycle, most template-dependent polymerases are highly active during this stage (Bjursell and Skoog 1980). However, most DSB repair occurs by NHEJ outside of S-phase. In fact, most repair takes place during the G1 phase of the cell cycle (Takata et al. 1998) when dNTP pools are significantly lower (McCormick et al. 1983). Thus, polymerases that participate in NHEJ face a significant challenge in performing DNA synthesis since the availability of nucleotide substrates may be a limiting factor. However, the polymerases involved in NHEJ appear to use extraordinary means to overcome low dNTP levels. For example, pol λ has an unusually high affinity for dNTPs as the K_d for dATP is ~30-fold lower than that for any other X-family member (García-Díaz et al. 2000b; Ruiz et al. 2003). This higher binding affinity means that pol λ can remain active even when dNTP levels are very low. Pol μ and TdT appear to employ an alternative strategy by using rNTPs rather than dNTPs. While most template-dependent polymerases display a 1,000-fold preference for dNTPs over rNTPs, both pol μ (Roychoudhury and Kössel 1971) and TdT (Boulé et al. 2001) show little discrimination between either substrate. This lack of discrimination is thought to occur due to the absence of amino acids that can function as a "steric gate" to prevent the utilization of rNTPs. In any event, it may be advantageous for these polymerases to use either ribo- or deoxyribose nucleotides since rNTP pools typically remain high throughout the cell cycle (Traut 1994). As such, the ability to incorporate rNTPs could allow pol μ and TdT to temporarily repair DSBs formed outside of the S-phase of the cell cycle where dNTP levels are precariously low. This "temporary fix" would allow the cell to survive rather than undergo cell death via apoptosis.

5.2.3 The Involvement of DNA Polymerases During V(D)J Recombination

The T- and B-cells of the adaptive immune system function to mount a rapid and robust protective response against foreign entities present in the systemic circulation. This is achieved by expanding the number of pathogen-specific T-cells after an antibody receptor binds an antigen (Papermaster et al. 1964). Once binding of an antigen and activation occurs, thousands of clones are generated over the course of a week to produce effector functions (Landau et al. 1987b; Lieber et al. 1988; Bertocci et al. 2006b). While the vast majority (90–95 %) of these activated T-cells undergo apoptosis (Haeryfar et al. 2008), a small population of T-cells persist in lymphoid and non-lymphoid tissues (Benedict et al. 2000; Baltimore 1974), and this population of cells scans the body for previously encountered pathogens. The impressive immunological memory displayed by the adaptive immune system provides long-term protection against subsequent infection, and this effect can last for several decades.

To increase acquired immunity against biological and chemical antigens, B- and T-cells use a genetic strategy known as V(D)J recombination (Beutler 2003). V(D)J recombination, also known as somatic recombination, is a mechanism for recombining genetic fragments during the initial stages of immunoglobulin and T-cell receptor production of the vertebrate immune system. This process occurs only in primary lymphoid tissue (bone marrow for B-cells and the thymus for T-cells). During this process, the variable (V), diversity (D), and joining (J) gene segments are randomly rearranged to increase the number and diversity of antigen receptors. By creating unique antibodies with distinct antigen specificity, this process creates a highly versatile and competent immune system (Schatz et al. 1992). The ability to cleave, rearrange, and rejoin the V, D, and J regions of germline immunoglobulin genes requires the concerted efforts of the three distinct enzyme activities that include nucleases, polymerases, and ligases (Fig. 5.3).

The early steps of V(D)J recombination occur during the RAG (recombination-activating gene) cleavage phase. This process is initiated by the introduction of a DSB at the edge of the selected gene segment and is catalyzed by the *RAG-1* and *RAG-2* proteins which selectively bind to specific recombination signal sequences (RSS) containing heptamer and nonamer elements with 12 or 23 bp spacer regions in between (Schatz 2004; Early et al. 1980). Recognition of complementary RSS allows the *RAG* complex to introduce a nick between the D and J coding segment as well as with the adjoining recombination signal sequence. The RAG complex also catalyzes the formation of hairpins at each coding end. This occurs using the 3′-OH group at each nick as the nucleophile to catalyze the reaction.

DSBs introduced during the RAG cleavage phase are subsequently repaired during NHEJ (Fig. 5.3). This second stage depends upon the activity of the Artemis:DNA-PK_{cs} complex which functions as a nuclease to trim 5′ and 3′ overhangs (Steen et al. 1996). This activity opens the hairpins present at the coding ends of the gene segment to produce palindromic nucleotides (P-nucleotides). TdT

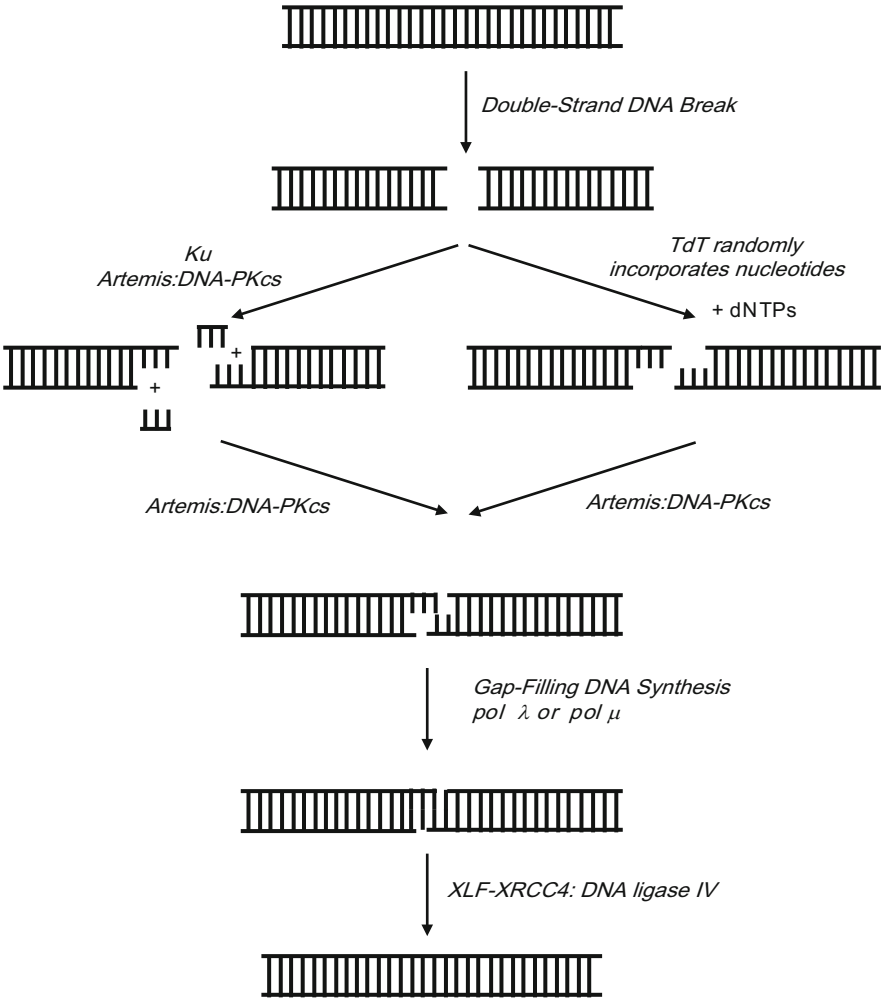

Fig. 5.3 Overview of V(D)J recombination. A simplified model for the RAG cleavage phase generating double-strand breaks and DNA repair through nonhomologous end-joining pathway during DJ gene segment assembly of the V(D)J recombination mechanism. Simplified overview of the enzymatic steps and the role of terminal deoxynucleotidyl transferase in lymphocyte gene rearrangement. The variability of the recombined gene segments is increased through the random addition of non-templated (N) nucleotides catalyzed by the terminal deoxynucleotidyl transferase prior to complementary pairing and extension by template-dependent DNA polymerases

participates at this stage by randomly incorporating nucleotides to an available 3′-OH. TdT is a template-independent DNA polymerase that is expressed only in T- and B-cells that are cells active in V(D)J recombination. This biological activity is designed to increase the diversity of the antigen receptor repertoire by mediating N-addition (nongermline encoded sequence addition) in receptor junctions. It is

likely that TdT activity is solely linked to V(D)J recombination as its expression pattern is restricted to only cells that are active in V(D)J recombination. Highly restricted expression of TdT is necessary since its template-independent activity would undoubtedly produces mutagenic consequences during general DSB repair in other cell types. As described later, certain forms of cancer show altered expression levels of TdT that correlate with pathophysiological cellular activity.

In vitro studies have shown that TdT can utilize all four natural dNTPs (Bollum 1960; Boulé et al. 2001). However, in vivo studies show a distinctive bias for preferential incorporation of dGTP and dCTP versus dATP or dTTP (Mickelsen et al. 1999; Feeney 1990; Bangs et al. 1991; Basu et al. 1983; Coleman et al. 1974a; Bollum 1979a; Cabaniols et al. 2001). This preference offers a molecular mechanism to explain the high G/C content that is present in immunoglobulin (Ig) and T-cell receptor (TCR) N-regions (Mickelsen et al. 1999). In addition, the average length of the N-nucleotide segment created by TdT is between 2 and 5 base pairs per coding joint (Bollum 1979a). While this length appears short, it is sufficiently long enough to allow the extended DNA strands to anneal using microhomology alignment via Watson–Crick base-pairing. Again, the role of TdT to increase immunological diversity has been also been validated by the near exclusive expression and localization of this polymerase in primary lymphoid tissues including thymus and bone marrow (Coleman et al. 1974a; Bollum 1979a). Furthermore, TdT-knockout mice display tenfold reductions in TCR diversity compared to wild-type mice (Cabaniols et al. 2001), further validating the role of this specialized polymerase in V(D)J recombination.

The last stage of V(D)J recombination requires the action of the Artemis:DNA-PK_{cs} complex which excises any unpaired bases that form during the annealing process (Ma et al. 2002). Gaps that exist in the recombined section of DNA are filled in by template-dependent DNA polymerases, including X-family members such as pol λ, pol μ, or replicative polymerases such as pol δ or ε. Ligation of the coding ends is performed by the XRCC4-DNA ligase IV complex (Sibanda et al. 2001).

Recently, it was shown that competition between TdT and pol μ is a biologically relevant reaction with important ramifications on efficient immune function. In this case, overexpression of pol μ leads to a reduction in the frequency of TdT-dependent N-addition in a plasmid-based assay for V(D)J recombination (Bertocci et al. 2003). In addition, mice lacking pol μ have a mild B-cell deficiency apparently arising as a result of deletions in V(D)J recombination sites at immunoglobulin light chain loci (Bertocci et al. 2003).

5.3 Terminal Deoxynucleotidyl Transferase

Chapter 2 in this book discusses the mechanism of other X-family polymerase members including pol λ and pol μ that can also perform template-independent DNA synthesis. As such, the remaining focus of this chapter will be on the mechanism and regulation of TdT activity.

5.3.1 Sources and Purification of TdT

There are numerous complexities in the number of different TdT isoforms that are present in mammalian systems. Discussions here are limited to human (*h*) TdT isoforms. Humans possess three alternative splice variants designated as *h*TdTS (short), *h*TdTL1 (long), and *h*TdTL2 (long) (Takahara et al. 1994; Thai and Kearney 2004, 2005). Although *h*TdTL1 and *h*TdTL2 both localize in the nucleus, *h*TdTL2 is expressed more abundantly in normal lymphocytes compared to *h*TdTL1 (Thai and Kearney 2005). The long isoforms of human TdT are both reported to possess $3' \rightarrow 5'$ exonuclease activity for nucleotide removal whereas the short isoform performs nucleotide elongation of the coding ends during V(D)J recombination (Takahara et al. 1994; Thai and Kearney 2004, 2005).

Overexpression of either *h*TdTS or *h*TdTL2 reduces the efficiency of V(D)J recombination greatly (Thai and Kearney 2005) while simultaneous overexpression of *h*TdTS and *h*TdTL2 results in normal recombination frequencies. In addition, the presence of all three human TdT variants during antigen receptor gene rearrangement drastically diminishes recombination frequencies. These results collectively suggest that *h*TdTL1 serves to modulate the activities of either *h*TdTL2 or *h*TdTS to maintain proper recombination.

TdT has been purified from a variety of different sources including calf thymus glands and cultured cell lines propagated from patients with acute lymphoblastic leukemia (Chang and Bollum 1971; Deibel and Coleman 1980a, b; Chang et al. 1982; Bollum and Chang 1981; Nakamura et al. 1981). In its purified form, hTdTL2 (designated from this point on simply as TdT) is a monomeric protein with a molecular weight of approximately 58 kDa (Nakamura et al. 1981). In general, large quantities of human TdT can be purified from cultured cells. However, this approach is generally cost prohibitive for most laboratories. To circumvent this complication, several attempts have been made to overexpress TdT in bacterial systems (Peterson et al. 1985; Boule et al. 1998). These efforts have generally been met with limited success, and the associated failures are attributed to differences in codon frequencies and tRNA pools in *Escherichia coli* versus eukaryotes as well as the low solubility of expressed TdT in these bacterial systems (Peterson et al. 1985; Boule et al. 1998). The production of soluble and active forms of TdT have been achieved by overexpressing a rare *argU* tRNA in the *E. coli* system coupled with growing cultures at a lower temperature (15 °C) to optimize protein folding (Boule

et al. 1998). Recombinant human TdT has also been overexpressed using a baculovirus expression system (Chang et al. 1988).

5.3.2 *Enzymatic Properties of TdT*

The ability of TdT to incorporate nucleotides in a template-independent manner has been investigated by several different research groups (Karkow and Kamen 1966; Kato et al. 1967; Chang et al. 1972; Roychoudhury 1972; Chang and Bollum 1980). In most reports, the template-independent activity of TdT was distinguished from that of template-dependent DNA polymerases by measuring polymerization activity with single-strand versus double-strand DNA. Under in vitro conditions, the replication of homopolymers by TdT requires an initiator chain of six or more nucleotides for poly(dA) and more than five nucleotides for poly(dT) (Kato et al. 1967). Interestingly, inactivating the exonuclease domain of the archaeal B-family DNA polymerase from *Sulfolobus solfataricus* results in the ability of this polymerase to robustly extend short single-strand DNA into several thousand bases using a hybrid mechanism of template-dependent and independent activities (Zuo et al. 2011). TdT, however, proves to be a unique DNA polymerase as it can catalyze the de novo synthesis of polynucleotides ranging in size from 2- to 15-mers when provided with dNTPs in absence of a primer (Chang et al. 1972). As indicated earlier, TdT can also utilize rNTPs, under in vitro conditions. In these instances, TdT can elongate a primer containing a 3′-terminal ribonucleotide 5′-monophosphate (rNMP) (Roychoudhury 1972). However, the addition of rNMPs to a DNA template can significantly impair the kinetics of elongation. For example, the addition of more than two rNMPs does not occur, and this lack of activity may reflect the ability of single-strand RNA to form secondary structures that resemble duplex DNA. As discussed earlier, most template-dependent polymerases utilize dNTPs 1,000-fold more efficiently than rNTPs. The ability of TdT to show little discrimination against rNTPs as potential substrates may have important ramifications for temporarily repairing DSBs under in vivo conditions.

Like all other DNA polymerases, TdT requires divalent metal ions as cofactors to catalyze nucleotide incorporation. However, TdT is again unique amongst all polymerases as it can perform polymerization with a wide number of different divalent cations including Co^{2+}, Mn^{2+}, Zn^{2+}, and Mg^{2+} (Chang and Bollum 1980). Under in vitro conditions, each metal ion produces a different effect on the efficiency and kinetics of nucleotide incorporation. For example, the presence of Mg^{2+} leads to the preferential utilization of dGTP and dATP whereas Co^{2+} leads to the preferential utilization of the pyrimidines, dCTP, and dTTP (Chang and Bollum 1980). Zn^{2+} behaves as a unique cofactor as it functions as a positive allosteric effector for TdT. This is based on experiments demonstrating that polymerization rates by TdT in the presence of Mg^{2+} are stimulated by the addition of micromolar quantities of Zn^{2+} (Chang and Bollum 1980). This rate enhancement could be caused by Zn^{2+} producing different conformational changes in TdT, each of

which displays higher intrinsic polymerization efficiencies (Chang and Bollum 1980). However, further work is needed to verify this proposal. Surprisingly, polymerization rates are lower in the presence of Mn^{2+} compared to Mg^{2+} (Deibel and Coleman 1980b). This is intriguing since opposite effects are observed with most template-dependent DNA polymerases. Indeed, Mn^{2+} has been shown by several groups to be a highly pro-mutagenic metal ion as its presence has a negative effect on polymerization fidelity, increasing both the frequency and rate of misincorporation events (Beckman et al. 1985).

5.3.3 Kinetic Mechanism of TdT

5.3.3.1 Order of Substrate Binding

Template-dependent DNA synthesis is achieved through the strictly ordered binding of DNA substrate to the polymerase prior to the binding of dNTP. From a biological perspective, the binding of DNA before dNTP is intuitively obvious as this order allows the polymerase to "sample" each dNTP prior to making a commitment to incorporate the correct nucleotide. The alternative scenario, a prerequisite binding of dNTP prior to DNA, is unattractive since this would allow the polymerase to bind the "correct" complementary nucleotide only 25 % of this time. This mechanism would be highly inefficient for cellular replication. In fact, forcing a polymerase to bind a dNTP substrate first often causes the formation of a dead-end complex that reduces polymerization efficiency. For example, the use of exceedingly high concentrations of dNTPs (>1 mM) can produce substrate-induced inhibition that is likely caused by the inappropriate binding of dNTP prior to DNA substrate (Zhang et al. 2011). However, TdT again appears to be the lone exception to this general rule as the order for substrate binding follows a rapid-equilibrium random mechanism as determined by initial velocity studies in the absence and presence of product inhibitors (Deibel and Coleman 1980b). There are several important ramifications for the ability of TdT to randomly bind substrates. Since the relative "lifetime" of blunt-end DNA formed during V(D)J recombination is likely to be very short, "preloading" TdT with a dNTP could increase the efficiency of template-independent synthesis and aid to produce random mutations. Alternatively, potential interactions with PCNA and Ku70/80, both of which participate in recombination, could also affect the kinetic mechanism of TdT (Hoek et al. 2011). For example, interactions with the clamp protein, PCNA, could force TdT to be more stably associated with DNA substrate and thus mandate an ordered mechanism for DNA binding prior to dNTP selection.

5.3.3.2 Conformational Changes During Catalysis

During template-dependent DNA synthesis, most DNA polymerases undergo at least one enzymatic conformational change after the binding of nucleotide to the polymerase-DNA complex. The existence of these conformational changes has been demonstrated using various kinetic, structural, and spectroscopic techniques (Echols and Goodman 1991; Kiefer et al. 1998; Hogg et al. 2004; Bloom et al. 1993; Hariharan and Reha-Krantz 2005; Johnson 1993; Dunlap and Tsai 2002). While it is clear that conformational changes occur during catalysis, there is still much debate regarding their role and importance during DNA polymerization. For example, conformational changes have been proposed to be a key step in maintaining replication fidelity by aligning the incoming dNTP into a precise geometrical shape with the templating nucleobase. In this model, proper alignment allows for efficient phosphoryl transfer. In addition, this step imposes discrimination against the misinsertion of an incorrect nucleotide as the geometry of the polymerase's active site perturbed to inhibit efficient phosphoryl transfer (Johnson 1993).

Since TdT uses only single-strand DNA, it is unclear if this template-independent DNA polymerase undergoes a rate-limiting conformational change to achieve efficient polymerization. This represents an important question since, as outlined above, this conformational step is often associated with a kinetic control point that plays a pivotal role in maintaining polymerization fidelity. Since TdT is designed to randomly incorporate nucleotides, it should not be limited by constraints imposed by fidelity. Thus, the lack of "faithful" polymerization displayed by TdT should negate any need for a conformational change step. As such, the phosphoryl transfer should be the rate-limiting step for nucleotide incorporation. Initial velocity studies performed by Coleman's group (Deibel and Coleman 1980b) provided evidence that TdT possesses a rapid-equilibrium random kinetic mechanism, a result consistent with phosphoryl transfer being the rate-limiting step for enzyme turnover. Indeed, similarly mechanistic deductions have been reported for "error-prone" polymerases that have lower constraints in fidelity (Dunlap and Tsai 2002).

5.3.3.3 Product Release

After nucleotide incorporation, template-dependent polymerases show an obligatory release in products in which pyrophosphate is first product to be released. Following pyrophosphate release, the polymerase can either remain bound to DNA and continue primer elongation (processive DNA synthesis) or dissociate from the extended primer to reinitiate DNA synthesis on another usable primer (distributive DNA synthesis). Again, since TdT follows a rapid-equilibrium random kinetic mechanism, it is likely that it uses a distributive mode for replication rather that a processive mechanism. While this mechanism is reasonable, it has yet to be

conclusively established especially within the context of other interacting recombination proteins such as PCNA and Ku70/80.

5.3.4 Mechanism of Nucleotide Selection

With most DNA polymerases, the binding of a dNTP is highly influenced by steric constraints and hydrogen-bonding interactions imposed by the presence of a templating strand (Beckman and Loeb 1993; Kool 2002; Lee and Berdis 2010). However, the molecular details regarding nucleotide binding and selection by TdT remain elusive since this polymerase does not rely on a templating strand for polymerization activity. At face value, the non-reliance on coding information predicts that TdT would utilize all four dNTPs with equal efficiency. This mechanism appears unlikely as work from the Coleman and Modak laboratories have independently demonstrated that TdT shows an unequal bias for incorporating nucleotides (Deibel and Coleman 1980b; Modak 1978). For example, TdT binds dGTP with a ~4-fold higher affinity than dATP (compare K_m values of 120 μM versus 540 μM, respectively) (Deibel and Coleman 1980b; Modak 1978). In addition, it was more recently demonstrated that recombinant TdT utilizes dGTP, dCTP, and dTTP much more efficiently than dATP (Berdis and McCutcheon 2007). Collectively, these studies indicate that TdT actively discriminates against utilizing dATP. At the molecular level, this could be achieved by the use of hydrogen-bonding information present on amino acids in the active site of TdT. This type of "negative selection" mechanism has been documented by various DNA polymerases (Urban et al. 2010; Beckman et al. 2007) and would be similar to that displayed by Rev1, an error-prone DNA polymerase that preferentially incorporates dCTP via direct interactions with an active site arginine (Nair et al. 2005). At the cellular level, "negative" selection against dATP could be necessary since the intracellular concentration of dATP is higher than that for the other three natural dNTPs. As a result, active discrimination against dATP utilization would compensate against a higher concentration of this nucleotide to ensure randomization. An alternative mechanism is that TdT may preferentially incorporate nucleotides that form complementary base pairs (G/C or A/T) which are predicted to facilitate annealing during recombination. Complementary pairing combinations of G and C are predicted since they would form more thermodynamically stable base pairs.

Several groups have used various nonnatural nucleotides lacking hydrogen-bonding functional groups to probe their importance for nucleotide recognition by TdT (Berdis and McCutcheon 2007; Arzumanov et al. 2000; Sosunov et al. 2000; Krayevsky et al. 2000a; Horáková et al. 2011; Jarchow-Choy et al. 2011; Motea et al. 2012). One recent example is the recent demonstration that TdT incorporates nonnatural analogs such as 5-nitroindolyl-2′-deoxyriboside triphosphate (5-NITP) (Motea et al. 2012). In this case, 5-NITP is incorporated by TdT with an overall catalytic efficiency equal to dGTP. These data suggest that the primary molecular

determinant for nucleotide binding is the triphosphate moiety of the incoming nucleotide which can favorably interact with positively charged amino acids lining the active site of TdT. However, this mechanism cannot fully account for productive binding interactions since it cannot adequately explain the significant differences observed in kinetic parameters for the utilization of various natural and nonnatural nucleotides (Berdis and McCutcheon 2007; Motea et al. 2012). In addition, replacement of an active site arginine residue (R336) that interacts with the triphosphate moiety of a dNTP with either glutamine (R336Q) or alanine (R336A) reduces the binding affinity for dGTP and dATP by only tenfold (Yang et al. 1994). This reduction indicates that ionic interactions between active site residues and the triphosphate of an incoming dNTP are important for binding. However, they are not essential for catalysis.

5.4 Structural Insights into Template-Independent DNA Synthesis

5.4.1 Primary Amino Acid Sequence Information

Sequence alignment of the C-terminus of the X-family DNA polymerases shows that they all possess fingers, palm, and thumb subdomains that are universally associated with members of the A-, B-, Y-, and RT-families of DNA polymerases (reviewed in Brautigam and Steitz 1998). TdT, pol λ, and pol μ are distinct from pol β as their N-terminal domain contains nuclear localization signal motifs as well as the breast cancer susceptibility protein BRCA1 C-terminal (BRCT) domains (Rodriguez and Songyang 2008) (Fig. 5.2). The BRCT domain typically functions to mediate protein/protein and protein/DNA interactions during DNA repair pathways and cell cycle checkpoint regulation. In addition, the BRCT domain of TdT, pol λ, and pol μ may interact with Ku70/86, a heterodimeric protein involved in recognizing and binding the ends of DSBs formed during V(D)J recombination (Morozov and Wawrousek 2008).

A more thorough comparison of the primary amino acid sequences reveals that these polymerases have very little overall identity with each other. In fact, while TdT and pol μ are the most closely related members of this family, they only share 42 % amino acid identity. Despite this low identity, TdT and pol μ appear to share a common ancestry as their template-independent activity coincides with the development of V(D)J recombination in mammals (Bartl et al. 2003).

5.4.2 Tertiary Structure of TdT

The structures of all template-dependent DNA polymerases characterized to date reveal a common molecular architecture resembling a right hand containing thumb, fingers, and palm subdomains (Fig. 5.4a) (reviewed in Morozov and Wawrousek 2008). The palm subdomain is viewed as the catalytic core since this is where the phosphoryl transfer reaction occurs. In the case of TdT, this subdomain contains three carboxylic acid amino acids, two of which are highly conserved amongst all DNA polymerases (Steitz 1999). These carboxylic amino acids coordinate metal ions that act as Lewis acids to lower the activation energy barrier needed for efficient phosphoryl transfer (Steitz and Steitz 1993). With template-dependent DNA polymerases, the fingers subdomain plays a large role in coordinating interactions between the templating base and the incoming dNTP. This subdomain mediates the conformational change step that allows for proper alignment of the nucleobases prior to phosphoryl transfer. Finally, the thumb subdomain of most template-dependent DNA polymerases serves a dual role by positioning duplex DNA for accepting the incoming dNTP as well as for polymerase translocation to the next templating base position after phosphoryl transfer.

The structure of TdT (Fig. 5.4a) shows remarkable similarities to template-dependent DNA polymerase as it also contains thumb, fingers, and palm subdomains. This model shows the presence of two Co^{2+} ions in the palm domain, and this stoichiometry of 2 is consistent with the "two divalent metal ion" mechanism proposed for phosphoryl transfer (Delarue et al. 2002). Finally, these metal ions are coordinated by the oxygens of the triphosphate moiety of the incoming dNTP as well as by three conserved aspartate residues.

Despite these similarities, however, there are several structural variations in TdT that distinguish it from conventional template-dependent polymerases. For example, TdT possesses a "lariat-like loop" that is not present in the structures of conventional template-dependent DNA polymerases (Fig. 5.4a) (Delarue et al. 2002). Superimposing the structure of TdT with the closed form of pol λ shows that the 16 amino acids forming the "lariat-like loop" in TdT would hinder the polymerase from interacting with duplex DNA. This unique structural element provides the physical basis for why TdT only replicates single-strand DNA. In addition, TdT contains an 8 kDa domain, referred to as the "index finger" domain that contacts the thumb subdomain to form a channel allowing dNTPs to diffuse into the enzyme's active site. Based on this structural model, Delarue and colleagues proposed that TdT bound with either DNA or ddATP resembles the "closed conformation" of pol β complexed with DNA and nucleoside (Delarue et al. 2002). This type of "closed" complex suggests that TdT can incorporate nucleotides without requiring a conformational change step. However, further structural and kinetic studies are needed to truly validate this proposal.

Despite having binary complexes of TdT bound with ddATP (PDB ID code 1KEJ or DNA [PDB ID code 1KDH (Delarue et al. 2002)], the molecular details accounting for nucleotide selectivity and specificity remain elusive. Inspection of

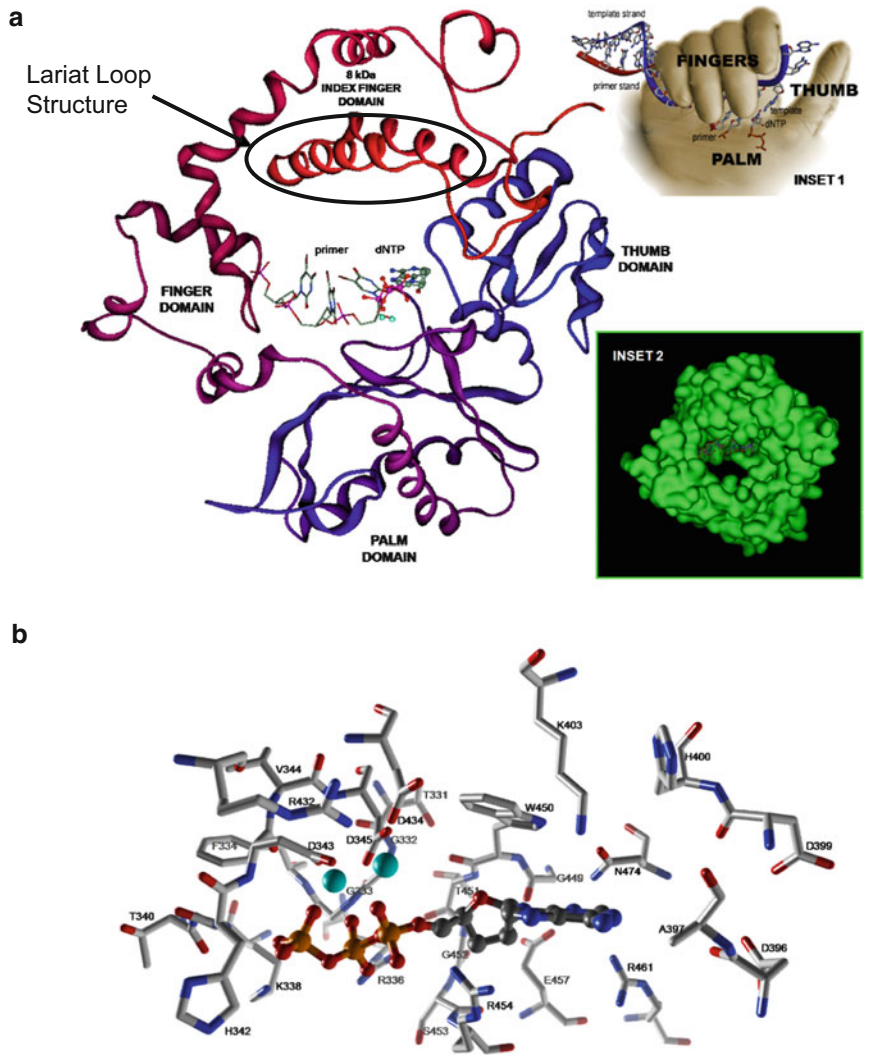

Fig. 5.4 (**a**) Crystal structure of terminal deoxynucleotidyl transferase. *Highlighted* are the fingers, thumb, palm, and index finger (8 kDa) subdomains that work synergistically to catalyze nucleotide incorporation. The ternary complex structure was prepared using the available binary crystal structures of murine TdT (Delarue et al. 2002) and the PDB ID codes 1KEJ (TdT•ddATP) and 1KDH (TdT•ssDNA). MOE (http://www.chemcomp.com) was used for all structural modeling. (**b**) The active site of TdT (PDB ID code: 1KEJ) as defined by amino acids that exist within 6 Å of the bound nucleotide substrate, ddATP. The incoming nucleotide is shown in *ball-stick* representation in CPK color scheme. The two cobalt ions are colored as *cyan*. This figure was prepared using the UCSF Chimera package (http://www.cgl.ucsf.edu/chimera). This figure was adapted from Motea and Berdis (2010)

amino acids that exist within a 6 Å radius of the bound ddATP (Fig. 5.4b) shows that three positively charged residues, K338, R336, and R454, point toward the triphosphate moiety. Based on their proximity and orientation in the active site, these amino acids likely function to neutralize the negatively charged phosphates of the incoming dNTP. Other interactions include the aromatic ring of W450 that is located only 3.6 Å away from the adenine and is positioned parallel to this nucleobase. The location and orientation of this amino acid could provide favorable pi–pi stacking interactions with the adenine base. The positively charged ε amino group of K403 lies only 4 Å away from the adenine base and thus could also provide favorable pi–cation interactions. Likewise, R454 may also participate in pi–cation interactions with the aromatic adenine to influence its binding. There are no amino acid side chains in close proximity with either the 2′ or the 3′ position of the ddATP sugar to provide any electrostatic or hydrogen-bonding interactions. The conspicuous absence of amino acids that could function as a "steric gate" to select against a 2′-OH could explain the rather promiscuous activity of TdT to utilize both ribo- and deoxyribonucleotides (Roychoudhury 1972). Finally, three conserved aspartates, D343, D345, and D434, exist in the catalytic palm subdomain that likely function to position the two metal ions for catalysis.

5.5 Regulation of TdT Activity

The activities of the enzymes involved in V(D)J recombination are tightly regulated, primarily since the formation of DSBs can produce adverse cellular effects such as apoptosis (Roos and Kaina 2012) or an increase in the frequency of genetic mutations (Nickoloff et al. 2008). TdT is regulated at multiple levels including transcriptional control, posttranslational modifications, and through protein–protein interactions. TdT expression is primarily confined to lymphoid tissues including bone marrow and the thymus (Coleman et al. 1974b; Bollum 1979b) in which its transcription is regulated by several factors including AP-1 (Peralta-Zaragoza et al. 2004). Posttranslational regulation of TdT comes primarily through phosphorylation. Ex vivo experiments proved that TdT can be phosphorylated in lymphoblastoid cells (Elias et al. 1982). In addition, recombinant human TdT can be phosphorylated in vitro by protein kinase C (Trubiani et al. 1995). Calf thymus TdT can be phosphorylated by beef heart cAMP-dependent protein kinase at multiple sites corresponding to S7 and T19 in human TdT (Chang and Bollum 1982). While TdT can be phosphorylated at several different sites, it is still unknown what the exact role of this posttranslational modification plays in regulating TdT activity.

Protein–protein interactions between TdT and other DNA-binding proteins can produce both positive and negative effects on TdT activity (Yamashita et al. 2001). For example, TdT interacting factors (TdiFs) can increase the polymerase activity

of TdT by ~4-fold through binding interactions with the C-terminus of TdT (Yamashita et al. 2001). In contrast, the binding of TdiF2 to TdT via its C-terminus causes a twofold decrease in polymerase activity.

PCNA can also physically interact with TdT (Ibe et al. 2001). The cellular role of PCNA is to coordinate several aspects of DNA metabolism including chromosomal DNA replication, DNA repair, translesion DNA synthesis, and recombination (Moldovan et al. 2007). PCNA appears to function similarly to TdiF2 as it reduces the polymerase activity of TdT by ~2-fold (Ibe et al. 2001). It is proposed that PCNA and TdiF1 compete for binding to the C-terminal region of TdT, and this competition for a single binding site can produce positive or negative effects against TdT. Other proteins including the Ku proteins and various DNA polymerases may also regulate TdT activity. For example, "N" regions generated by TdT are unusually longer when Ku80 is knocked-out compared to when Ku80 is present (Sandor et al. 2004). As described earlier, TdT activity can also be indirectly regulated by other X-family polymerase family members. For example, the ability of pol μ to compete with TdT for the same binding site on DNA can inhibit "N" region synthesis catalyzed by TdT (Bertocci et al. 2003).

5.6 TdT in Disease

5.6.1 The Involvement of TdT in Cancer

Alterations in TdT activity and/or its expression level can play a significant role in the initiation and progression of various cancers as well as in the response of these cancers to chemotherapy. For example, TdT overexpression is a common feature in both *a*cute *l*ymphocytic *l*eukemia (ALL) and in *a*cute *m*yelocytic *l*eukemia (AML) (Greaves et al. 1980; Hoffbrand et al. 1977; Kung et al. 1978; Venditti et al. 1997; McCaffrey et al. 1983). Greater than 80 % of ALL patients present with significant higher levels of TdT expression in addition to displaying multiple TdT isoforms in their blast cells (Greaves et al. 1980; Hoffbrand et al. 1977; Kung et al. 1978; Venditti et al. 1997). Although TdT overexpression is less frequent in patients with AML (~20 %), levels of TdT are still significantly higher than those found in other lymphoid malignancies such as *c*hronic *l*ymphocytic *l*eukemia (CLL) (Venditti et al. 1997). In addition, higher levels of TdT activity correlate with a poor prognosis due to suboptimal responses to chemotherapy. Prognosis and survival studies of patients with ALL show that remission rates are nearly twofold higher in TdT-negative (61 %) versus TdT-positive (36 %) patients.

Based on these epidemiological data, several attempts have been made to develop anticancer agents that target TdT activity (McCaffrey et al. 1983; Spigelman et al. 1988; Kodama et al. 2000). One important example is the nucleoside analogue, cordycepin (3′-deoxyadenosine) (Fig. 5.5). Since this analogue lacks

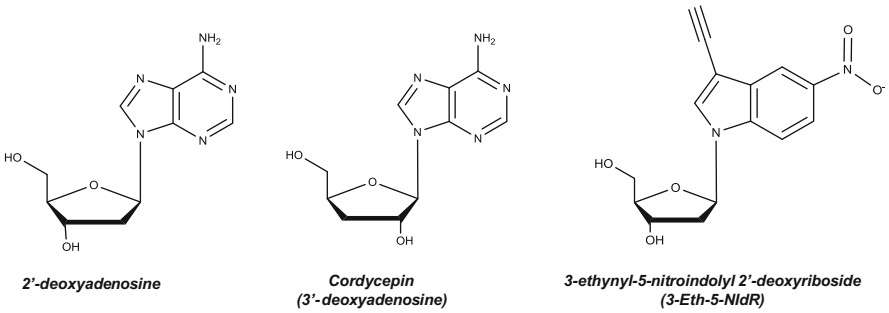

Fig. 5.5 Comparison of the chemical structures for natural nucleotide substrate, dATP, with TdT inhibitors including cordycepin and 3-Eth-5-NITP

a 3′-OH, it can function as a chain terminator after the corresponding nucleoside triphosphate is incorporated into DNA. Unfortunately, cordycepin is not a truly selective inhibitor of TdT as it can be utilized by several template-dependent DNA polymerases involved in chromosomal DNA synthesis (Plunkett and Gandhi 2001). This off-target activity can produce adverse side effects including immunosuppression, fatigue, nausea, and vomiting (Plunkett and Gandhi 2001).

To overcome this problem, Motea et al. (2012) recently developed a nonnatural nucleotide, designated 5-nitroindolyl-2′-deoxynucleoside triphosphate (5-NITP) (Fig. 5.4). The advantage of this nonnatural nucleotide is that it is efficiently utilized by TdT while poorly utilized by conventional template-dependent DNA polymerases. The addition of an ethynyl moiety at the 3-position of the 5-NITP produced a chemical probe that could be used to visualize and quantify replication catalyzed by TdT (Motea et al. 2012). The corresponding "theranostic nucleotide," designated 3-ethynyl-5-nitroindolyl-2′-deoxynucleoside triphosphate (3-Eth-5-NITP), functions as an efficient and potent chain-terminating nucleotide for TdT. The K_m for 3-Eth-5-NITP is ~200 nM while the inhibition constant for terminating in vitro replication catalyzed by TdT is ~350 nM. This group also demonstrated that the corresponding nucleoside, 3-ethynyl-5-nitroindolyl-2′-deoxynucleoside (3-Eth-5-NIdR), produces both cytostatic and cytotoxic effects against leukemia cells that overexpress TdT. Finally, the strategic placement of the ethynyl moiety allows the incorporated nonnatural nucleotide to be efficiently and selectively tagged with an azide-containing fluorophore via "click" chemistry. The application of Cu^{2+}-catalyzed "click" chemistry allows one to quantify the amount of nucleotide incorporation. Using this technique, it was shown that the anticancer effects of the corresponding nonnatural nucleoside could be quantified, and a dose-dependent relationship on the cytostatic effects of the analog was established. Furthermore, a distinct correlation between therapeutic activity and cellular levels of TdT were demonstrated. Collectively, these studies highlight the development of a first-in-class "theranostic" agent against TdT-positive ALL. The use of this agent can hopefully improve the accuracy of dosing regimens and thus accelerate clinical decisions regarding therapeutic intervention in leukemia patients.

5.6.1.1 The Involvement of TdT in Other Cancers

Merkel cell carcinoma is a rare form of skin cancer that primarily affects patients over the age of 60 (Pectasides et al. 2006). While this form of skin cancer accounts for less than 1 % of all cutaneous malignancies (Albores-Saavedra et al. 2010), Merkel cell carcinoma can display aggressive biological behavior and rapidly metastasize to regional lymph nodes and other organs including liver, bones, lungs, and brain. The most frequent change is the loss of heterozygosity caused by deletions or chromosomal translocations (Ronan et al. 1993).

Differential diagnosis of Merkel cell carcinoma historically involves histochemical validation to distinguish it from small cell carcinomas that originate in other organs and hematological malignancies. These include acute lymphoblastic lymphoma, acute myeloid leukemia, and cutaneous natural killer (NK)/T-cell lymphoma and hematodermic CD56/CD4 neoplasms. Several recent studies have shown that TdT is expressed in Merkel cell carcinoma. This unusual feature provides a new biomarker to further diagnose Merkel cell carcinoma and avoid diagnostic pitfalls associated with identifying other small round cell tumors presenting in cutaneous and soft tissues. An early study performed by Sur et al. (2007) reported that 8 of 15 cases (53 %) of Merkel cell carcinomas displayed strong yet diffuse TdT nuclear staining. A subsequent study by Buresh et al. (2008) expanded upon these results to shown that nuclear TdT immunoreactivity was present in 19 of 26 (73 %) cases of Merkel cell carcinoma. Furthermore, TdT expression was not observed in the epidermis, the epithelium of cutaneous appendages, endothelial cells, or stromal cells, and thus difference provides a biomarker for selectivity. Finally, Sidiropoulos et al. (2011) showed that 28 of 40 (70 %) cases of Merkel cell carcinoma were positive for nuclear staining of TdT. Equally important, they showed that only 2 of 30 (7 %) cases of small cell lung carcinoma (SCLC) were positive for TdT. In addition, positive staining for TdT was not detected in pulmonary carcinoid tumors (0/6). Collectively, these findings provide an important way to differentiate Merkel cell carcinomas from SCLC carcinomas, both of which are very aggressive neuroendocrine cancers and that display high mortality rates. While TdT can be used in the differential diagnosis of Merkel cell carcinoma, it has yet to be demonstrated if TdT can be targeted for therapeutic intervention against this malignancy.

5.6.2 Biochemical Applications of TdT

The ability of TdT to incorporate a wide variety of nucleotide analogs (Arzumanov et al. 2000; Sosunov et al. 2000; Krayevsky et al. 2000a; Horáková et al. 2011; Jarchow-Choy et al. 2011; Motea et al. 2012) has led to the development of a very effective method for in vivo labeling of DNA broken ends. Perhaps the most widely used technique is the *T*dT-mediated d*U*TP-biotin *n*ick *e*nd-*l*abeling (TUNEL) assay

(Gorczyca et al. 1993). This assay is based on the ability of TdT to efficiently incorporate biotinylated dUTP into single-strand DNA at sites of DNA breaks. Biotin dUTP that is incorporated at the ends of broken DNA can be visualized using fluorescently labeled avidin or streptavidin. The application of this technique allows the number and location of DNA breaks to be directly quantified. This technology is widely used to detect apoptosis, a form of programmed cell death, in eukaryotic cells.

TdT is also used ubiquitously to label the 3′-termini of synthetic oligonucleotides with a radioactive nucleotide or with various fluorescent probes (Krayevsky et al. 2000b). The labeled primers can then be annealed to a complementary strand as used as substrates for various enzymes involved in nucleic acid metabolism including restriction endonucleases, DNA glycosylases, and, of course, template-dependent DNA polymerases.

5.7 Conclusion

Several DNA polymerases including polymerase μ, polymerase λ, and TdT display unique catalytic activity as they can perform DNA synthesis in the absence of a DNA template. This unusual activity defies that displayed by conventional DNA polymerases that are charged with maintaining genomic fidelity by replicating undamaged DNA or that properly replicate various forms of damaged DNA. While this template-independent polymerase activity can produce mutagenic consequences, it also plays an indispensible role in the efficient and proper repair of double-strand DNA breaks. In fact, the activity of TdT during V(D)J recombination highlights the importance of template-independent DNA synthesis in generating immunological diversity that is needed for an efficient immune system. While kinetic and structural studies have provided significant insights into the catalytic mechanisms of these DNA polymerases, there is clearly much more work needed to completely understand their biological roles, especially during the repair of damaged DNA. Again, TdT provides an important example of this feature as its unregulated activity is linked with various cancers including acute lymphoblastic leukemia and Merkel cell carcinoma. It is highly likely that dysfunctional or unregulated activity of either polymerase μ and polymerase λ also play key roles in genetic diseases such as cancer.

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Chapter 6
Archaeal DNA Polymerases: Enzymatic Abilities, Coordination, and Unique Properties

Michael A. Trakselis and Robert J. Bauer

Abstract DNA polymerases from archaea coordinate both replication and repair activities under extreme conditions. Traditional DNA polymerase families B and Y are represented, but an entire phylum of archaea contains members from the unique D-family. These model archaeal polymerases from each family have be exploited for a variety of biotechnology applications but have also provided great insight into kinetic mechanisms, structural properties, conformational changes, and protein interactions with DNA polymerases from other domains. Interestingly, individual polymerase members have revealed some unique features including template uracil recognition, more open active sites, novel motifs, and direct polymerase interactions that modulate replication and repair in these simpler organisms. How multiple polymerases coordinate synthesis on the leading and lagging strands and replication or repair duties is an ongoing fundamental question in archaea. Nevertheless, their intrinsic enzymatic properties are fascinating and continue to shape and influence research avenues for all DNA polymerases. The ability to regulate robust and accurate DNA synthesis through specific and loosely associate oligomeric states and protein interactions maintains a high degree of genomic stability in spite of harsh environmental conditions.

Keywords DNA replication • DNA repair • Fidelity • Archaea • PCNA • Polymerase • Holoenzyme

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Abbreviations

BER	Base excision repair
NER	Nucleotide excision repair
NTP	Nucleotide triphosphate
Pab	*Pyrococcus abyssi*
PCNA	Proliferating cellular nuclear antigen
Pfu	*Pyrococcus furiosus*
Pho	*Pyrococcus horikoshii*
Pol	Polymerase
PP_i	Pyrophosphate
RFC	Replication factor C
Sac	*Sulfolobus acidocaldarius*
Sso	*Sulfolobus solfataricus*
Tgo	*Thermococcus gorgonarius*
UV	Ultraviolet

6.1 Introduction

Efficient DNA replication relies on both precise and uninterrupted DNA synthesis at the replication fork. As such, DNA replication polymerases coordinate accurate synthesis on the leading and lagging strands with error-prone polymerases when damage is encountered. Most organisms contain members from multiple DNA polymerase families including Archaea which provide a relevant model system for assessing the kinetics, dynamics, structure, and interactions of multiple DNA polymerases.

Studies over the past decade of DNA replication machinery have revealed that many components from Eukaryotes have evolved from a common ancestor in Archaea (Yutin et al. 2008). In particular, the archaeal DNA replication machinery is essentially a simplified eukaryotic vestige and provides an excellent experimental system for deciphering mechanisms of enzymatic action and evolutionary relationships. Although there are a number of similarities in sequence, structure, and function between Archaea and Eukaryotes, the link is not absolute, as Archaea also contain bacterial- and archaeal-specific features.

A number of DNA replication systems from divergent archaeal species have been examined enzymatically and structurally to gain insight into fundamentally conserved metabolic processes as well as for the development of a variety of ubiquitous biotechnology tools. In this chapter, we will describe and compare the current findings on the relationship and function of archaeal DNA polymerases, their importance in deciphering DNA replication and repair mechanisms, as well as interactions that promote elevated enzymatic properties.

6.2 Archaeal DNA Polymerase Families and Function

The archaeal domain is subdivided minimally into several phyla with the largest two: Crenarchaeota (crenarchaea) and Euryarchaeota (euryarchaea), containing replication systems with high homology to those found in eukaryotic systems. DNA polymerases have been classified into at least six different families. Compared with the 15 human DNA polymerases, archaeal organisms generally have two to four DNA polymerases from two different families. Crenarchaea contain members from both the B- and Y-families, while euryarchaea contain those belonging to B- and D-families (Table 6.1). DNA polymerases employed by archaeal organisms have high sequence, structural, and functional similarities to those found in eukaryotes, yet they have adapted for optimal function under extreme conditions, most notably high temperatures. The ease of purification, high conservation to eukaryotes, and adaptation for biotechnology applications have made archaeal polymerases models for studying processivity, protein interactions, lesion bypass, polymerase–exonuclease shuttling, and polymerase switching mechanisms essential to all domains of life.

6.2.1 Crenarchaeal B-Family DNA Replication Polymerases

B-family polymerases are typically robust and accurate enzymes, containing an N-terminal 3′–5′ exonuclease and a C-terminal polymerase domain (Kim et al. 2008; Savino et al. 2004). The exonuclease domain increases selective nucleotide incorporation efficiencies generally by a factor of 10^2 up to 10^8 in total (Kunkel and Bebenek 2000). The polymerase domain is similar in structure to a right hand with fingers, thumb, and palm subdomains that act to bind the DNA template, orientate the incoming nucleotide, and catalyze polymerization through conformational changes between domains (Fig. 6.1). The enzymes are typically not highly processive on their own, but possess the ability to form complexes with their respective processivity clamps (PCNAs), allowing for the incorporation of >10,000 nucleotides in a single binding event (Jeruzalmi et al. 2002). As a result, B-family enzymes are also thought to be the main replication polymerases in crenarchaea.

Crenarchaea possess three B-family polymerases. PolB1 has robust synthesis activity, high nucleotide fidelity, and an included exonuclease domain (Pisani et al. 1998; Zhang et al. 2009). PolB1 is evolutionary related to eukaryotic B-family polymerases (α, δ, ε, ζ) (Prakash et al. 2005) but seems to have arisen after PolB2 and PolB3 in a gene duplication event (Table 6.1) (Edgell et al. 1998). PolB1 has a typical right-hand conformation but includes two extra α-helices in the N-terminal domain that contact the fingers domain (Fig. 6.1). It is proposed that these helices strengthen the contacts between the N- and C-terminal domains for catalysis at high temperatures (Savino et al. 2004). Alternatively, they could also play a role in promoting protein complex formation as seen for the trimeric PolB1 complex discussed below (Mikheikin et al. 2009).

Table 6.1 Archaeal polymerase family members

	Archaeal phyla				
Family	Crenarchaeota	Euryarchaeota	Nanoarchaeota	Thaumarchaeota	Korachaeota
A					
B	PolB1			PolB	PolBI
C	PolB2				
	PolB3	PolB	PolB		PolBII
D		PolD	PolD	PolD	PolD
X	PriSL[a]	PriSL[a]	PriSL[a]	PriSL[a]	PriSL[a]
Y	PolY			PolY	

[a]AEP family of DNA primases with homology to X-family polymerases

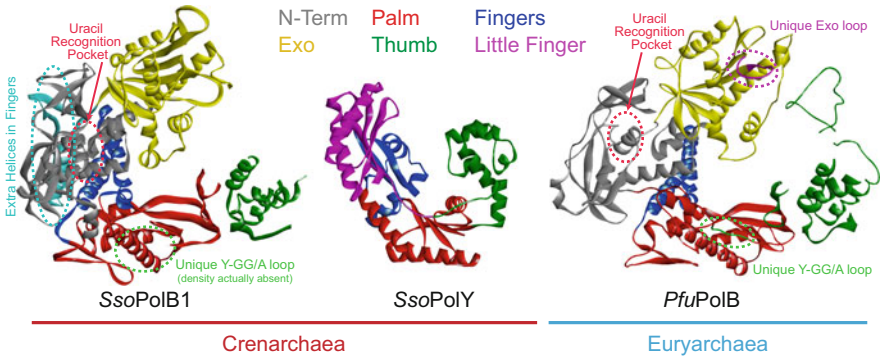

Fig. 6.1 Structures of crenarchaeal, *Sso*PolB1 (PDB: 1S5J), *Sso*PolY (PDB: 1JX4), and euryarchaeal *Pfu*PolB (2JGU) DNA polymerases highlighting conserved N-terminal (*gray*), exonuclease (*yellow*), palm (*red*), thumb (*green*), fingers (*blue*), and little finger (*pink*) domains. The extra α-helices in the fingers of *Sso*PolB1 are highlighted in *cyan*; the unique Y-GG/A loop is highlighted in *green*; the unique exonuclease (Exo) loop is highlighted in *pink*; and the uracil recognition pocket is circled in *red*

The exonuclease domain of *Sso*PolB1 contains an inherent 3′–5′ proofreading ability that enhances the fidelity 14-fold (Zhang et al. 2009) (Fig. 6.2). Although PolB1 utilizes an induced fit mechanism for nucleotide incorporation (Brown and Suo 2009), occasionally, it can make a mistake. The shuttling between the multiple active sites (pol and exo) has been examined in great detail in phage organisms with B-family polymerases and involves multiple steps including long-range movement of the separated primer strand from the polymerase active site to the exonuclease site (Fidalgo da and Reha-Krantz 2007). In crenarchaeal B-family polymerases, control of these two catalytic activities occurs intramolecularly, mediated by a flexible loop (Y-GG/A) in the palm domain (Truniger et al. 1996; Bohlke et al. 2000) (Fig. 6.1). Most likely, the polymerase is able to efficiently achieve this feat by maintaining contact with their respective processivity clamps and allowing alternative holoenzyme conformations (discussed below). Mutation of the conserved aspartates in the exonuclease domain was instrumental in measuring the inherent fidelity of PoB1

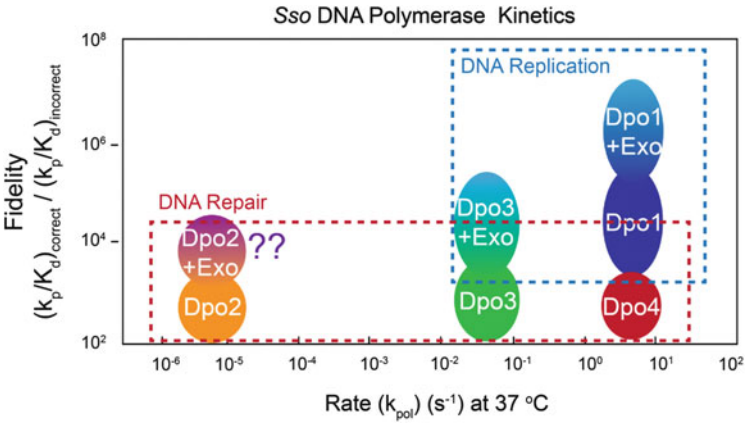

Fig. 6.2 Representation of the kinetics and fidelity of all four DNA polymerases in *Sso*. The fidelities for both the polymerase and exonuclease domains are shown. *Highlighted* are regions required for efficient DNA replication or DNA repair calculated from the total genome size (2.99 Mb) (She et al. 2001), number of origins of replication (3) (Robinson and Bell 2005), and S-phase time (1.5 h) of *Sso* (Duggin et al. 2008)

(Zhang et al. 2009) as well as other archaeal B-family polymerases (Bauer et al. 2012). Surprisingly, without an active exonuclease domain, PolB1exo$^-$ also exhibited a masked ability to extend ssDNA with a template-independent and template-dependent terminal transferase activities (Zuo et al. 2011). Short 20 nucleotide ssDNA templates are extended in a template-independent fashion initially adding three to five bases on the 3′ end. Newly added DNA is then wrapped around and stabilized intramolecularly through incomplete base paring interactions before template-dependent slipping extension creates products greater than 7 kb in a mechanism akin to what has been shown for human X-family DNA polymerase μ and λ (Dominguez et al. 2000; Maga et al. 2005).

Crenarchaeal PolB2 is not as well characterized and has been postulated to be inactive based on bioinformatic analysis of unconserved active site residues (Rogozin et al. 2008). PolB2 has high sequence conservation with PolB1, and it is thought that duplication of PolB2 gave rise to PolB1. Intriguingly, expression of *Sso*PolB2 is upregulated in response to UV exposure prompting many to hypothesize a role in bypassing cyclobutane thymine dimers (Frols et al. 2007, 2009; Gotz et al. 2007). Recently, *Sso*PolB2 has been shown to have some basic polymerase activity, but it is the least active of the four *Sso* polymerases (Fig. 6.2) and actually lacks the in vitro ability to bypass thymine dimers (Choi et al. 2011). Rather, PolB2 was able to bypass uracil, hypoxanthine, and 8-oxoguanine. The binding affinity of PolB2 to DNA is weak compared to PolB1 and limits its polymerase and exonuclease activities. It is speculated that PolB2 participates either in oxidative DNA lesion bypass or in short patch repair of UV-induced DNA damage after excision of the damaged bases, and additional protein interactions may be required to form stable complexes on DNA for efficient activity.

Bioinformatic analysis suggests that crenarchaeal PolB3 and its homolog from euryarchaea, PolB, are actually the original archaeal polymerases (Iwai et al. 2000). Further studies indicate PolB3 may have evolved into the inactive polymerase or exonuclease domain found near the C-terminus of eukaryotic ortholog pol ε (Tahirov et al. 2009). In PolB3, the highly conserved active site YxDTD motif, which is generally responsible for the coordination of the two active site Mg^{2+} ions for catalysis, has diverged significantly. While it had been previously shown that both aspartic acid residues in this motif were required for polymerase activity (Bernad et al. 1990; Copeland and Wang 1993), *Sso*PolB3 has been found to be moderately active despite having a mutated motif (LAN-D) (Choi et al. 2011; Bauer et al. 2012). As a consequence, the polymerase activity and DNA-binding ability of PolB3 are surprisingly low and similar to PolB2. The kinetics and fidelity of PolB3 are moderate, placing it squarely between values for replication or repair polymerases (Fig. 6.2). Although the exact metabolic role of PolB3 has not yet been uncovered, it can bypass cyclobutane dimers in vitro more efficiently than any of the other polymerases in *Sso* including PolY, indicating a potential role in UV-induced damage repair (Choi et al. 2011).

The number, conservation, and biochemistry of B-family polymerases in crenarchaea present the possibility that they are utilized similarly to those of eukaryotes (Edgell et al. 1997; Iwai et al. 2000), with PolB1 acting as the leading strand replicase and either PolB2 or PolB3 as the lagging strand replicase. The fidelities of B-family polymerases are generally greater than Y-family members providing for highly accurate and robust synthesis on the leading and lagging strands. On the other hand, genetic ablation of PolB2 or PolB3 has no effect on viability of *Sulfolobus* (Steve Bell, personal communication). More likely is that PolB1 acts as both the leading and lagging strand polymerases or can compensate for the loss of either PolB2 or PolB3 during replication. It may be that PolB2 and/or PolB3 will have a more specialized unrecognized role in DNA damage repair, potentially complimenting or providing redundancy to the function of the Y-family polymerase. The combination of specificities, fidelities, kinetics, and lesion bypass abilities of the four DNA polymerases in *Sso* encompass a broad range of complimentary activities for efficient replication and repair (Fig. 6.2).

6.2.2 *Euryarchaeal B-Family DNA Replication Polymerases*

In euryarchaea, there is typically only a single chromosomally encoded PolB. *Pyrococcus furiosus* (*Pfu*) and *Thermococcus gorgonarius* (*Tgo*) PolB have been characterized extensively and are commonly used in PCR applications due to their robust accurate activities and high thermostabilities (Lundberg et al. 1991; Hopfner et al. 1999). Many euryarchaeal PolB polymerases contain inteins which are protein sequences that facilitate their excision from flanking polypeptides while catalytically ligating the remaining segments splicing together the catalytic polymerase active site (Perler et al. 1992). The resulting spliced amino acid sequence is similar

between crenarchaeal and euryarchaeal PolBs. The crystal structure of euryarchaeal B-family polymerases shows a right-hand conformation with only slight differences in the loop regions compared to crenarchaeal PolB. One unique loop structure was revealed within the exonuclease domain responsible for regulating the polymerase and nuclease activities of these polymerases (Fig. 6.1) (Bohlke et al. 2000; Hashimoto et al. 2001). This exonuclease loop is not observed in structures of B-family polymerases from other organisms like RB69 and appears to be unique to euryarchaea PolB. Mutation of the exonuclease loop results in a conformational change in the editing cleft caused by altered interactions between the loop and the thumb domain decreasing exonuclease activity and making the polymerase more amenable for PCR reactions (Kuroita et al. 2005). Control and regulation of the polymerase and nuclease activities is important for the accurate and efficient replication necessary for the maintenance of the archaeal genome. While, in crenarchaea, PolB1 is thought to perform both leading and lagging strand synthesis, euryarchaeal PolB was found to function on only the leading strand during chromosomal replication (Henneke et al. 2005), although recent conflicting data indicates that PolB may not be essential to the replication process (Sarmiento et al. 2013; Cubonova et al. 2013).

6.2.3 Archaeal-Specific D-Family Polymerases

The D-family polymerase (PolD), ubiquitously found in all euryarchaeal species, was first discovered in cellular extracts from *P. furiosus* (*Pfu*) (Cann et al. 1998) but is also found in nanoarchaea (Waters et al. 2003), thaumarchaea (Hallam et al. 2006), and korarchaea (Elkins et al. 2008), but not members of the crenarchaeal phyla nor within the genome of any bacterial or eukaryotic organism. The presence of D-family polymerases in so many phyla of Archaea suggests that PolD may have been at least partly responsible for the replication of the genome of the last common ancestor of Archaea (Matsui et al. 2011; Tahirov et al. 2009). Archaeal-specific PolD enzymes are heterotetramers composed of two large (DP2) and two small (DP1) subunits. DP2 is the catalytic subunit of the polymerase, while DP1 is the catalytic subunit of an Mre11 like 3′–5′ exonuclease (Cann et al. 1998). Although DP2 shares some of the common motifs found in other DNA polymerases, it has no global similarities to any other proteins found in the database justifying a new family of DNA polymerases (D-family). The exonuclease region in DP1 is homologous to a C-terminal region in the small noncatalytic subunits (B subunits) of multisubunit eukaryotic B-family polymerases α, δ, and ε (Cann et al. 1998; Ishino et al. 1998; Yamasaki et al. 2010; Shen et al. 2004). Additional homology has been established between zinc fingers found in D-family polymerase and those found near the C-terminus in all eukaryotic B-family polymerases (Tahirov et al. 2009).

Studies performed with *Pyrococcus horikoshii* (*Pho*) PolD illustrated that strong polymerase and exonuclease activities were obtained only when both subunits are

present (Uemori et al. 1997). In *Pyrococcus abyssi* (*Pab*), PolD was unable to bypass abasic sites and maintained error rates comparable to *Pab*PolB (Palud et al. 2008). Importantly, D-family polymerases genes have been found to be essential to the survival of the euryarchaeal cell (Berquist et al. 2007; Cubonova et al. 2013). This, in tandem with their high fidelity, implicates them in a primary role in the replication process. *Pab*PolD has been found to be efficient in the extension of RNA-primed DNA, potentially involving it in the initial elongation of RNA-primed DNA prior to replacement by the B-family polymerase (Rouillon et al. 2007). It is possible that B- and D-family polymerases in euryarchaea may perform coordinated DNA synthesis much like the eukaryotic polα primase and pols ε and δ (Hubscher et al. 2002). Intriguingly, *Pab*PolD is also implicated as the lagging strand replicase, due to its ability to displace downstream complementary RNA/DNA duplexes required for Okazaki fragment processing by facilitating excision of the RNA primer by FEN-1 like nucleases (Henneke et al. 2005). In *Thermococcus kodakarensis*, it has been found that only PolD is required for genomic DNA replication, and it is speculated that while the tandem replication by both PolB and PolD may be preferable, PolD itself may be the primary replication in archaea as a whole (Cubonova et al. 2013).

Many of the D-family polymerases also contain inteins. All catalytic elements for intein splicing and subsequent ligation lie within the intein and flanking peptide regions (Paulus 2000; Perler 2005). In *Thermococcus litoralis* splicing was found to control production of the mature form of a polymerase (Perler et al. 1992). In *Pab*, an intein sequence is found within the Dp2 subunit (Mills et al. 2004). It is possible that these sequences serve as a means to regulate polymerase expression levels in vivo. Intein sequences are also present in euryarchaeal B-family polymerases (Perler et al. 1997) but are not common in crenarchaea. Both B- and D-family polymerases were found to contain inteins in *Thermococcus fumicolans* that when expressed separately, possess nuclease activity. The two intein nucleases have different substrate specificities and metal cofactor requirements (Saves et al. 2000), indicating that inteins may not only serve in a regulatory capacity for protein maturation but also potentially play a role in the maintenance of the archaeal genome.

6.2.4 Archaeal Y-Family Lesion Bypass Polymerases

Archaeal Y-family polymerases are found primarily in crenarchaea but are not universally conserved throughout the phyla and are proposed to only be present in those organisms exposed to UV light (Kelman and White 2005). Interestingly, Y-family polymerases do not share sequence identity to any of the other polymerase families (A, B, C, D, X) and also lack the 3′–5′ exonuclease domain present in the archaeal B- and D-families (Ling et al. 2001). Despite these differences, the structure of PolY is similar to that of polymerases in the A- and B-families, possessing the usual palm, fingers, and thumb subdomains (Fig. 6.1). However, in addition to the

usual domains, PolY possesses an additional "little finger" subdomain and linker, primarily important for DNA binding. Archaeal Y-family polymerases have served as models for understanding lesion bypass mechanisms and specificities as related to the eukaryotic orthologs of the same family: pol η, pol ι, and pol κ.

As has been observed in bacteria and eukaryotes, archaeal Y-family polymerases have a much more specialized role in the maintenance of the archaeal genome. Y-family polymerases have error rates 100–1,000-fold higher than B-family polymerases (Fig. 6.2) (McCulloch and Kunkel 2008). This is due not only to the lack of a proofreading exonuclease domain but also to a larger, more accommodating active site which allows for binding of incoming nucleotides in additional orientations, prevented by steric clashes with residues in the active sites of polymerases in other high-fidelity polymerase families (Perlow-Poehnelt et al. 2004). Multiple dNTP orientations in the active site allow for potential base pairing with a damaged template base and the concurrent bypass of these sites of damage. The Y-family polymerases from *Sso*(Dpo4) and *Sulfolobus acidocaldarius* (*Sac*Dbh) are two of the best characterized polymerase enzymes and have been found to bypass a large number and variety of DNA lesions including: abasic (Fiala et al. 2007; Ling et al. 2004a), (deoxyguanosin-8-yl)-1-aminopyrene (Sherrer et al. 2009), benzo[*a*]pyrene diol epoxide (Ling et al. 2004b), 8-oxoguanine (Rechkoblit et al. 2006; Zang et al. 2006), methylguanine and benzylguanine (Choi et al. 2011), and thymine dimers (Johnson et al. 2005; Boudsocq et al. 2001). Active site metal ion composition has also been shown to be important in determining which lesions can be bypassed by Y-family polymerases. While typically Mg^{2+} is the metal cofactor most associated with nucleotide binding and incorporation, it has been observed that when replaced by Mn^{2+}, *Sso*Dpo4 exhibits increased catalytic efficiency yet reduced fidelity, with an ability to efficiently bypass otherwise unfavorable substrates such as abasic sites and cyclopyrimidine dimers (Vaisman et al. 2005).

Y-family polymerases are able to bypass lesions through error-free and error-prone means (Fig. 6.3). For example, *Sso*Dpo4 is able to correctly incorporate cytosine across from the aminofluorene adduct of guanine; however, base deletions or substitutions occur directly after the lesion (Rechkoblit et al. 2010). When bypassing an abasic site, Dpo4 is known to employ either the "A-rule" (Strauss 1991), where an adenosine is added opposite a noninstructional template lesion, or a template slippage loop-out mechanism where the template lesion is looped out and replication continues opposite the next base resulting in a −1 frameshift (Fiala and Suo 2007; Wu et al. 2011). Y-family polymerases are also known to be able to induce deletions of a single base through a template slippage mechanism (Wu et al. 2011). While *Sso*Dpo4 is able to bypass most lesions, the ability to bypass cyclopyrimidine dimers is limited and instead speculated to be performed by *Sso*Dpo3 (Choi et al. 2011).

The structural mechanism of lesion bypass involves a large conformational change in the little finger domain of *Sso*Dpo4 upon formation of the polymerase–DNA binary complex, with a 131° rotation relative to the palm, fingers, and thumb domains (Wong et al. 2008). A third little finger orientation,

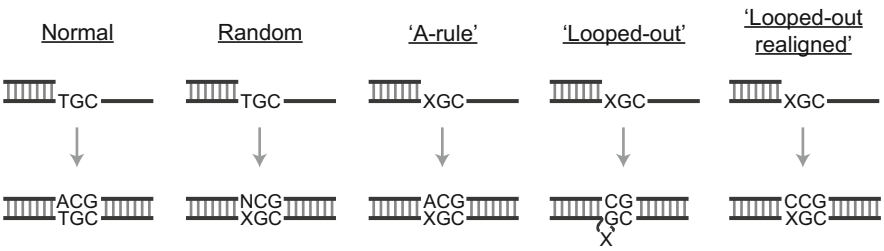

Fig. 6.3 Lesion bypass mechanisms for archaeal Y-family DNA polymerases. Random incorporation includes partial base pairing to the template strand for preferential incorporation of nucleotides dependent on the lesion type. The "A-rule" is utilized primarily when no templating base is available and preferentially incorporates adenine over the other three bases. The "looped-out" mechanism utilizes base pairing interactions at the +1 site of the template strand while excluding the lesion and results in a −1 frameshift. The looped-out mechanism can also realign to avoid the frameshift albeit with much less frequency

distinct from the DNA bound and apo forms, is observed upon polymerase binding to PCNA (Xing et al. 2009). It was found that the linker region between the little finger and thumb domain is important in the interactions between *Sso*Dpo4, DNA, and possibly PCNA (Sherrer et al. 2012). Generally, the activity and lesion bypass ability of *Sso*Dpo4 is much greater than for *Sac*Dbh and has been attributed to the sequence of the little finger linker domain, which contributes to the strength of DNA binding and processivity (Boudsocq et al. 2004; Wilson et al. 2013). Interestingly, knockouts of *Sac*Dbh resulted in no changes to growth rates or sensitivity to a variety of tested DNA damaging agents (Sakofsky et al. 2012). However, an increase in the number of spontaneous mutations was observed, much like that which is seen upon deletion of *E. coli* pol IV (DinB) in cells in a stationary phase (Nowosielska et al. 2004). This indicates that while the Y-family polymerase is not absolutely required for the bypass of lesions, its absence results in the bypass being performed in an even more error-prone fashion possibly by the other archaeal DNA polymerases.

One consequence of the more spacious solvent accessible Y-family polymerase active site is the potential incorporation of ribonucleotides. Selective incorporation of only dNTPs is essential for the DNA replication process, and much like polymerases from other families, Y-family members still maintain a high level of selectivity in spite of their more relaxed active site. This selectivity is preserved through the presence of a highly conserved tyrosine residue termed the "steric gate" (Fig. 6.1) (Delucia et al. 2003). The steric gate blocks binding of rNTPs through steric clash with the 3′OH of the incoming nucleotide (Kirouac et al. 2011). The steric gate also seems to be conserved in a variety of polymerases from the A, B, X, Y, and RT families to provide specificity for deoxyribonucleotides (Brown and Suo 2011).

Another feature identified in *Sso*Dpo4 but not *Sac*Dbh is the ability to perform pyrophosphorolysis or reattachment of the pyrophosphate (PP_i) to the 3′ end of a nucleotide monophosphate, reversing the NTP incorporation mechanism.

Particularly upon misincorporation of dGTP or dCTP across from template dT, pyrophosphorolysis can occur in *Sso*Dpo4 to remove the misincorporated nucleotide (Vaisman et al. 2005). Pyrophosphorolysis is related to the strength of the interaction between the little finger and DNA. Exchange of the little finger from *Sso*Dpo4 with the weaker binding motif from *Sac*Dbh abolished pyrophosphorolytic activity. It may be that *Sso*Dpo4 and other exonuclease-deficient archaeal polymerases utilize pyrophosphorolysis as a means of error checking favored by the slow release of PP_i after nucleotide misincorporation to increase fidelity further.

6.2.5 *PriSL Primase, a Possible X-Family Polymerase Homolog*

The eukaryotic-like archaeal primase, PriSL, is a heterodimeric protein composed of a large regulatory subunit (PriL) and a small catalytic subunit (PriS) that is conserved across the archaeal domain. It is proposed to be responsible for the *de novo* synthesis of short RNA primers to initiate DNA replication on the leading and lagging strands. PriSL has the remarkable ability to synthesize DNA and RNA in a template-dependent and template-independent manner producing oligonucleotide products from 2 bases to greater than 7,000 (Lao-Sirieix and Bell 2004; Le Breton et al. 2007). The PriS subunit shares significant structural and sequence homology to eukaryotic X-family polymerases, most notably polymerase β (Augustin et al. 2001; Lao-Sirieix et al. 2005). PriSL can also synthesize across discontinuous templates (Hu et al. 2012) in a similar manner to that of eukaryotic pol μ, which is known to be involved in both base excision repair and double-strand break (DSB) repair. Archaea do not have a direct polymerase homolog from the X-family but the biochemical data suggests that PriSL may be a functional homolog (Table 6.1).

6.3 Archaeal DNA Polymerase Holoenzymes

DNA polymerases by themselves are traditionally not very processive and require complexation with their respective clamps to replicate long stretches of DNA without dissociating. In Archaea, the proliferating cell nuclear antigen (PCNA) clamp is loaded onto DNA by the replication factor-C (RFC) clamp loader in an ATP-dependent manner to facilitate recruitment of the DNA polymerase to the holoenzyme complex. This minimal DNA polymerase holoenzyme complex will be active on both the leading and lagging strands during replication as well as participate in lesion bypass mechanisms of exchange at DNA damage sites.

B-family polymerases from both crenarchaea and euryarchaea have been found to interact with their processivity clamps through a motif called the PCNA-interacting peptide (PIP) box (Castrec et al. 2009; Pisani et al. 2000). This motif has a consensus sequence of Qxxhxxaa, where x is any amino acid, h is a hydrophobic residue, and a is an aromatic residue (Warbrick 1998). PCNA loading on DNA allows for the binding of the polymerase and formation of a replicative holoenzyme similar to those seen in both prokaryotes and eukaryotes. The association of the PCNA clamp with PolB results in a highly processive complex that limits dissociation and allows synthesis of greater than 10 kb products in a single binding event. The structure of euryarchaeal (*Pfu*) DNA polymerase holoenzyme has provided significant insight into the holoenzyme assembly mechanism and conformational changes required for both polymerization, editing, and switching (Fig. 6.4a) (Mayanagi et al. 2011; Nishida et al. 2009; Bunting et al. 2003). *Pfu*PolB was found to possess an additional site of contact with PCNA (standby), proposed to be important for pivoting between the editing (locked-down) and polymerization (tethered) modes. This flexibility provided through multiple interactions between PCNA, and the polymerase allows for uninterrupted and dynamic, error-free DNA synthesis and may also be important for polymerase switching during replication.

Euryarchaeal D-family polymerases also interact with the homotrimeric PCNA at the PIP site to increase processivity (Tori et al. 2007; Henneke et al. 2005). Like B-family polymerases, PolD also possesses a PIP box (Castrec et al. 2009); *Pab*PolD has been shown to have two separate sites for interaction with PCNA, one at the C-terminus and a separate palindromic PIP box at the N-terminus of the large subunit. Both sites effect PolD binding to PCNA; however, while mutation of the N-terminal PIP site reduced processivity, mutation of the C-terminal PIP had no effect. It was also shown that both PIP sites interact with the same site on PCNA, raising the question of whether the binding site changes depending on the desired activity between polymerization and editing as seen for euryarchaeal PolB (Castrec et al. 2009).

Unlike the euryarchaeal homotrimeric PCNA, the crenarchaeal PCNA is a heterotrimeric complex comprised of three separate proteins (PCNA1, PCNA2, and PCNA3). PolB1 from *Sulfolobus solfataricus* has been found to interact specifically with PCNA2 subunit within the heterotrimer to increase replication rate (Dionne et al. 2003). We have recently found that the *Sso* DNA polymerase holoenzyme synthesizes DNA somewhat distributively and is weakly processive unlike other well characterized systems (Bauer, Trakselis et al, submitted). Specific contacts between the C-terminal PIP motif in *Sso*PolB1 and *Sso*PCNA2 is responsible for actively re-recruiting the polymerase during replication to maintain a dynamic processivity. Using specific contact points and measured distances (Trakselis, unpublished data) between the *Sso*PCNA123 and *Sso*PolB1, we have created a DNA polymerase holoenzyme model to highlight interactions in the polymerization mode (Fig. 6.4b). By having three separate proteins, crenarchaeal PCNA123 can also specifically interact with additional proteins, such as flap endonuclease (PCNA1) and DNA ligase (PCNA3) in close proximity to the polymerase to efficiently process Okazaki fragments in a “tool-belt” fashion

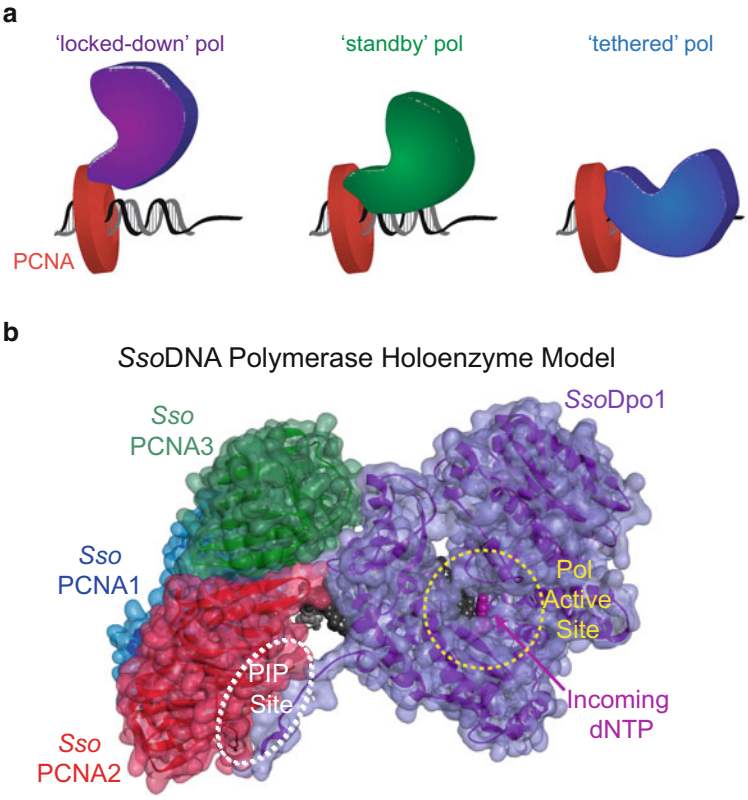

Fig. 6.4 (**a**) Complexation of the DNA polymerase with the PCNA clamp can occur in different conformational states for assembly or activation of exonuclease or polymerase activities. (**b**) DNA polymerase holoenzyme model from Sso showing the specific interactions of the C-terminal PIP domain of SsoDpo1 (*purple*) with the interdomain connecting loop (ICL) of SsoPCNA2 (*red*) as well as adjacent interactions with SsoPCNA3 (*green*) modeled from the PfuPolB1–PCNA cocrystal structure (PDB ID: 3A2F). Model is built from specific detected interactions of PCNA123 (PDB ID: 2IX2) and SsoPolB1 (PDB ID: 1S5J) simulating the tethered complex. The polymerase active site is *highlighted* with the incoming dNTP base (*pink*) pairing to the primer–template junction prior to catalysis. Primer–template DNA was modeled in the active site from analogous interactions with the RB69 polymerase-DNA structure (PDB ID: 1IG9)

(Beattie and Bell 2012; Pascal et al. 2006; Indiani et al. 2005). The retention of additional DNA replication accessory factors as well as multiple copies of DNA polymerases in high local concentration at the replication fork as coordinated by PCNA provides for uninterrupted DNA synthesis ability.

Lesion bypass polymerases from the Y-family are also known to interact with the crenarchaeal PCNA1 subunit (Xing et al. 2009). Much like the B-family polymerases, those of the Y-family exhibit increased processivity and replication rates in the presence of the clamp (Grúz et al. 2001; Dionne et al. 2008). Although much is known about the mechanism of lesion bypass for these Y-family DNA

polymerases, the influence of PCNA on this activity is not known. PolY from euryarchaeal *Methanosarcina acetivorans* is unique among Y-family pols as when it is complexed with PCNA; it can synthesize extremely long products, greater than 7.2 kb (Lin et al. 2010). Typically mutagenic lesion bypass polymerases are not able to synthesize such long products presumably due to the effect their low fidelities would have on the genome. Therefore, molecular access to PCNA must be regulated.

The interaction between the polymerases and differing subunits of PCNA has led to the proposal that both B-family and Y-family polymerases can be retained simultaneously for dynamic polymerization in the presence of lesions (Furukohri et al. 2008; Heltzel et al. 2009). If the replicative polymerase (PolB) encounters a DNA damage site, it can shuttle between polymerase and exonuclease sites destabilizing binding in favor of PolY binding (Maxwell and Suo 2013). After switching, the Y-family polymerase bypasses the damage site while maintaining PolB within the holoenzyme. After the lesion has been successfully bypassed, the B-family polymerase can return, reestablishing accurate and normal DNA synthesis. Of course, this would require that multiple polymerases are intimately associated in and around the replisome.

6.4 Polymerase Coordination During DNA Replication and Repair

The DNA-binding specificity for all DNA polymerases is primarily afforded by the 3′OH on the primer strand but also includes significant contacts with both the dsDNA and the ssDNA templates. When multiple DNA polymerases are present in a single organism with similar DNA substrate specificities, a question arises as to how polymerase binding to DNA is regulated to ensure accurate DNA replication and efficient DNA repair. Regulation of polymerase binding and access to DNA in the cell utilizes multiple biophysical strategies including kinetics, thermodynamics, transcriptional and translational regulation, interactions with accessory proteins, and oligomer formation.

6.4.1 Oligomeric DNA Polymerase Complexes

In *E. coli*, three DNA polymerase III cores are coordinated within the replisome by the clamp-loader complex (McInerney et al. 2007). Two of the three polymerase cores are proposed to be involved in the formation of alternating Okazaki fragments, while the other consistently synthesizes DNA on the leading strand. Other polymerases including T4 gp43 (Ishmael et al. 2003), and Klenow (Purohit et al. 2003), have been found to interact with DNA in a dimeric state. In Archaea,

*Sso*PolB1 can assemble into a unique trimeric complex to increase both replication rate and processivity of the enzyme (Mikheikin et al. 2009). Trimeric *Sso*PolB1 is observed at temperatures ranging from 10 to 70 °C where processivity values increase with temperature and routinely exceed 1,000 bases (Lin et al. 2012). As discussed above, DNA polymerases are generally nonprocessive with synthesis of only 20 bases before dissociation from the template and require interactions with their respective clamps to achieve extremely large processivity values. As such, we have suggested that the large processivity value for trimeric *Sso*PolB1 is a consequence of direct interactions between subunits, effectively encircling the DNA template akin to the structural role of the clamp proteins (Fig. 6.5a). Similarly, the Y-family polymerase, *Sso*PolY, also forms oligomeric complexes on DNA in a concentration-dependent manner as highlighted in a variety of crystal structures (Fig. 6.5b). The function of oligomeric PolY complexes is not known and no known enzymatic enhancement has been noted, but this interaction may be used to keep high concentrations of polymerases at the replication fork. In addition, direct interactions between the replication (*Sso*PolB1) and lesion bypass (*Sso*PolY) polymerases have also been detected highlighting another contact point for polymerase exchange (De Felice et al. 2007).

In all, these results suggest that polymerase action may be more complicated than previously thought, utilizing both intimately and loosely bound polymerase molecules in the replisome. In T4 and T7, DNA polymerase molecules can exchange freely at the site of catalysis during replication (Yang et al. 2004; Loparo et al. 2011). This "dynamic polymerase processivity," along with the variety of different possible homo- and heteroligomeric complexes that can form between *Sso*PolB1 and *Sso*PolY, highlights the possibility that the archaeal cell utilizes these complexes in a variety of yet unknown genomic maintenance functions. The regulation of oligomeric polymerase complex formation may be controlled through subtle changes in equilibria at the site of action to afford specific genome maintenance functions.

6.4.2 Thermodynamic DNA Polymerase Selection

Although DNA polymerase binding can be quantified using a number of methods, the most complete way to quantify the binding specificity is from a measure of the heat capacity change (ΔC_p^o). For thermophilic DNA polymerases binding to DNA, there is a broad temperature range that can be explored generally giving large and negative ΔC_p^o values. These negative ΔC_p^o values highlight strong structural complementarity between the polymerase and DNA instead of the more traditional explanations of DNA sequence specificity. In comparing the ΔC_p^o values for B- and Y-family polymerases from *Sso*, we have found that *Sso*PolB1 and *Sso*PolY have similar strong enthalpy–entropy compensation in binding over a large temperature range giving rise to smaller fluctuations in free energy (ΔG^o) up to 50 °C

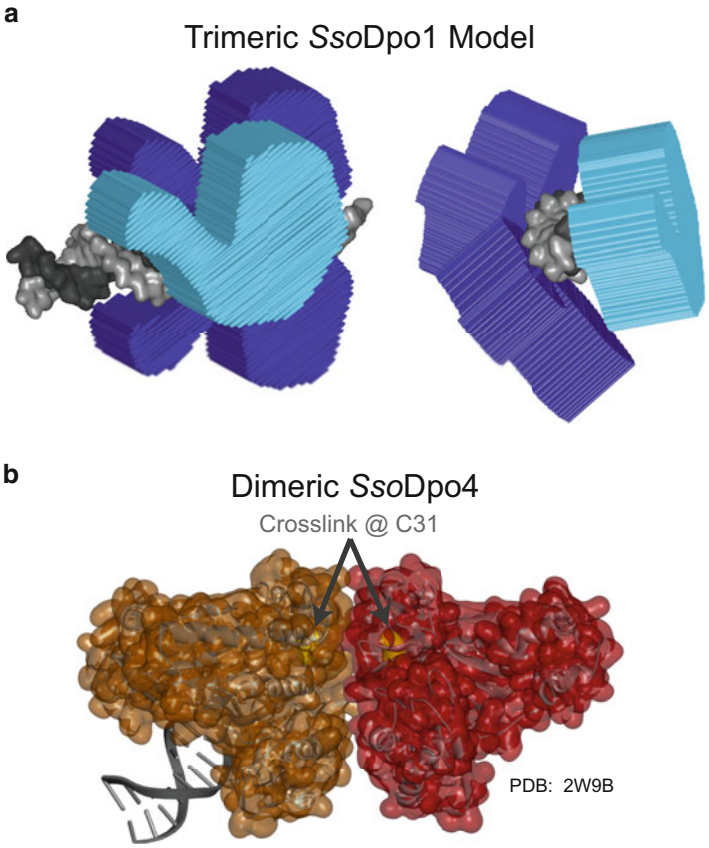

Fig. 6.5 (**a**) Model of the trimeric *Sso*Dpo1 polymerase bound to DNA. The *encircled* conformation is proposed based on binding and footprinting on a small primer–template DNA substrate. (**b**) Crystal structure (PDB: 2W9B) of dimeric *Sso*Dpo4 consistent with chemical cross-linking at cysteine 31

(Lin et al. 2012). Above 50 °C, there is higher affinity of binding for *Sso*PolB1 over *Sso*PolY providing thermodynamic discrimination of monomeric binding at high physiological temperatures. In addition, there are similar sequential binding events dependent on local concentration and temperature that modulate oligomeric *Sso*PolB1 and *Sso*PolY coupled equilibria (Lin, Trakselis et al., unpublished). There are extreme differences in the free energies (ΔG^{o}) of binding oligomeric *Sso*PolB1 over *Sso*PolY that are exaggerated with temperature providing an additional level of thermodynamic selection for oligomeric B-family polymerases on DNA during normal replication in the absence of damage (Lin et al. 2012). The collective results suggest that binding of both polymerases to DNA is not necessarily dictated by specific interaction differences between replication and repair polymerases and DNA, but rather thermodynamic selection of more stable oligomeric DNA polymerase complexes at high temperatures to promote accurate synthesis.

6.4.3 *Uracil Read-Ahead Function for Archaeal B-Family Polymerases*

Another unique property of archaeal B-family polymerases is their ability to detect uracil in the downstream DNA sequence (Greagg et al. 1999). The presence of uracil in DNA usually arises due to the deamination of cytosine; however, as a result of uracil base pairing to thymine, this deamination results in a transversion to a C-G base pair after replication. A uracil recognition pocket was identified in the N-terminal region of *Tgo*PolB (Hopfner et al. 1999) and *Sso*PolB1 (Savino et al. 2004) that stalls the polymerase four bases from the primer–template junction (Fig. 6.1). Mutations in the recognition pocket of *Pfu*Pol restored the ability to replicate through uracil, confirming that this region is responsible for the uracil "read-ahead" function (Fogg et al. 2002). A Mg^{2+} ion (exchangeable with Mn^{2+}) has recently been identified just below the uracil recognition pocket in *P. abyssi* (*Pab*) PolB that guides the DNA to the binding site (Gouge et al. 2012). In *Pfu*PolB, sensing of uracil (+4 on the template) seems to stimulate the unwinding of the 3′ end of the primer and exonuclease proofreading, trimming the primer to maintain distance from the uracil (Richardson et al. 2013). It is noteworthy that this uracil binding pocket is not present in eukaryotic B-family polymerases, including those with high homology to archaeal B-family polymerases. As a result, it is speculated that the uracil read-ahead function serves as a means to recognize DNA damage prior to replication in organisms with incomplete base excision repair (BER) or nucleotide excision repair (NER) pathways.

6.4.4 *Polymerase Participation in DNA Repair*

Archaea maintain a level of genomic stability equivalent to or slightly better than other microorganisms. This is somewhat surprising due to the environments that many of these organisms thrive in where oxidations and deaminations would be common. Therefore, Archaea must possess very robust mechanisms by which DNA damage is repaired (Grogan et al. 2001). In bacteria and eukaryotes, DNA is typically repaired through several means including error-prone lesion bypass, BER, NER, and mismatch repair. B-family and D-family polymerases contain exonuclease domains and would shuttle between incorporation and excision events at sites of DNA damage. Most Archaea also possess Y-family polymerases capable of performing the error-prone lesion bypass processes in spite of DNA damage; however, this ability may not be essential as knockouts of PolY are unaffected by DNA damaging agents (Sakofsky et al. 2012).

As the error-prone polymerases are not universally conserved, DNA repair in Archaea must include alternative mechanisms. Traditionally, BER and NER pathways would fulfill this role, but many Archaea are missing many of the proteins homologs to either the eukaryotic or bacterial versions making identification

difficult (Kelman and White 2005). Therefore, it is still not understood how Archaea maintain stable genomes even in spite of intense environmental stress, but it is certain that polymerases in Archaea will have both specific and redundant roles in repair processes.

6.5 Conclusion

DNA polymerases from Archaea have both unique and conserved biochemical features compared with polymerases from other domains. Because of their high homology, similar structures, and conserved catalytic features, they are considered to be relevant model systems for understanding mechanisms of action and protein interactions involved in DNA replication and repair. Cells that contain multiple DNA polymerases need a mechanism to regulate individual recruitment to the replication fork or DNA damage site for specialized nucleotide incorporation. As we learn more about the specificities and kinetics of each DNA polymerase, the molecular interactions with accessory proteins, and the dynamics that occur to coordinate their activities, we can better comprehend how their activities are regulated. This is of great importance for understanding how genomes of all organisms are accurately maintained in spite of intense environmental stressors that are mercilessly acting the DNA. Only after revealing how multiple DNA polymerases dynamically orchestrate their specificities can we attempt to modulate this activity to promote evolutionary diversity of new function or restrict cancer cell growth for specialized therapies.

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Chapter 7
Engineered DNA Polymerases

Roberto Laos, Ryan W. Shaw, and Steven A. Benner

Abstract This chapter reviews the methods used to generate variants of DNA polymerases that have improved ability, in particular to accept unnatural nucleotides, focusing especially on in vitro and directed evolution methods. Several natural families of DNA polymerases have independently evolved for millions of years to accept their natural nucleotide substrates with high fidelity and the ability to exclude closely related structures, such as ribonucleoside derivatives. However, polymerases that can accept unnatural nucleotide substrates would have many applications in biotechnology. Directed evolution may be an efficient method to produce new DNA polymerases capable to do so. Directed evolution relies on methods to create a library of sequence diverse polymerases starting with a gene for a parent polymerase. These methods are reviewed here, as well as examples of their application to produce variant polymerases. An evolutionary rationalization is offered to explain some mutations produced by directed evolution experiments.

Keywords Nucleic acids • DNA polymerases • AEGIS • PCR • CSR • Phage display • In vitro evolution

Abbreviations

AEGIS	Artificially expanded genetic information system
CSR	Compartmentalized self-replication
ddGTP	Dideoxy guanosine triphosphates
ddNTPs	Dideoxy nucleotides triphosphates
dNTPs	Deoxy nucleotides triphosphates
ePCR	Error-prone PCR

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PCR	Polymerase chain reaction
REAP	Reconstructed evolutionary adaptive paths
Sf	Stoffel fragment
Taq	*Thermus aquaticus*
Taq pol I	*Taq* DNA polymerase
TBD	Thioredoxin-binding domain

7.1 Introduction

DNA polymerases are enzymes responsible for catalyzing the template-directed synthesis of DNA. Over billions of years, they have evolved to have the speed, specificity, and accuracy required for them to transmit valuable genetic information from and to living organisms, with just enough infidelity to support Darwinian evolution. These properties allow polymerases to be used routinely in biotechnology. This use increased with the invention of the polymerase chain reaction (PCR) by Kary Mullis, who was awarded the Nobel Prize in chemistry in 1993 for his accomplishment.

PCR iterates a relatively simple cycle of strand-strand dissociation, primer annealing, and polymerase extension to produce, in an exponential amplification, millions of copies of product DNA from just a few molecules of starting nucleic acid (DNA or RNA). This technique was improved dramatically since it was first reported (Saiki et al. 1985). Initially, PCR was performed using *Escherichia coli* DNA polymerase I. However, this enzyme is unstable at the temperature used for duplex strand dissociation, typically 95 °C. This made it necessary to add extra polymerase for each cycle.

Three years later, the introduction of a homolog of DNA polymerase I from the thermophilic bacterium *Thermus aquaticus*, *Taq* polymerase (*Taq* pol I), was reported to overcome this limitation (Saiki et al. 1988). *T. aquaticus* grows naturally at 70 °C. Therefore, *Taq* pol I has evolved to remain active at high temperatures, and this thermostability allows it to survive the repeated heating steps required for PCR. The availability of *Taq* polymerase and of PCR thermal cyclers made PCR automated and routine.

Currently, many DNA polymerases are used to support PCR and other procedures that involve the copying of nucleic acids. These include multiplexed PCR, nested PCR, reverse transcription PCR, and DNA sequencing among others. Polymerases are also used to incorporate modified nucleotides, including those that tag, report, or signal the presence of product DNA. These allow nucleic acids to be amplified from complex samples, including blood, saliva, forensic traces, and fossil remains. The choice of a particular polymerase depends on the specific need, especially for processivity and fidelity, temperature of initiation, or ability to accept unnatural nucleotide analogs.

However, despite the large number of commercially available polymerases with their particular attributes, the need for additional polymerase variants, especially

those with specialized attributes, shows no sign of diminishing. This is especially true as new architectures for amplifying and detecting nucleic acids emerge and with the growing interest in the use of unnatural nucleic acids.

7.2 Direct Design of New Polymerases with Novel Properties

The availability of high-quality crystal structures for many polymerases, as well as advanced computational tools, has encouraged many to alter the structure of natural polymerases by design. Two categories of direct design have achieved special importance.

7.2.1 Fully Guided Modifications to the Sequences of DNA Polymerases Themselves

The first efforts to modify a DNA polymerase to obtain variants better able to incorporate unnatural nucleotide substrates were motivated by the need to use dideoxynucleotides triphosphates (ddNTPs) in Sanger-type DNA sequencing. Native *Taq* pol I can incorporate ddNTPs, but at considerably lower rates than it incorporates natural deoxynucleotides triphosphates (dNTPs). In addition, this enzyme incorporates each ddNTP with a different rate, producing uneven DNA sequence signals.

Using a guided approach, Tabor and Richardson started with three DNA polymerases that belong to the same Family A (Braithwaite and Ito 1993). They noticed that bacteriophage T7 DNA polymerase incorporated ddNTPs better than the homologous polymerases from *E. coli* and *T. aquaticus*. Of course, the T7 polymerase has evolved to grow at 37 °C and does not have the thermostability required for PCR. However, Tabor and Richardson noticed that T7 polymerase had a tyrosine at a position (numbered 526) homologous to positions that held a phenylalanine in the *E. coli* and *Taq* polymerases (positions numbered 762 and 667, respectively). They hypothesized that this single amino acid difference was responsible for the different levels of discrimination against ddNTPs among the three polymerases.

Based on this hypothesis, Tabor and Richardson replaced the phenylalanine in the *Taq* polymerase by a tyrosine. The result was a variant *Taq* (F667Y) that retained the thermostability of the *Taq* parent but had improved ability to accept ddNTPs. Similar improvement was seen when the analogous replacement was made in the polymerase from *E. coli*. The mutant *Taq* (F667Y) became one of the first specialized polymerases used for DNA sequencing (Tabor and Richardson 1995).

Subsequently, Li et al. (1999) studied the crystal structures of Klentaq1, a derivative of *Taq* DNA polymerase that lacks an exonuclease domain. In separate structures, protein crystals binding ddNTPs were observed to have closed ternary complexes, where a conformational change upon substrate binding was associated with a large shift in the position of the side chain of residue 660 in the O helix. Comparing the open and closed structures with ddGTP, Li et al. concluded that the selective interaction of arginine 660 with the O6 and N7 atoms of the G nucleobase provided the structural grounds for better incorporation of ddGTP by *Taq* polymerase. Guided by these observations, Li et al. then replaced amino acids at residue 660 in Klentaq1 already holding the Tabor–Richardson (F667Y) replacement and then studied the resulting variants. Among the variants, the double mutant *Taq* (F667Y; R660D) showed superior performance in DNA sequencing architectures that used ddNTPs (Li et al. 1999).

7.2.2 *Fused Polymerases for Increased Processivity*

These engineering efforts have modified the sequence of the polymerase itself. However, a separate class of modifications appends polymerases to proteins that are not polymerases.

Most DNA polymerases responsible for large-scale DNA copying in their natural environments have evolved to use accessory proteins to enhance their processivity. These are, however, generally not used in biotechnology, because of the complexity of the assembled combination. Indeed, *Taq* polymerase and other enzymes are used by themselves for PCR because of their simplicity, a simplicity arising from their physiological roles in lagging strand replication and DNA repair. For this natural function, *Taq* need not be particularly processive compared to, for example, polymerase III, which carries out most leading strand DNA synthesis.

Thus, it is sensible to consider adding to simple polymerases one or two of the factors used in complex polymerases that makes them processive. This would create a simple system that is processive, with various advantages for in vitro DNA replication.

Following this rationale, Wang and collaborators covalently fused the double-stranded DNA-binding protein *Sso*7d from *Sulfolobus solfataricus* at the N-terminus of *Taq* polymerase (S-*Taq*) and to the fragment of *Taq* polymerase that results from the deletion of the first 289 amino acids which lacks the exonuclease domain (S-*Taq*(Δ289)). The average primer extension of *Taq*(Δ289) was increased from 2.9 to 51 nucleotides in S-*Taq*(Δ289). The full-length *Taq* polymerase which is intrinsically more processive than *Taq*(Δ289) improves its average primer extension from 22 (*Taq*) to 104 (S-*Taq*) nucleotides. Similarly they fused the *Sso*7d domain to the C-terminus of the polymerase from *Pyrococcus furiosus*, *Pfu* polymerase (*Pfu*-S). Just as in the case of *Taq* polymerase, the fusion of the *Sso*d7 domain leads to an increase of the average primer extension, from 22 nucleotides for *Pfu* to 104 for *Pfu*-S (Wang et al. 2004).

Another example of increased processivity of *Taq* polymerase uses the insertion of the T3 bacteriophage DNA polymerase thioredoxin-binding domain (TBD) in the thumb domain of *Taq* DNA polymerase, deleting amino acids 480–485 (Davidson et al. 2003). The rationale behind this modification is that T7 DNA polymerase forms a complex with *E. coli* thioredoxin. Upon binding, the processivity increases from 15 to 2,000 nucleotides as well as increasing the affinity to the primer/template by 80-fold. The TBD of T3 bacteriophage differs only in one amino acid from the TBD of T7 bacteriophage. The resulting polymerase remains thermostable, and its processivity is 20–50 times higher than the original *Taq* pol.

7.3 Protein Engineering Methods for Evolved DNA Polymerases

These successes of "design" in polymerase engineering are not frequent and for good reason. First, the design required crystal structures of enzymes that are closely homologous to the enzyme that starts the engineering. These crystal structures are useful to guide engineering only if they have bound substrates or substrate analogs. With the Tabor–Richardson analysis, the guidance also relied on the fortunate discovery of a homolog having the desired properties. This kind of information and understanding is not always available.

Further, the demands placed on a polymerase to incorporate unnatural triphosphates in Sanger sequencing procedures are much lower than the demands placed on a polymerase to repeatedly copy, as in PCR, DNA molecules containing unnatural nucleotides. In particular, the fidelity required by a DNA polymerase to support PCR with unnatural nucleotides must be very high. Further, the structural differences between a DNA polymerase that makes one error per thousand nucleotides and one error per million can be quite subtle, involving geometric differences that would not be necessarily distinguished even in a high-resolution crystal structure. Thus, the design of a high-fidelity DNA polymerase from a medium-fidelity polymerase is largely beyond current structure theory, making it impossible to get polymerases with the desired high-level behaviors from fully guided protein engineering.

As a consequence, many investigators use protein engineering to select for polymerases with certain properties improved with respect to a desired function, starting from libraries of polymerase variants. This approach, often called "directed evolution," is today considered by many to be the method of choice for protein engineering (Bornscheuer and Pohl 2001; Yuan et al. 2005; Leemhuis et al. 2009; Turner 2009) although other complementary methods also exist.

7.3.1 Directed Evolution

In a directed evolution experiment, a "starting" enzyme is first identified as having (at least) some of the properties desired in the enzyme that is desired as the end product. The gene of this parent enzyme is then altered to create a library encoding variant forms of the enzyme, some of which might be able to catalyze the desired transformation better than the parent enzyme. The members of the library are then not screened, a process whereby the members of the library are tested individually to find those having the desired properties. Rather, in directed evolution, the collection of variants is processed in bulk, where this "selection" generates extracts enriched in variants that better allowed them to survive. If the selection is properly constructed, the survivors will have mutations that confer the desired properties. This process is shown schematically in Fig. 7.1. With iteration, the process mimics natural evolution, except that the selective pressures applied come from the bioengineer, rather than nature.

7.3.1.1 Library Creation and Protein Sequence Space

The success of a directed evolution exercise depends on the success of two key steps (a) the generation of a collection of variants of the protein (called a library) that include members having the desired properties and (b) a selection strategy that allows survivors to actually have the properties that are desired in the end product. As we consider the first of these, it is useful to understand the magnitude of the challenge.

The size of the "protein sequence space" that might be explored by a directed evolution experiment is immense. As discussed by Smith (1970), the behavior of all possible proteins of length n with respect to a measurable behavior can be represented by a space in n dimensions, where each dimension can have 1 of 20 values, representing the 20 natural amino acids. Each protein sequence is represented by a point in that space. Two points are neighbors in that space if one can be converted into another by a single amino acid substitution. With 20 amino acids, each point in the sequence space has $19n$ neighbors. The measurable behavior is a real number displayed in the nth + 1 dimension.

Different sequences have different functions, and moving from a sequence having a function to another functional sequence can proceed via intermediates that either have or lack function. This is illustrated in Fig. 7.2 with a word game used by Smith, where functional protein sequences are analogous to strings of letters with a meaning in English. In Smith's (somewhat imperfect) analogy, the sequence of letters in the word "WORD" is converted to the sequence of letters in the word "GENE" by exchanging one letter at the time, with one path having a sequence of letters with a meaning (WORE, GORE, and GONE), illustrated by solid lines. Pathways that proceed via words lacking meaning are illustrated by broken lines (e.g., WOND, GOND, and GEND).

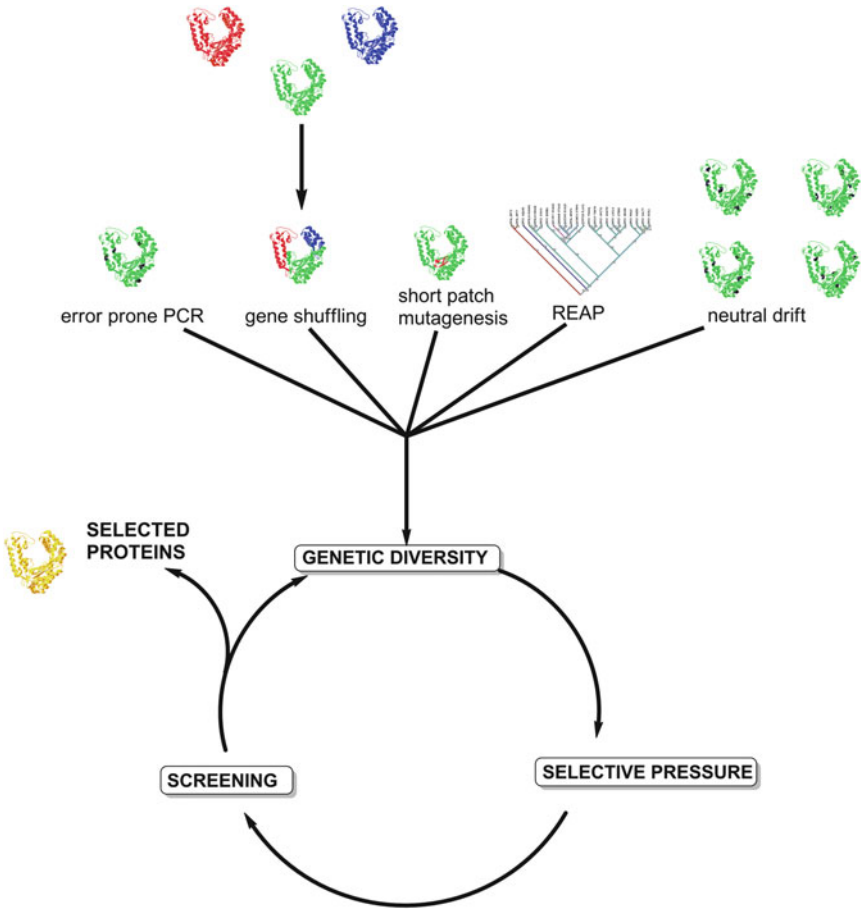


Fig. 7.1 Directed evolution experiments start with genetic diversity created by, for example, error-prone PCR (ePCR); gene shuffling; short-patch mutagenesis or design, including design methods that rely on evolutionary information such as reconstructed evolutionary adaptive paths (REAP); or neutral drift libraries. A selective pressure is imposed on the variants so that only polymerases with desired properties are enriched in a collection of polymerases, which may then be screened. This is most appropriate if the desired behavior is sparsely distributed within the library. Alternatively, genes for the variants that survived the selection can then be the starting points for processes that create new genetic diversity. Iteration of this process eventually leads to enzymes displaying properties produced by the selective pressures imposed on the experiments

In this example, meaningfulness is an analog of fitness, which provides the *n*th +1 dimension to the surface, a "fitness landscape" (Wright 1932). The landscape is portrayed like in a topographic map with peaks, marked with a (+) for optimal sequences. The absence of function is depicted as dips, marked with a (−). Smith proposed that natural evolution proceeds along paths only if all intermediates are functional. Nonfunctional sequences are removed by "purifying" selection. Thus,

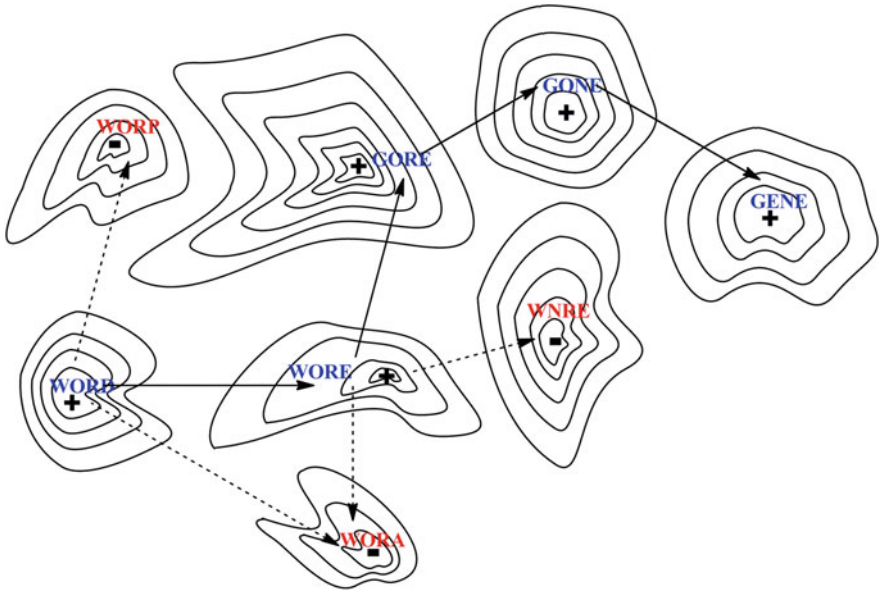


Fig. 7.2 Evolution can be modeled as a walk across a fitness landscape, here presented as a two-dimensional representation of a multiple dimensional hypersurface; analogous to a topographic map, peaks (*plus symbols*) indicate the locations where function exists, while dips (*minus symbols*) represent regions with lack of function. Illustrated through an analogy to a word game, a meaningful (functional) string of letters (here "word") must be reached starting from another string ("gene") via stepwise replacement of single letters, where every intermediate along the path must itself also be a functional word. *Solid arrows* indicate a path of accepted mutations, while *dashed arrows* illustrate deleterious mutations that produce nonfunctional proteins

the only valid pathways to explore a sequence space proceed via functional sequences, just as the evolution of words can proceed only via meaningful words.

Sequence space is vast to the point of being incomprehensible. For example, a 100-amino acid protein can be arranged in 20^{100} different ways. Typical polymerases, eight times longer, are found in a space with 20^{800} points. Both numbers are astronomical. No experiment can sample this space effectively, even if all the atoms of the known universe were available.

Accordingly, a search of sequence space can be successful only if one of three things is true: (a) the fitness landscape is "smooth," meaning that a single useful protein can be obtained starting with any sequence via a path that encounters only other functional proteins or, if not, then (b) useful functional proteins can be obtained no matter where one starts the search, as the surface is littered with many of them or, if not, then (c) the library is guided so as to start the search in a region of the astronomical enormity of sequence space where usefully functional proteins reside. Presumably, selecting a parent invariably does (c), at least in part.

7.3.1.2 Completely Unguided Library Creation by Random Variation

Cast in this way and given our ignorance of the "smoothness" of protein sequence spaces in general, it is clear that the directed evolutionist should give some thought to how a library might be generated. Given that selection of a parent polymerase is likely to start in a region of the fitness landscape that is already quite elevated, the simplest approach is to simply randomly mutate the gene of the parent polymerase. Various tools are available to do so. One of these exploits PCR, under conditions where the polymerase performing the copying makes more mutations than typical. Known as "mutagenic" or "error-prone" PCR (ePCR), this approach takes advantage of the inherent propensity of *Taq* polymerase to introduce mistakes into the copies of DNA under certain conditions. The frequency of mismatching is often increased by introducing manganese Mn^{2+} along with the natural cofactor Mg^{2+} (Vartanian et al. 1996). Other additives such as alcohols or unbalanced concentrations of nucleotides can also be used to introduce mutations through PCR.

Error-prone PCR produces mutations that are "unguided," in the sense used in this review. Nevertheless, they do not produce a truly random set of amino acid replacements, for a variety of reasons:

(i) *Taq* pol tends to replace purines (adenine and guanine) by purines and pyrimidines (thymidine and cytidine) by pyrimidines; these changes are called transitions. The exchanges of a purine by a pyrimidine and vice versa are called transversions. The tendency of the polymerase for transitions leads to produce libraries with amino acid replacements that are nonrandom with respect to the parent protein.

(ii) Even if ePCR introduced transitions and transversions entirely randomly, the resulting amino acid replacements would not be random. Most amino acids are encoded by more than one codon; most of them cannot be interconverted by a single-nucleotide change. Further, similar codons code for amino acids having similar chemical properties (Wong et al. 2007). For instance, the valine codon (GTN[1]) is converted by a single-nucleotide replacement to a phenylalanine (TTY[2]), leucine (CTN), isoleucine (ATN), aspartate (GAY), or glycine (GGN) codon. To gain access to other amino acids and, consequently, more dramatically altered chemical properties, two or three nucleotide replacements are required. For example, with valine, two nucleotides must be replaced to get a codon for proline (CCN). Typical ePCR introduces no more than four to six mutations per 1,000 nucleotides; higher mutation rates tend to disrupt protein function. Under these conditions it is very unlikely to introduce two or three consecutive mutations at the DNA level to fully explore amino acid sequence space.

[1] N is any nucleotide.

[2] Y is a pyrimidine.

Reetz et al. (2008) applied an approach where a subset of the 64 standard codons is introduced in specific sites by one or two degenerated codons: NNK and NDT. Here, D is adenine, guanine, or thymine, K is guanine or thymine, and N is any nucleobase. With the NNK degenerate codon, all 20 amino acids are covered by just 32 ($= 4 \times 4 \times 2$) of the 64 codons possible with standard nucleotides. With the 12 ($= 4 \times 3 \times 1$) codons covered by the NDT degenerate codon, only a sample of the standard amino acids can be encoded. This sample includes, however, a representative set of nonpolar, aromatic hydrophobic, hydrophilic, and charged amino acids.

Further thought generates further considerations about what is meant by "random" when discussing amino acid replacements. With 20 amino acids, a fully random sequence might have, on average, 5 % of each of the natural amino acids in bulk. However, this distribution is not found in natural proteins nor is it expected in proteins encoded by random genes. Because some amino acids (like serine, with six codons) are encoded by more codons than other amino acids (like tryptophan, with just one codon), a gene with a truly random nucleotide sequence would give proteins with a codon-weighed distribution of amino acids. Even this might not be the desired goal of an unguided approach to library generation, since some amino acids (aspartate and glutamate, e.g., each with two codons) appear in natural proteins more abundantly than is expected from their few codons. Thus, an "ideal" library might arguably be one where amino acids are replaced by a process that leaves the naturally observed overall composition of the protein unchanged. Finally, given our ignorance on the shape of function landscapes generally, as well as our ignorance of the local topography around any individual parent sequence, a perfectly random mutagenesis tool need not be the most useful approach for a directed evolution experiment.

7.3.2 Libraries Made by Gene Shuffling or Molecular Breeding

One obvious deficiency of random mutagenesis of a parent gene is that it fails to use all of the available information, information that is especially abundant in a post-genomic world. In particular, nature has already provided many homologs of a parent protein having many amino replacements relative to the parent sequences. Most of these homologs are functional, identifying other points in the vast sequence space that are elevated on the fitness landscape. It would be desirable to use the information that these homologs provide.

Gene shuffling was introduced by Stemmer (1994) more than a decade ago to directly use these homologs. Here, the starting point is a family of genes that share enough sequence similarity that they can be recombined. Using a modified PCR protocol, gene chimeras are produced. These are then often screened for

recombinants that have the desired activity, although a collection of recombinants can also be the starting point for a selection step.

Those using shuffling in protein evolution assume, of course, that sequence space is more efficiently searched by combining the outcomes of two historically successful searches of a particular region of sequence space, than a search that simply replaces single amino acids starting from a single parent. These historical searches delivered the two functioning proteins whose genes are being shuffled. Here, the landscape is assumed to be such that specific paths between two elevated points are also similarly elevated.

This would be a more compelling hypothesis if natural evolution were observed to use shuffling. Natural evolution does, of course, have access to mechanisms that shuffle parts of genes. Natural evolution uses these mechanisms to rearrange, for example, the order of independently folded units in multiunit polypeptides. This is famously done in the evolution of multiunit proteins involved in metazoan signal transduction, where a regulatory protein might contain one "src homology domain 1" unit (SH1, a protein kinase), a few SH2 units, and a few SH3 units (Benner et al. 1993). Evolutionary analysis shows that these are all obtained by shuffling, implying that shuffling is an efficient way to search sequence space when no protein folding unit is disrupted.

However, natural evolution does not provide many examples where polypeptide chains within a *single* folded unit are shuffled. This is presumably because the buried contacts binding collections of secondary structural units are finely tuned to permit packing. Changing a single hydrophobic side chain in a packed protein fold often converts a core that is (typically) as densely packed as an organic crystal into a "molten globule." Thus, these biophysical realities would make it surprising to expect that shuffling explores sequence space more effectively than point mutation. Such expectations rely, of course, on the view that natural evolution exploits the most effective ways to search sequence space.

This notwithstanding, various individuals have used shuffling to generate polymerase libraries for directed evolution experiments. For example, d'Abbadie et al. (2007) shuffled the genes of the polymerases from three *Thermus* species (*aquaticus*, *thermophilus* and *flavus*) to generate libraries to start a directed evolution experiment to identify DNA polymerases that can extend single, double, and quadruple mismatches, process noncanonical primer-template duplexes, and bypass hydantoins and abasic sites (d'Abbadie et al. 2007). They applied these to PCR-amplify cave bear DNA from remains ca. 50,000 years old. These experiments showed that the polymerases obtained by directed evolution applied to these libraries outperformed Taq DNA polymerase, and were therefore better able to solve a biotechnological problem, here the sequencing of ancient damaged genomes.

In another example from the Holliger laboratory, Baar et al. (2011) shuffled the genes from eight different polymerase orthologs from the genus *Thermus* to generate libraries for their directed evolution system (Baar et al. 2011). Here, polymerases were sought that were not inhibited by various complex environmental inhibitors. For example, a polymerase resistant to inhibition by a broad spectrum of

complex inhibitors (humic acid, bone dust, peat extract, clay-rich soil, cave sediment, and tar) was found that contained parts of the sequence of DNA polymerases from *Thermus aquaticus*, *Thermus oshimai*, *Thermus thermophilus*, and *Thermus brockianus*. Again, these polymerases might be useful in analyzing archaeological samples. However, as before, these experiments do not help us understand whether shuffling is a more effective way to search sequence space within compact single protein folds.

7.3.3 Evolution-Based Approaches to Search Sequence Space Around Parent Sequences

Alternative approaches now exist to create libraries that search sequence space around parent sequences (Lutz and Patrick 2004; Jackel et al. 2008; Lutz 2010). One class of these exploits the divergence of sequences in homologs of the parent enzyme. For example, Gaucher, Benner, and their coworkers introduced an approach, called the reconstructed evolutionary adaptive paths (REAP) approach, to create libraries that were hypothesized to more efficiently explore local sequence space (Chen et al. 2010). REAP begins with a phylogenetic analysis of homologous sequences, seeking signatures of functional divergence (Cole and Gaucher 2011). For example, an amino acid at a site may be entirely conserved in one branch of a phylogenetic tree, while not conserved at all in a second branch. This pattern of divergence, called *heterotachy*[3] (Lopez et al. 2002), indicates that the purifying selective pressures operating in the first branch at this site are different (and stronger) than those in the second branch. This, in turn, means that the function of the proteins within the first phylogenetic branch is different from the function in the second branch.

The variation observed in natural history was, of course, only rarely responsive to the specific adaptive changes desired by today's biotechnologist. Ancient polymerases, for example, were most likely *not* evolving to become, for example, resistant to heparin. The rationale is more subtle. The phylogenetic information used in a REAP analysis identifies sites that have been historically involved in *some* adaptive event. Because *some* changes are involved, the amino acid at the site cannot be *absolutely* required for core function. Conversely, the REAP-identified sites are not likely to be those whose amino acid *never* has a phenotypic impact. The rationale is that sites that have in the past been involved in an adaptive event without losing core function are sites that might be productively examined to identify sites that might adapt the protein to the *new*, biotechnologist-demanded function.

REAP is based on the hypothesis that the most productive sites to replace in a protein engineering experiment are *neither* sites whose amino acids contribute to a

[3] Different speed in Greek.

core function (as indicated by their absolute conservation) *nor* sites where the choice of amino acid is incidental to function (as indicated by their easy variability). By identifying sites where replacement *might* have phenotypic impact *without* destroying core function, REAP is proposed to have an advantage compared to other methods in the generation of libraries with productively altered behaviors. The advantage of the REAP approach relies on the fact that nature has already tested several amino acid sites and these modifications on these sites produce enzymes that retain the original activity. Searching for new variants in a REAP library gives the advantage of having several parent enzymes.

Chen et al. have applied REAP to generate DNA polymerases able to accept unnatural triphosphates modified on their sugar units. Using REAP, they identified 35 sites having heterotachous pattern of divergence, after filtering for sites where additional information from evolutionary history, structural biology, and experiments was exploited. They then asked which replacements improve the ability of *Taq* polymerase to accept reversible terminating triphosphates, where the 3′-OH unit of the nucleoside triphosphate had been replaced by an $–ONH_2$ unit, which prevents continued primer extensions. A single modification (L616A) appears to open space behind Phe-667, allowing the enzyme to accommodate a larger 3′-substituent (Chen et al. 2010).

7.3.4 Neutral Drift Libraries

REAP focuses the bioengineer on sites that have historically been involved in adaptive events. It does, perforce, exclude sites whose amino acids must be specific for core function. Deleterious mutations, those that diminish the fitness of the host by damaging core function, are expected to be removed by "purifying selection."

A conceptually simpler way to focus the bioengineering experiment considers only sites that are able to vary without impact on core function, variation that is often called "neutral." Neutral drift is traditionally defined as a natural process whereby genetic changes accumulate over geological time without having any impact on the fitness of the host organism. More recently, the concept has been expanded to include nucleotide substitutions that have perhaps only small impacts on fitness. It has been argued that most mutations that accumulate during natural history are neutral.

Here, the rationale holds that mutations that do not damage core function might nevertheless allow the enzyme to perform in a biotechnologically more useful way. Thus, a "neutral drift library" might be expected, as a starting point for a directed evolution experiment, to be already depleted in proteins lacking core function. Under this rationale, a bioengineer should start by creating a collection of variants that are all active under (approximately) natural conditions. This smaller but functional library might be more useful than a large randomized library where the large majority of the population lacks of function.

Neutral drift libraries have not been applied to DNA polymerases, but have been successfully used for other enzymes. For instance, Frances Arnold and her collaborators began an engineering experiment by selecting for active cytochrome P450 enzymes among a random library of enzyme variants. Screening the recovered mutants, they found enzymes able to catalyze oxidations of five other substrates different from the original (Bloom et al. 2007b). The use of neutral drift libraries has been further discussed by Tawfik and his coworkers (Gupta and Tawfik 2008; Bershtein et al. 2008). A particularly interesting example applied neutral drift libraries to create a beta-galactosidase from a beta-glucuronidase via two different evolutionary pathways (Smith et al. 2011).

7.4 Directed Evolution Methods Involving Genotype–Phenotype Linkage

The variants generated by shuffling, REAP, and other approaches can be (and have been) the starting points for simple screens, where the behaviors of individual proteins are examined one at a time. This was done in the early papers of Stemmer and in the work with REAP on DNA polymerases.

Screening variants requires a certain level of confidence that the approach used to vary the parent has a high probability of producing a variant that has the desired properties. If that confidence is low, or if the desired behavior is only sparsely distributed within a library of variants, screening is too slow to identify variants with the desired activities. Rather, processes are needed to be applied to all members of a large library that will generate extracts enriched in variants that have those activities. Further, if the first library is unable to deliver variants with improved activities, but not the desired levels of activities, it would be desirable to have the first extract serve as the library for subsequent selections.

In such systems, the protein must remain linked to the gene that encodes it. Further, the protein cannot have an opportunity to become linked with a gene that encodes an inferior enzyme. This linking can be done if, for example, the gene is within a bacterium whose fitness is enhanced by an enzyme that catalyzes the desired transformations. These strategies for linking genotype and phenotype are, however, problematic, as the selection often ends up being focused not on the enzyme of interest, but rather on some of the many other enzymes that are important to the survival of the host bacterium.

Accordingly, a variety of techniques have been developed to provide a physical genotype–phenotype linkage in the laboratory without requiring a complex host taken from natural biology. Two techniques used for polymerase directed evolution are compartmentalized self-replication (CSR) and phage display.

7.4.1 Compartmentalized Self-Replication

Compartmentalized self-replication (CSR) is a method that has been frequently used to link genotype and phenotype in polymerase development. Developed first by Tawfik and Griffiths, CSR holds proteins and genes together in water droplets suspended in oil emulsions. These generally receive the gene–protein pair from a single *E. coli* cell that is encapsulated within individual droplets (Tawfik and Griffiths 1998).

CSR was first adapted a decade ago for the directed evolution of DNA polymerases in the Holliger laboratory (Ghadessy et al. 2001). A library of different polymerase genes was delivered in plasmids to create clones in *E. coli* cells. These cells were dispersed into emulsified water droplets containing the primers and buffers needed to perform a PCR amplification of the polymerase gene. Approximately ~10^8–10^9 compartments are formed per milliliter of emulsion; ideally each compartment contains a single variant. PCR cycling was then performed, with the first heat step lysing the *E. coli* cell to present its expressed thermostable polymerase and its encoding plasmids to the primers. Polymerases that functioned under the conditions imposed by the experiment were able to make copies of their own genes (Fig. 7.3). After 20 rounds of PCR, the emulsions were broken to give a pool of PCR products enriched in the genes that encoded the polymerase variants able to replicate their own genes. These genes could be directly used, or be introduced in cells for another round of selection.

The Holliger laboratory applied CSR to develop polymerases that remained active in the presence of heparin. Further, as noted above, they applied CSR to polymerase libraries generated by shuffling to avoid inhibition by materials contained in samples of archaeological DNA. In other examples, CSR was applied to obtain DNA polymerases that accept unnatural sugars (Pinheiro et al. 2012). A summary of these and other results is in Table 7.1.

The Benner laboratory has also attempted to use CSR to copy DNA that is constructed from artificially expanded genetic information systems (AEGIS) (Piccirilli et al. 1990; Geyer et al. 2003). AEGIS is a species of DNA having additional nucleobases that present alternative hydrogen bonding patterns, allowing its members to form mutually exclusive pairing schemes. Some members of AEGIS are used in the clinic to monitor the viral load of patients infected with human immunodeficiency virus and hepatitis C virus (Collins et al. 1997).

While many natural polymerases are able to synthesize duplex DNA containing AEGIS nucleobase pairs, the efficiency of the synthesis is typically less than with completely natural DNA. This efficiency includes pausing at sites containing AEGIS nucleotides. This might be expected, given two unnatural features of the AEGIS pyrimidine components:

(a) Some are C-glycosides, having a nucleobase joined to the carbohydrate ring via a C–C bond, rather than the C–N bond joining natural pyrimidine nucleoside. This is expected to distort the conformation of the carbohydrate ring, a distortion that the polymerase might reject as “unnatural.”

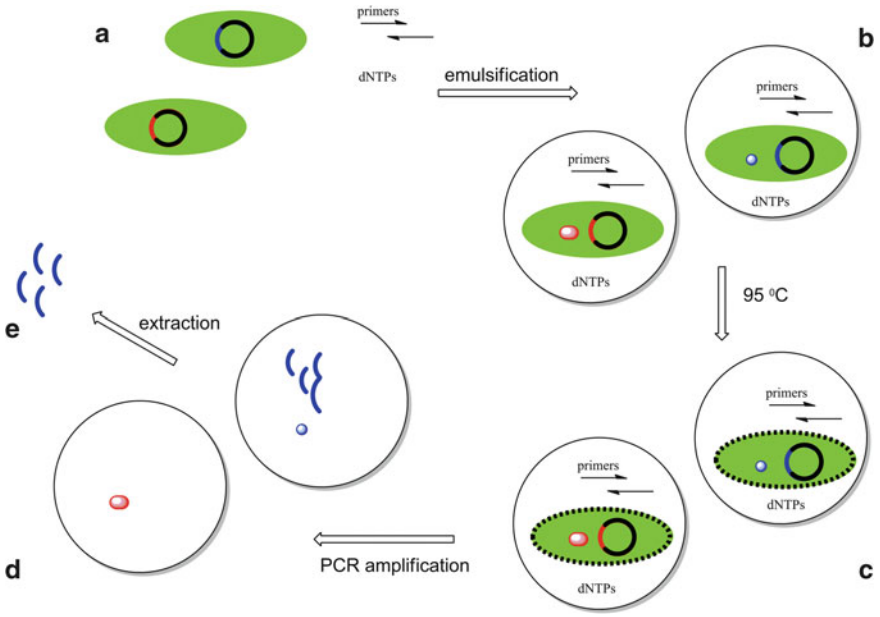
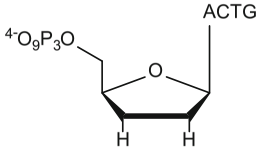
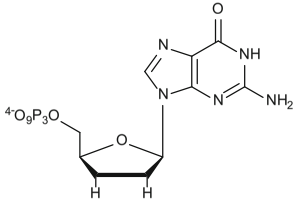
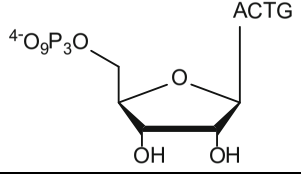


Fig. 7.3 Compartmentalized self-replication system (CSR) experiments start with the creation of a library of genes encoding variants of a polymerase. Members of this library are introduced into *E. coli* cells by electroporation. Here, just two variant genes (*red* and *blue*) are represented. These genes drive the expression of mutant polymerases in each *E. coli* cell, each of which is isolated in its own water-in-oil-emulsion droplet. (**b**) The first cycle of PCR breaks the cell wall of the *E. coli*, exposing the expressed polymerase molecules and their gene to the contents of a water droplet containing all of the necessary components necessary for a PCR amplification (1) primers, (2) dNTPs, (3) a mutated gene of the polymerase, and (4) the enzyme expressed by this gene (**c**). During PCR, any polymerases active under the selective pressure (*blue*) amplify their respective genes, enriching the pool of mutants having the desired properties; inactive polymerases (*red*) fail to do so (**d**). The emulsion is then broken and the amplified genes enriched in those encoding polymerases having the desired behaviors are extracted and inserted in a plasmid vector (circular DNA) (**e**). These then enter the cycle of selection again (**a**). After repeating these cycles, an enriched pool of variants of the original gene are produced

(b) Some lack electron density in the minor groove. This is believed to be a key element for recognition by polymerases (Joyce and Steitz 1994; Morales and Kool 1999).

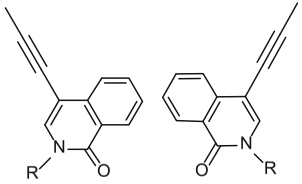
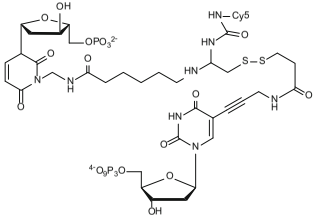
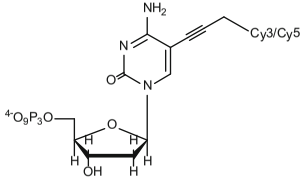
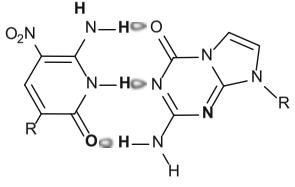
We applied the water–oil emulsions to select for polymerases that copied a pair (the Z:P pair) (Fig. 7.4) where both components present electron density to the minor groove, but where one component (Z) is a C-glycoside. The library was generated by ePCR and short-patch saturated mutagenesis of the O helix. Two polymerase variants emerged that had particularly interesting properties, including diminished stalling at the point where the Z:P pair is synthesized in in vitro copying. Even more interestingly, several of the amino acid replacements were at sites

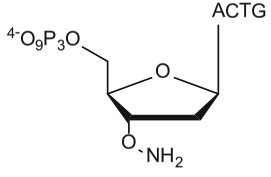
Table 7.1 Some DNA polymerases developed for specific purposes

Target/mutation	Pol	Nucleotide or analog(s)	Characteristics	Comments	Reference
F667T	Taq		Increased rates of dideoxynucleoside triphosphate (ddNTP) incorporation	Obtained by rational design	Tabor and Richardson (1995)
F667T R660D	Taq		Incorporates ddGTP at rates similar to the other dideoxynucleotides	Obtained by rational design	Li et al. (1999)
R660S	Taq	Standard	Reduces T → C transitions		Yoshida et al. (2001)
T664P	Taq	Standard	Works like WT for correct nucleotides, enlarges catalytic pocket for misincorporation		Tosaka et al. (2001)
E531K A597T A600T W604G A608S L609V I614T E615G	Stoffel fragment (Δ289 *Taq*)		Incorporate ribonucleoside triphosphates	Obtained by phage display	Xia et al. (2002)

(continued)

Table 7.1 (continued)

Target/mutation	Pol	Nucleotide or analog(s)	Characteristics	Comments	Reference
P2: Q489H F598I I614F	Stoffel fragment (Δ289 *Taq*)		Incorporates hydrophobic nucleobases analog propynylisocarbostyril (PICS) self-pair		Leconte et al. (2005)
V618I L619M V631A I638V T640K M646V M658L A661I T664A I665V L670M F700Y T756S A757G	Taq		Incorporates deoxynucleotides triphosphates-bearing fluorophore attached through cleavable linkers		Leconte et al. (2010)
V93Q V337I E399D N400D R407I Y546H Chimeric polymerases	Pfu-exo-		Incorporates Cy3-dCTP and Cy5-dCTP	Obtained by CSR	Ramsay et al. (2010)
N577S L625V E829V	Stoffel fragment (Δ289 *Taq*)		Incorporation of a third nucleobases pair with alternative hydrogen bonding pattern	CSR	Laos R. (unpublished results)

A597T E615G	Stoffel fragment (Δ289 *Taq*)	Ribonucleotides	DNA polymerase mutated into an efficient RNA polymerase	Obtained by phage display	Xia et al. (2002)
A597T W604R L605Q I614T E615G	Stoffel fragment (Δ289 *Taq*)	Ribonucleotides	DNA polymerase mutated into an efficient RNA polymerase	Obtained by phage display	Xia et al. (2002)
N/A	Taq-T3 chimera	Standard	Converts *Taq* DNA pol to a highly processive enzyme	Fused polymerase	Davidson et al. (2003)
L33P E76K D145G P552S E775G M777T	*Thermus thermophilus* (*Tth*)–*Thermus aquaticus* chimera (*Tth–Taq*)	Standard	Capable of bypassing DNA lesions	Obtained by CSR	d'Abbadie et al. (2007)
E76K E91Q D145G R336Q A448T I616M V739M E744G	*Tth–Taq* chimera	Standard	Capable of bypassing DNA lesions	Obtained by CSR	d'Abbadie et al. (2007)
L616A	*Taq* pol		Incorporates reversible terminators having 3′ hydroxyl blocked with a $-ONH_2$ moiety	REAP	Chen et al. (2010)
(81 mutations from the Taq consensus)	Chimera of: *Taq* (1–109), *Tos* (110–388), *Tth* (389–456), *Tbr* (457–471), *Taq* (472–834)	Standard	Resistance to environmental inhibitors: clay-rich soil; tar-impregnated bone material	Obtained by CSR	Baar et al. (2011)
L322M L459M S515R I638F S739G L789F E773G	*Taq*	Standard	Increased reverse transcriptase activity	Randomized library and screening	Kranaster et al. (2010) Sauter and Marx (2006)

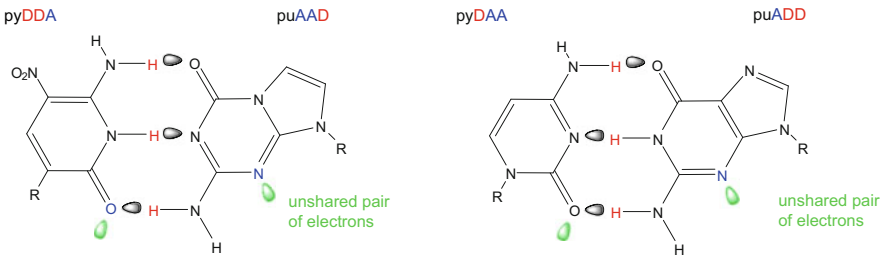


Fig. 7.4 The Z:P pair from an artificially expanded genetic information system (AEGIS, *left*) showing in *green* orbitals containing the unshared electron pairs presented to the minor groove of DNA. The natural C:G pair (*right*) showing these unshared pairs of electrons in the standard C:G pair. The pattern of hydrogen bonding is indicated A, hydrogen bond acceptor, and D, hydrogen bond donor

previously identified as heterotachous by the REAP method on *Taq* polymerase, even though the starting point was a randomized library (Laos et al. 2013).

7.4.2 Phage Display

An alternative way to link a polymerase and its encoding gene through a selection step is to join them in a single viral particle. In this technique, the protein of interest is co-expressed in the coat of a virus, keeping the genotype linked with phenotype. The Romesberg laboratory has been especially active in generating useful polymerases using this technique. They have evolved an RNA polymerase from a DNA polymerase (Xia et al. 2002), found polymerases having an improved ability to incorporate the self-pairing hydrophobic nucleobases analog propynylisocarbostyril (PICS) (Leconte et al. 2005), and more recently found polymerases having an improved ability to incorporate modified dUTP with a fluorophore (dUTP-Fl) that can be used for sequencing by synthesis. Leconte and his coworkers generated a library of Stoffel fragment (Sf) which is *Taq* DNA polymerase minus the first 289 amino acids. This fragment conserves the polymerase activity but lacks the exonuclease domain. The library was done by shuffling the genes of six homologous polymerases: *Thermus aquaticus*, *Thermus thermophilis*, *Thermus caldophilus*, *Thermus filiformis*, *Spirochaeta thermophila*, *and Thermomicrobium roseum*. The three most active polymerase mutants were as follows: **Sf168** (F598I, I614F, V618I, L619M, L622F, I638V, T640A, A643G, M646V, A661T, T664V, I665V, L670M, A691V, F700Y, F749A, V753I, A757G, Q782H), **Sf197** (V618I, L619M, V631A, I638V, T640K, M646V, M658L, A661I, T664A, I665V, L670M, F700Y, T756S, A757G), and **Sf267** (I614F, L619M, L622F, T640E, M658K, T664A, M751T, V753I, T756S, A757G, L760I). These mutants had between 10- and 50-fold increase in efficiency for dUTP-Fl incorporation compared with

Table 7.2 Some heterotachous sites in *Taq* polymerase reported in the literature from directed evolution experiments

Mutation	Method	Comments on site	Reference
D144G	CSR	Viral clade presents only G	Ghadessy et al. (2004)
A597T	Phage display	Viral clade presents only T	Xia et al. (2002)
F598L	CSR	Viral clade presents only L	Ghadessy et al. (2004)
A600T	Phage display	T is sparsely present in both clades. Viral clade presents also contain V, S	Xia et al. (2002)
I614K	Genetic complementation and screening	Viral clade presents L, Q, I, G, E; nonviral has I	Patel et al. (2001)
I614N	Genetic complementation and screening	Viral clade presents L, Q, I, G, E; nonviral has I	Patel et al. (2001)
I614T	Phage display	Viral clade presents L, Q, I, G, E; nonviral has I	Xia et al. (2002)
I614E	Phage display	Viral clade presents L, Q, I, G, E; nonviral has I	Fa et al. (2004)
E615G	Phage display	Viral clade presents E, D, Q, I, V, T; nonviral has E	Xia et al. (2002)
L616I	Genetic complementation and screening	Viral clade presents I, P, A, V, D, Q; nonviral has L and few M	Patel et al. (2001)

These amino acid positions have been identified by the REAP method as heterotachous sites

wild-type Sf (Leconte et al. 2010). Therefore, phage display seems to be an alternative for directed evolution experiments.

7.5 Outlook

The literature on DNA polymerase engineering includes examples representative of a range of strategies and tactics used more widely to generate enzymes with unnatural behaviors, including the ability to act on unnatural substrates. The demand for engineered polymerases is certain to grow, as many scientists seek to copy DNA constructed from alternative genetic alphabets (Geyer et al. 2003), highly tagged substrates (Hollenstein et al. 2009), modified backbones (Pinheiro et al. 2012), and other unusual structures (Hirao et al. 2007; Fa et al. 2004; Leconte et al. 2005). Further, we (and many others) are seeking to develop living systems that implement a "synthetic biology" based on unnatural DNA analogs. These have the potential for being "biosafe" platforms for artificial metabolisms, fermentations, diagnostics, and therapeutic tools, inter alia (Schmidt 2010).

The literature teaches that in some cases, simple downstream screening can obtain polymerases with the needed properties. This is illustrated in particular with efforts by Tabor and Richardson (1995) and Chen et al. (2010) to create polymerases that accept various 3′-terminating groups. Their combination of structural biology and evolutionary biology analyses was sufficiently powerful to ensure

that regions of sequence space small enough to be screened contained polymerases having the desired properties. In each case, screening began with rich sets of variants extracted from the sequence space around a deftly chosen parent, allowing a relatively simple one-at-a-time inspection to get useful enzymes.

For less effectively constructed libraries of variants, including those generated by shuffling and by undirected mutagenesis, various selection tools stand to pick up where screening cannot possibly go. Here, CSR and phage display have been especially useful. These have yielded polymerases that are able to read through highly damaged DNA that might be extracted from forensic and archaeological samples, as well as polymerases that support the copying of entirely different genetic systems. Other approaches are possible. For example, neutral drift libraries (Amitai et al. 2007; Bloom et al. 2007a, b) have yet to be applied as starting points for directed evolution experiments targeted against polymerases. These have, however, been found to be useful for other enzyme systems.

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Chapter 8
Reverse Transcriptases

Stuart F.J. Le Grice and Marcin Nowotny

Abstract Reverse transcriptases (RTs) catalyze the multi-step process that converts the single-stranded viral RNA genome to double-stranded integration-competent DNA, a process that is essential for the proliferation of retroviruses and retrotransposons. The former are released from cells as infectious particles and include human immunodeficiency virus (HIV) and hepadnaviruses such as hepatitis B virus (HBV). In contrast, retrotransposons are restricted to intracellular proliferation cycle and are very common in eukaryotic genomes. The N-terminal DNA polymerase domain of RTs structurally resembles other nucleic acid polymerases and assumes the topology of a right hand, with subdomains designated fingers, palm, and thumb. RTs exhibit low fidelity and processivity and are capable of both intra- and intermolecular strand displacement synthesis. A distinguishing feature of RTs is the presence of a C-terminal ribonuclease H (RNase H) domain in enzymes from viruses and long terminal repeat (LTR)-containing retrotransposons. The general role of this domain is sequence-independent removal of RNA from the RNA/DNA hybrid intermediates of reverse transcription, although precise cleavage is also required to mediate key steps such as (+) strand primer selection and DNA strand transfer. In LTR retroelements the RNase H domain is located immediately following the thumb subdomain, while in retroviruses an RNase H-like "connection domain" devoid of catalytic activity is followed by the catalytically competent C-terminal RNase H domain. DNA polymerase and RNase H activities are coordinated, but RNA/DNA hybrid hydrolysis is significantly slower than nucleotide

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incorporation. Whether this reflects simultaneous or individual active site occupancy of the nucleic acid substrate remains controversial.

Keywords Human immunodeficiency virus • Reverse transcription • DNA polymerase • Ribonuclease H • Antiviral therapy

8.1 Overview

Reverse transcription is a unique process through which single-stranded RNA is converted into double-stranded DNA. During its course, genetic information flows from RNA to DNA which is opposite to the "central dogma" of molecular biology and hence the term "reverse." This process is essential for mobile genetic elements such as viruses and retroelements (also called retrotransposons), for which at some stage of their proliferation, the genetic information is stored in RNA. Reverse transcription is catalyzed by multifunctional enzymes designated as reverse transcriptases (RTs), discovered in 1970 by Temin and Baltimore (Baltimore 1970; Temin and Mizutani 1970). These enzymes possess RNA- and DNA-dependent DNA polymerase activities and a ribonuclease H (RNase H) activity responsible for the hydrolysis of RNA in RNA/DNA hybrid intermediates of reverse transcription (Telesnitsky and Goff 1997).

RT-containing viruses belong to two groups: retroviruses such as human immunodeficiency virus 1 (HIV-1), most of which store their genetic information in single-stranded RNA, and caulimoviruses and hepadnaviruses (an example of the latter is hepatitis B virus, HBV) with a double-stranded DNA (dsDNA) genome and an RNA replication intermediate. The main difference between retroviruses and retrotransposons is that the former contain *env* genes coding for viral envelope proteins that allow the virus to leave the cell and spread, while retrotransposons lack this gene and are restricted to an intracellular life cycle. Retrotransposons thus have to integrate into the germ line to be passed on to the next generation. They are quite diverse and can be divided into two broad classes depending on whether or not their genetic information is flanked by long terminal repeats (LTRs). The diversity of retrotransposons suggests that they are the ancestral form from which the viruses evolved by acquisition of an *env* gene (Eickbush and Jamburuthugoda 2008). The DNA polymerase domain of RT is the only common element of all retrotransposons and related viruses (Eickbush and Jamburuthugoda 2008).

Retrotransposons are ubiquitous and ~42 % of the human genome corresponds to retroelements, mainly non-LTR long interspersed nuclear elements (LINE-1 or L1) (Lander et al. 2001; Cordaux and Batzer 2009). In maize 75 % of the genome reflects multiplication of retroelements, mostly from the LTR group (SanMiguel et al. 1998). Retroelements are also present in bacteria (Simon and Zimmerly 2008) and include retrons which utilize an RT to synthesize chimeric RNA/DNA structures (designated multicopy single-stranded DNA—msDNA) of unknown function (Lampson et al. 2005). The other RT-containing bacterial elements are type II introns (Simon and Zimmerly 2008). Discussion of the bacterial elements is outside of the scope of this chapter and the reader is referred to review articles on this topic.

The most extensively studied RTs are the dimeric enzyme from HIV-1 due to its clinical importance and monomeric RT from the gammaretrovirus Moloney murine leukemia virus (Mo-MLV) (Moelling 1974; Roth et al. 1985; Cote and Roth 2008). Enzymes from retroelements have also been purified and studied, e.g., from LINE-like elements (Gabriel and Boeke 1991; Ivanov et al. 1991), human L1 element (Mathias et al. 1991), yeast LTR transposons Ty1 (Wilhelm et al. 2000) and Ty3 (Bibillo et al. 2005a, b), and ORF from Penelope-like retroelements (Pyatkov et al. 2004). Here we will focus on HIV-1 RT, but will include data for other enzymes where available.

8.2 Reverse Transcription and Viral DNA Synthesis in LTR Elements

Converting the single-stranded (+) RNA genome of retroviruses and LTR-containing retrotransposons to integration-competent dsDNA is a multi-step process catalyzed by the virus-coded RT. However, while the integrated provirus is flanked by the hallmark LTRs, these encode sites for initiation and termination of transcription. The (+) RNA genome from which the provirus is synthesized therefore harbors incomplete LTR copies at its 5′ and 3′ termini, requiring their regeneration during RNA- and DNA-templated DNA synthesis (Fig. 8.1).

(a) tRNA-primed (−) DNA synthesis. Reverse transcription initiates from a host-coded tRNA molecule, whose 3′-terminal 18 nucleotides are complementary to a sequence immediately adjacent to U5 and designated the primer binding site (PBS). Examples of tRNA primer usage include $tRNA^{Lys,3}$ (HIV-1, HIV-2), $tRNA^{Trp}$ (Rouse sarcoma virus), $tRNA^{Pro}$ (Moloney murine leukemia virus), and $tRNA^{iMet}$ (Ty1 and Ty3) (Le Grice 2003). An exception to priming with tRNA is the Gypsy group of retrotransposons. In Tf1 retrotransposon from the fission yeast *Schizosaccharomyces pombe*, the first 11 bases of the (+) RNA genome are used for priming (Lin and Levin 1997). For HIV-1, current evidence suggests $tRNA^{Lys,3}$ is packaged into virions complexed with a component of the host translational machinery, namely, lysyl-tRNA synthetase (LysRS) (Cen et al. 2001). Whether primer annealing occurs pre- or post-virus assembly/budding remains to be determined.

Establishing a productive initiation complex occurs subsequent to RT binding to the tRNA/viral RNA duplex. A combination of chemical and enzymatic probing studies suggest this RNA/protein complex assumes a complex tertiary structure that controls early steps of initiation (Isel et al. 1995, 1998, 1999). Besides the tRNA/PBS duplex, additional interactions with the HIV-1 RNA genome control early events of (−) strand DNA synthesis, with one model suggesting that a primer activation sequence (PAS) on the viral genome downstream of the PBS interacts with an "anti-PAS" sequence of the tRNA TΨC stem (Beerens and Berkhout 2002), and an alternative suggests an A-rich sequence in the U5-IR stem interacts with the U-rich $tRNA^{Lys,3}$ anticodon loop (Isel et al. 1993). Initial steps of (−) strand DNA synthesis in HIV-1 are characterized by pausing and premature termination of the

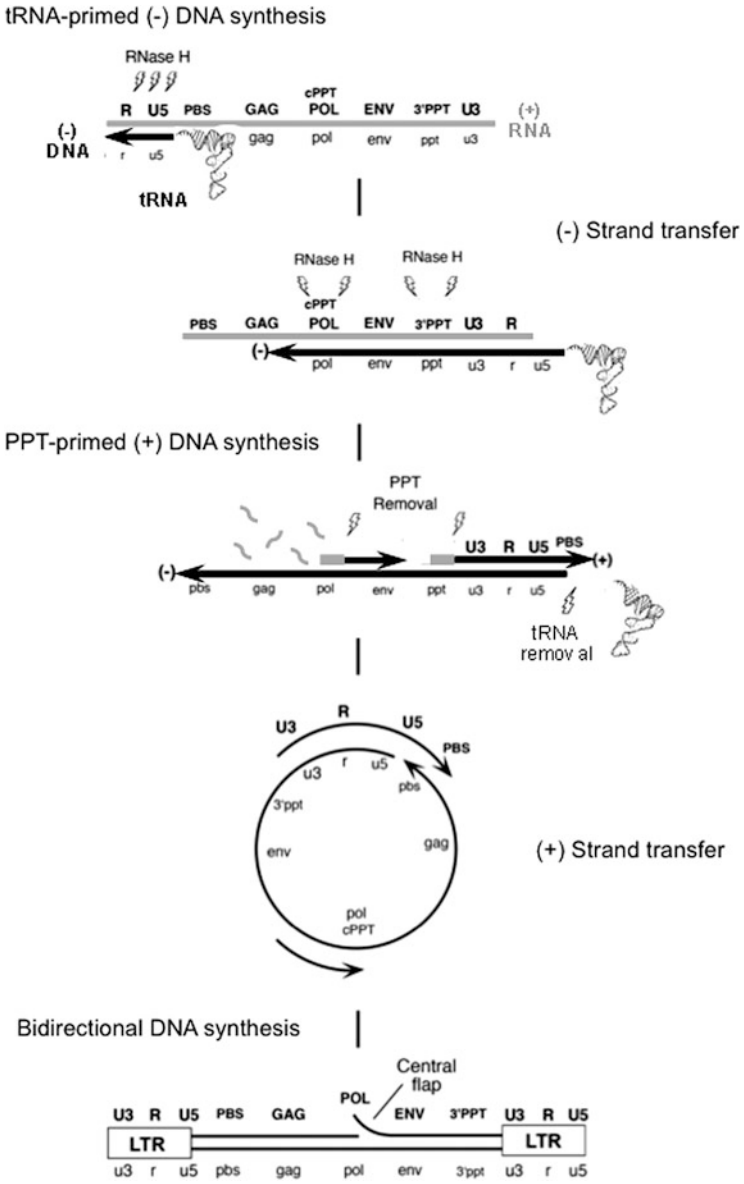

Fig. 8.1 RT-catalyzed synthesis of double-stranded, integration-competent HIV-1 DNA from the (+) strand RNA genome. RNA and DNA strands are depicted in *gray* and *black*, respectively. Note that for HIV-1 and related lentiviruses, the (+) strand of the DNA duplex is discontinuous, reflecting a second site for initiation of DNA synthesis at the center of the genome. Individual steps of DNA synthesis are described in the text

replication machinery, followed by a transition into a processive polymerization mode. This initiation "program," variations of which can be demonstrated for feline immunodeficiency and equine infectious anemia viruses, may represent a control mechanism that prevents premature reverse transcription prior to virus budding.

(b) (−) Strand strong-stop DNA synthesis and strand transfer. tRNA-primed DNA synthesis continues through U5 and R regions of the genome to the 5′ terminus, producing (−) strand strong-stop (−ss) DNA. The template RNA in RNA/DNA hybrid created by this process is hydrolyzed by RNase H activity of both the polymerizing enzyme and additional enzymes that reassociate with the substrate, producing short (+) strand oligoribonucleotides that spontaneously dissociate from nascent (−) strand DNA. Complementarity between the newly synthesized (−) DNA and the R region at the 3′-end of the genome facilitates the first DNA strand transfer event. (−) Strand DNA transfer can be both intra- or intermolecular, while inter-strand DNA transfer facilitates recombination, an essential process that maintains virus diversity and fitness. (−) Strand DNA transfer proceeds via two distinct mechanisms. In one scenario, transfer takes place between the termini of the full-length (−) strand DNA and the 3′-end of the viral RNA genome. An alternative mechanism invokes strand invasion, where an internal stretch of single-stranded DNA is accessed by the corresponding complement in the viral RNA, followed by branch migration and subsequent completion of (−) ss DNA synthesis (Kim et al. 1997).

(c) Polypurine tract-primed (+) strand DNA synthesis. Following (−) strand DNA transfer, RNA-dependent DNA synthesis resumes, accompanied by hydrolysis of (+) RNA of the ensuing RNA/DNA hybrid. However, a polypurine tract (PPT), located near the 3′ terminus, is refractory to hydrolysis, providing the primer for (+) strand, DNA-dependent DNA synthesis. A second, central PPT (cPPT) is a controversial feature of several lentiviruses and will be discussed later.

Since 3′ PPT-primed (+) strand DNA synthesis defines 5′ LTR sequences critical to the successful integration of viral dsDNA, its selection from the RNA/DNA replication intermediate and removal from nascent (+) DNA thus requires a considerable degree of precision. Although the structural basis for PPT selection remains elusive, X-ray crystallography (Sarafianos et al. 2001) and chemical footprinting (Kvaratskhelia et al. 2002) indicate the presence of locally altered base pairing, and, while speculative, the unusual conformation of this RNA/DNA hybrid may promote an "induced" fit to position the scissile PPT/U3 phosphodiester bond in the RNase H active site. Initiation of (+) strand DNA synthesis produces a PPT RNA/(+) DNA chimera hybridized to (−) DNA, and the unique architecture of the RNA/DNA junction, demonstrated by NMR spectroscopy (Fabris et al. 2009), may likewise mediate its recognition for accurate primer removal.

(d) tRNA primer removal and (+) strand DNA transfer. Prior to (+) strand DNA transfer, 3′ PPT-primed (+) strand DNA synthesis is templated by both DNA and RNA, namely, U3, R and U5 DNA, together with 18 terminal of the covalently linked tRNA primer. For HIV, the replication complex pauses at a modified $tRNA^{Lys,3}$ base (1-methyladenosine 58), placing the (−) DNA–tRNA junction in

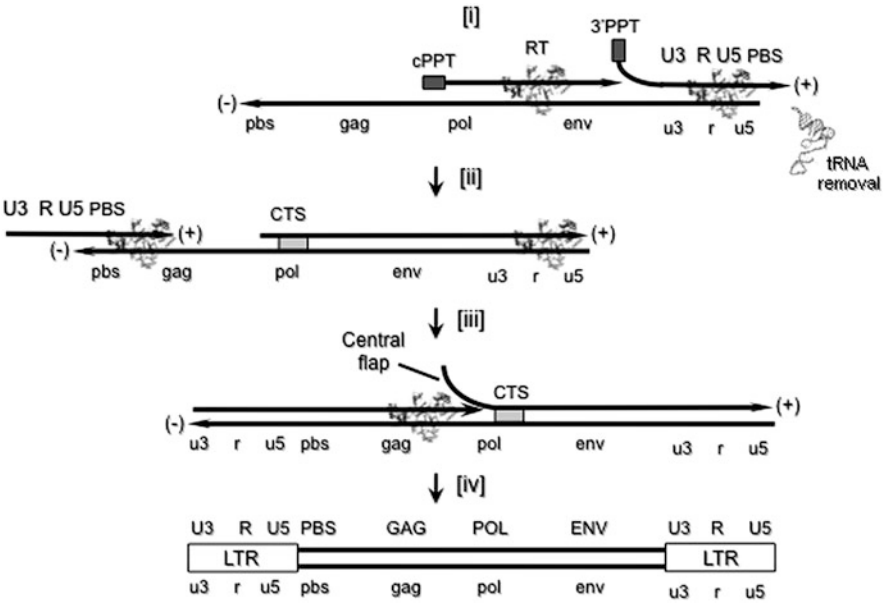

Fig. 8.2 Proposed mechanism for the termination of HIV-1 (+) strand DNA synthesis near the cPPT and generation of the "central flap." cPPT and 3′ PPT RNA primers are depicted in *gray* and (+) DNA in *black*, respectively. HIV-1 RT is represented by the 2-color dimer. *CTS* central termination sequence. Individual steps A–D are described in the text

the RNase H active site to facilitate tRNA removal. Although the tRNA–DNA junction is the logical RNase H target site, HIV-1 RT cleaves the penultimate ribonucleotide bond (Furfine and Reardon 1991a; Pullen et al. 1992). RNase H activity subsequently removes additional ribonucleotides from the tRNA 3′ terminus, promoting its dissociation and freeing homologous (+) and (−) strand PBS sequences for intramolecular (+) strand DNA transfer.

(e) Central termination of DNA synthesis. Following (+) strand DNA transfer, bidirectional DNA-dependent DNA synthesis would suffice to produce double-stranded, integration-competent viral DNA. However, a (+) strand discontinuity mapping near the center of the HIV-1 genome suggested a second initiation site *and* a unique termination mechanism (Hungnes et al. 1991; Charneau et al. 1992). Since the HIV-1 IN coding region contains a duplication of the 3′ PPT (designated the central, or cPPT), this provided the additional initiation site, and termination of (+) strand DNA synthesis was explained by the nearby *central termination sequence*, or CTS (Fig. 8.2).

3′ PPT-primed (+) strand DNA synthesis creates (+) strong-stop DNA containing the PBS complement necessary for (+) strand DNA transfer. However, cPPT-primed (+) strand DNA lacks a PBS sequence, precluding its involvement in strand transfer and thereby establishing one component of the discontinuous (+) strand. Following (+) strand DNA transfer and resumption of DNA-dependent

DNA synthesis, the replication complex ultimately reaches the duplex product of cPPT-primed DNA synthesis. After displacing ~100 nucleotides, the CTS is encountered in the form of duplex DNA, a prominent feature of which is phased dA:dT tracts that induce minor groove compression and "dislocation" of the replication complex (Berdis et al. 2001). Central termination also produces a "flap" of displaced DNA that is later removed and repaired by host-coded enzymes to create the replication-competent DNA duplex. Although the role of the central flap remains controversial, equivalent elements are present in the equine infectious anemia (Stetor et al. 1999) and feline immunodeficiency virus genomes (Whitwam et al. 2001).

8.3 Retrotransposition Mechanism of Non-LTR Elements

The mechanism of non-LTR retrotransposition is very different from that of LTR retroelements and retroviruses described above and is best characterized for the R2 retroelement from *Bombyx mori*. A key component in this process is the R2 protein, which contains an RT DNA polymerase domain flanked by N-terminal DNA-binding domain and C-terminal DNA-binding/endonuclease domain. In the proposed mechanism of "target-primed reverse transcription," R2 dimer binds ends of R2 transcript and the target DNA sequence in the 28S rRNA gene (Christensen et al. 2006). The R2 subunit bound upstream from the target sequence cleaves the DNA and the liberated 3′-OH terminus is used to prime reverse transcription of R2 element RNA (Luan et al. 1993). After first strand reverse transcription is completed, the second subunit of the R2 dimer cleaves the second DNA strand to initiate DNA synthesis from the other end to produce dsDNA. The RNA is either degraded by cellular RNases or displaced during second strand DNA synthesis (Christensen et al. 2006). R2 RT has higher strand displacement activity (Kurzynska-Kokorniak et al. 2007) and processivity than retroviral RTs which is likely important for the mode of action of this protein.

The large ORF of another non-LTR element, the retrotransposon L1, was demonstrated to possess RT activity (Mathias et al. 1991) and to catalyze target-primed reverse transcription, if nicks are present in the DNA (Cost et al. 2002). The N-terminal nuclease domain fused to L1 RT belongs to apurinic/apyrimidinic endonuclease family, whose role is to create a nick to prime reverse transcription (Christensen et al. 2000; Anzai et al. 2001).

8.4 RT Subunit Organization

The compact genomes of retroviruses exploit a small number of proteins and polyproteins that form repeating structures to enclose space, minimizing the requirement for a large protein-coding capacity. In this context, their RTs share a

common property of maturation from larger gag-pol polyprotein precursors by the virus-coded protease. In many retroviruses, synthesis of the gag-pol polyprotein involves a frameshift (−1 frameshifting in the case of lentiviruses) or termination suppression (e.g., for gammaretroviruses), often between gag and pol proteins (Herschhorn and Hizi 2010). Since frameshifting or termination suppression occurs with a defined frequency, this ensures the proper excess of gag protein, which is further processed to structural proteins of the virus. An interesting exception is spumaretroviruses in which the RT is produced from single mRNA (Yu et al. 1996).

RTs exhibit significant diversity in quaternary structure. For HIV-1 and HIV-2 RT, the biologically relevant enzymes are 66 kDa/51 kDa (HIV-1) or 68 kDa/55 kDa heterodimers (HIV-2), the smaller subunit resulting from protease-mediated cleavage of p66 or p68 (Telesnitsky and Goff 1997). Both subunits of the heterodimer thus share similar subdomains, designated (by analogy to a right hand) fingers, palm, thumb, and connection, while p66 and p68 retain the C-terminal RNase H domain. Despite primary sequence identity, subdomains of the heterodimer adopt significantly different conformations, i.e., the p66 and p68 polymerase domains exhibit an open, extended structure with a large active-site cleft, while the equivalent subdomains of the small subunit are closed, compact structures devoid of catalytic activity. The p51 subunit of HIV-1 RT is essentially rigid, having nearly the same conformation in numerous crystal structures of HIV-1 RT, while the p66 subunit undergoes large-scale motions (Bahar et al. 1999).

Proposed roles for the p51 HIV-1 subunit include providing a structural support and facilitating p66 loading onto nucleic acid. The recent structure of HIV-1 RT containing a non-PPT RNA/DNA hybrid (Lapkouski et al. 2013) has highlighted two novel features of the p51 C-terminus that facilitate accommodation of the duplex and correct presentation of the RNA strand in the RNase H active site. The first of these is a peptide spanning connection residues Phe416–Pro421, comprising the β-20–α-M connecting loop, which interacts with the DNA strand, crosses the shallow minor groove (where the hybrid bends), and forms van der Waals contacts with the RNA strand 3 nt from the scissile phosphate. In addition, Tyr427 of α-M is involved in hydrogen bonding with Asn348, a residue whose mutation to Ile has been associated with increased resistance to nucleoside and nonnucleoside RT inhibitors (Radzio and Sluis-Cremer 2011).

RTs of alpharetroviruses (avian leukosis virus (ALV) and Rous sarcoma virus (RSV)) are also heterodimers of p63 and p95 proteins. In this case, the 95 kDa β subunit retains the integrase domain of the gag-pol precursor, and the 63 kDa α subunit specifies the DNA polymerase and RNase H domains. Site-directed mutagenesis studies suggest that both enzymatic activities of the α/β heterodimer are conferred by the α subunit (Werner and Wohrl 2000).

In contrast to alpharetroviral and lentiviral RTs, the counterpart enzymes of gammaretroviruses (Moloney murine leukemia virus and xenotropic murine leukemia viruslike retrovirus—XMRV) and deltaretroviruses (bovine leukemia virus (Perach and Hizi 1999)) are monomeric, with molecular masses of ~70 kDa. Another feature distinguishing gamma- and deltaretrovirus RTs from lentiviral enzymes is the inclusion of an additional α-helix (designated the basic protrusion)

in their C-terminal RNase H domain, i.e., they closely resemble RNase H of *Escherichia coli* (see below). One of the main structural differences between monomeric and dimeric RTs is the positioning of their RNase H domains. In dimeric enzymes it is relatively rigidly positioned on the platform of the non-catalytic subunit. In contrast, the RNase H domain of monomeric enzymes is tethered to the polymerase domain through a flexible linker. As shown by recent small-angle X-ray scattering data, this C-terminal domain is very mobile in the absence of the nucleic acid but becomes organized on the substrate when it is bound by polymerase domain (Nowak et al. 2013).

8.5 DNA Polymerase Domain: Structure and Mechanism

RTs constitute a separate class of polymerases and are most closely related to telomerases (see Chap. 9) and viral RNA-dependent RNA polymerases. Although the overall shape and spatial arrangement of subdomains is similar across various classes of polymerases, their order in the primary sequence varies. In RTs the N-terminal region contains the palm subdomain with two insertions forming the fingers, followed by the thumb subdomain (Fig 8.3a).

The only RTs for which structural information is available are the dimeric HIV-1 enzyme (Kohlstaedt et al. 1992; Huang et al. 1998) and two nearly identical monomeric RTs from Mo-MLV and XMRV (Das and Georgiadis 2004; Zhou et al. 2012; Nowak et al. 2013). Although the structural description below is based on the information available for these enzymes, the high degree of amino acid sequence conservation of the palm and fingers subdomains (Xiong and Eickbush 1988, 1990) suggests that for other RTs at least those two subdomains are very similar. The thumb subdomain is more divergent in amino acid sequence and its full structural characterization across different groups of RTs awaits the solution of further structures.

At the center of the DNA polymerase domain of HIV-1 RT is the palm subdomain (Fig 8.3b, c), characteristic features of which are two three-stranded β-sheets and two long α-helices. The fingers subdomain comprises an α-helix and a two-stranded β-sheet, while the thumb contains three or four α-helices. The DNA polymerase active site is located in the palm subdomain and binds two metal ions, termed here A and B, which are essential for catalysis (Fig 8.3c, d). The preferred catalytic ion is Mg^{2+}, while Mn^{2+} can also support DNA synthesis. Metal ion B is coordinated by all three phosphate groups of the incoming nucleotide and a backbone carbonyl (Val111 in HIV-1 RT). Metal ion A is coordinated by two essential aspartates (Asp110 and 185 in HIV-1 RT) by the 3′-OH group of the primer, which is the attacking nucleophile in the nucleotidyl transfer reaction. Two carboxylates (Asp185 and Asp186 in HIV-1 RT) form the active-site motif -Tyr-X-Asp-Asp-, of which X denotes a hydrophobic residue. The two aspartic acid residues are absolutely essential for DNA polymerase activity (Kaushik et al. 1996), also for retrotransposon RTs. For example, for Ty1 RT substituting

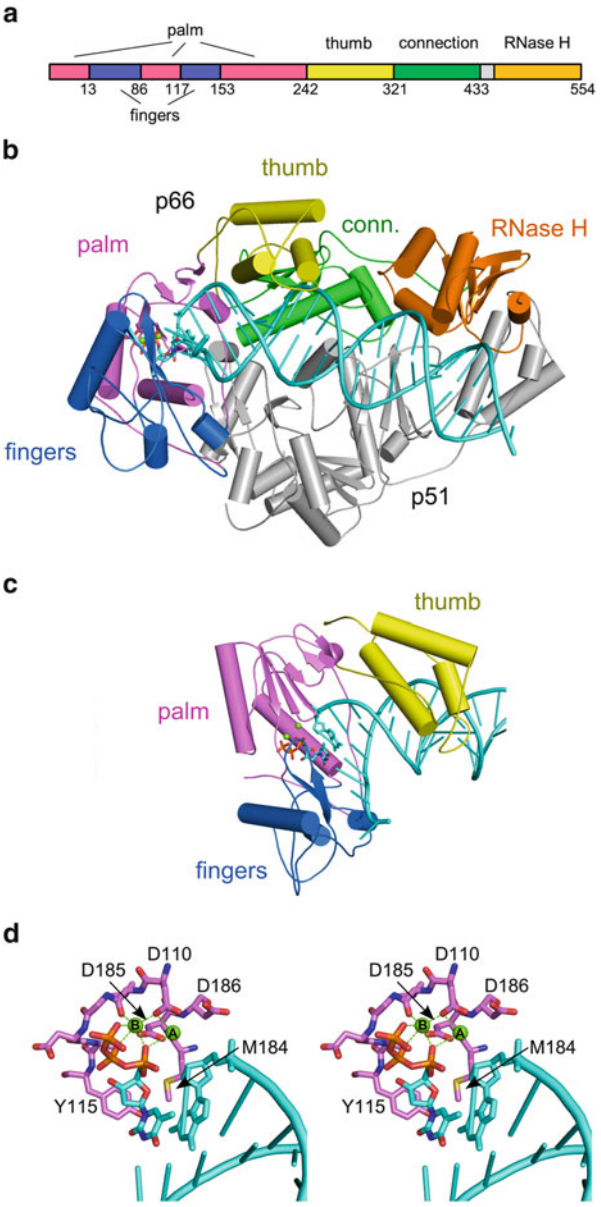

Fig. 8.3 Structure of HIV-1 RT. (**a**) Domain composition of the p66 subunit. The residue numbers at the boundaries of the domains are given. (**b**) Overall structure of HIV-1 RT in complex with dsDNA substrate (PDB ID: 1RTD). p66 subunit is shown in *color* and p51 in *gray*. The nucleic acid substrate is shown in *cyan ladder*. (**c**) Polymerase domain (fingers, palm, and thumb subdomains). The incoming nucleotide at the active site is shown in *sticks* and the metal ions (Mg^{2+}) as *green spheres*. (**d**) Close-up view of the active site (stereoview). Residues forming the active site (from palm domain) are shown in *stick* representation and metal ion coordination in *green dashed lines*. Note that the substrate present in the structure is missing the attacking 3′-OH group to inhibit the polymerization reaction

the first Asp with Asn abolished DNA polymerase function, and for both Ty3 and Ty1 RTs, mutating the second Asp only reduced DNA activity in vitro but abolished retrotransposition in vivo (Uzun and Gabriel 2001; Pandey et al. 2004; Bibillo et al. 2005a).

Another key element of the DNA polymerase active site is the pocket binding the incoming nucleotide, designated the N site. For HIV-1 RT the γ-phosphate of the dNTP interacts with Lys65 and Asp113, while the base stacks with Arg72. With respect to the ribose ring, the 3′-OH group interacts with Gln151 and the backbone amide of Tyr115. The side chain of the latter residue also forms a very important stacking interaction with the ribose ring forming a so-called steric gate in common with other DNA polymerases (Cases-Gonzalez et al. 2000; Brown and Suo 2011). The stacking is only efficient when the 2′-OH group is absent from the ring, selecting for the incoming dNTP and conferring DNA polymerase specificity. To investigate how structural changes of Tyr115 affect nucleotide incorporation by HIV-1 RT, Klarmann et al. substituted this residue with a variety of nonnatural tyrosine analogues (Klarmann et al. 2007). This approach demonstrated that aminomethyl-Phe115 RT incorporated dCTP more efficiently than the WT enzyme and was also resistant to the chain terminator 3TCTP. Molecular modeling suggested that the aminomethyl-Phe115 substitution provided new hydrogen bonds through the minor groove to the incoming dNTP and the template residue of the terminal base pair, which might contribute to the increased efficiency of dCTP incorporation. Substituting the equivalent steric gate residue in Mo-MLV RT, Phe155, with valine resulted in enhanced ribonucleotide incorporation (Gao et al. 1997).

The polymerization rate of RTs compared to other polymerases is slow—between 1 and 100 nucleotides per second in pre-steady state when released from a stalled state (Kati et al. 1992) and approximately 170 nucleotides per second during processive incorporation (Ortiz et al. 2005). The reaction starts with the binding of primer-template duplex such that the primer 3′ terminus is located at the active site (Jacobo-Molina et al. 1993; Huang et al. 1998; Sarafianos et al. 2001). Substrate binding is accompanied by a change in position of the thumb subdomain, which moves away from the substrate binding cleft (Rodgers et al. 1995; Hsiou et al. 1996). The incoming dNTP is next bound, leading to re-positioning of the fingers subdomain to align the attacking 3′-OH of the primer and the α-phosphate of the dNTP (Kati et al. 1992; Reardon 1992). The movement of the fingers subdomain is thought to be the rate-limiting step of nucleotide incorporation. Following dNTP binding, the reaction follows an S_N2-like mechanism shared by all polymerases, which involves the two divalent metal ions (Steitz and Steitz 1993; Steitz 1998; Yang et al. 2006). The primer 3′-OH, which is positioned and activated by metal ion A, catalyzes nucleophilic attack on the α phosphorus atom of the incoming dNTP, releasing pyrophosphate, and nascent DNA subsequently translocates to position the next 3′-OH group at the active site. Studies with pyrophosphate analogue foscarnet indicate that translocation occurs through a thermal ratchet mechanism in which the enzyme spontaneously switches between pre- and post-translocated states (Marchand et al. 2007).

RTs have a higher error rate than other replicative DNA polymerases, due in part to the fact that they lack proofreading exonuclease activity. HIV-1 RT is in fact among the least accurate RTs, making one error in 1,700 to 4,000 polymerized

nucleotides (Preston et al. 1988; Roberts et al. 1988). Mo-MLV MuLV RT, for example, has significantly higher fidelity, making 1 error per 30,000 nucleotides (Roberts et al. 1988, 1989). A critical residue that contributes to fidelity is the hydrophobic amino acid of the ^{183}-Tyr-X-Asp-Asp-^{186} active-site motif. In HIV-1 RT this is Met184, whose substitution to Val or Leu resulted in increased fidelity (Bakhanashvili et al. 1996; Pandey et al. 1996; Wainberg et al. 1996; Oude Essink et al. 1997), while replacing it with Ala caused a four- to eightfold reduction in fidelity (Pandey et al. 1996). In Mo-MLV and Ty1 RTs the equivalent hydrophobic residue is Val which could explain their higher fidelity (Kaushik et al. 2000; Boutabout et al. 2001). Interestingly, substitutions of HIV-1 Met184 which increase fidelity lead to resistance to nucleoside analogue inhibitors of polymerase activity (NRTIs) which is likely caused by higher discrimination against incorporation of incorrect incoming nucleotides (Wainberg et al. 1996; Oude Essink et al. 1997).

RTs are low-processivity polymerases and can synthesize up to several hundred nucleotides in a single binding event depending greatly on the nucleic acid sequence (Huber et al. 1989; Avidan et al. 2002). An element important for DNA synthesis processivity is the "pin" structure guiding the template strand opposite of the active site (Kim et al. 1999; Gu et al. 2001; Nowak et al. 2013) (see below). A unique feature of RTs is DNA synthesis with concurrent displacement of nucleic acid hybridized to the template upstream from the active site (Huber et al. 1989; Fuentes et al. 1996). Such strand displacement synthesis is important at several steps during viral DNA synthesis, for example, during removal of RNA fragments left over by RNase H activity or during the termination of reverse transcription and creation of the central flap (see above). For non-LTR enzymes, which lack RNase H activity, displacement synthesis must be essential for the completion of retrotransposition. Displacement synthesis is approximately threefold slower than non-displacement synthesis (Kelleher and Champoux 1998; Whiting and Champoux 1998). It has been shown that for retroviral RTs, an aromatic residue in the fingers subdomain (Trp24 in HIV-1 and Tyr64 in Mo-MLV) is important for displacement synthesis (Agopian et al. 2007; Paulson et al. 2007). This residue forms a stacking interaction with bases in the single-stranded template region ahead of the active site (Winshell and Champoux 2001; Nowak et al. 2013).

HIV-1 RT DNA polymerase activity, along with viral integrase and protease, is the prime target for development of antiretroviral drugs. In fact, the first drug used to treat AIDS was DNA polymerase inhibitor—the nucleoside analogue azidothymidine (AZT). Since then multiple nucleoside analogues have been developed. Nonnucleoside RT inhibitors (NNRTIs) are a second class of HIV-1 DNA polymerase activity inhibitors which bind to an allosteric pocket in the vicinity of the active site. The description of polymerase inhibitors and evolution of drug resistance is outside of the scope of this chapter and the reader is referred to reviews on this topic (Ilina and Parniak 2008).

8.6 The Connection and RNase H Domains

A unique feature present only in retroviral RTs is the connection subdomain between the DNA polymerase and RNase H domains. The connection adopts a structure similar to the RNase H domain, but the catalytic residues are absent. Based on amino acid sequence, the RNase H domain from retrotransposons and retroviruses are different, with retroviral RNases H resembling more closely the cellular enzymes. It was therefore proposed that retroviruses acquired a new RNase H domain from the host, while the ancestral one became the connection domain and lost catalytic activity (Malik and Eickbush 2001).

RNase H activity of RTs is essential for reverse transcription and inactivating mutations of this domain inhibit virus infectivity (Tisdale et al. 1991). Cellular RNases H are present in all eukaryotes and are divided into two classes: type 1 and type 2. The RNase H domain from RTs is a type 1 enzyme. Cellular RNases H1 are implicated in the (1) maintenance of genomic stability by processing R-loops which arise when the mRNA hybridizes with the DNA from which it was transcribed (Broccoli et al. 2004), (2) removal of RNA/DNA hybrids which can promote genomic instability (Wahba et al. 2011), and (3) removal of ribonucleotides from DNA, which is essential for genome integrity and development (Reijns et al. 2012). The general role of RNase H activity of RTs is to nondiscriminately degrade (+) genomic RNA during (−) strand, DNA-dependent DNA synthesis. RNase H also performs specific cuts at several stages of reverse transcription, namely, excising the tRNA primer and generating and removing the PPT primer of (+) strand DNA synthesis (Rausch and Le Grice 2004; Schultz and Champoux 2008).

The RT-associated RNase H domain adopts a typical RNase H fold with the central β-sheet of five strands (Fig. 8.4a). The first three strands in the primary sequence run antiparallel to each other and the last two are shorter and parallel to the first. The central β-sheet is flanked by α-helices. The single C-terminal helix is located on one side of the sheet and three or four helices are on the other. If four helices are present, two of them, together with the following loop, form an element designated the basic protrusion, which participates in substrate binding (Kanaya et al. 1991; Keck and Marqusee 1996; Haruki et al. 1997; Nowotny et al. 2007). RNase H both containing and lacking a basic protrusion are evident in RTs, most likely depending on the architecture of the enzyme. For example, dimeric HIV-1 RT lacks this element but it is present in the monomeric Mo-MLV and XMRV enzymes.

Currently no structures are available for retroviral RNases H interacting with nucleic acid substrate. The mechanism of RNase H-mediated RNA hydrolysis has been elucidated from crystal structures of substrate complexes of cellular RNases H1 from the bacterium *Bacillus halodurans* (Nowotny et al. 2005) as well as the human enzyme (Nowotny et al. 2007). The minor groove of the hybrid is bound by conserved carboxylate and amide side chains (equivalents in HIV-1 RNase H are Glu449, Asn474, and Gln475). The RNA backbone forms a network of interactions with the protein, many mediated by the main chain. In particular 2′-OH groups of

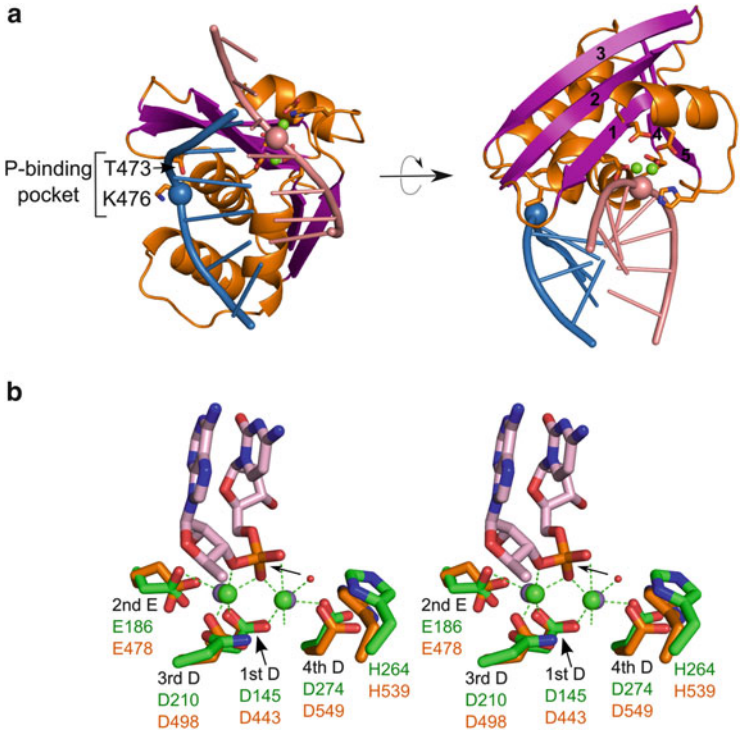

Fig. 8.4 HIV-1 RNase H domain. (**a**) Two views of the model of RNase H domain of HIV-1 RT interacting with RNA/DNA hybrid substrate and metal ions. The model was created by superimposing the structure of HIV-1 RNase H onto the structure of the substrate complex structures of very similar human and bacterial (*B. halodurans*) RNases H1. HIV-1 RNase H structure is shown in *cartoon* representation with the central β-sheet of the RNase H fold shown in *purple* and the strands labeled as they appear in the sequence. RNA and DNA from the human RNase H1 structure are shown in *light blue* and *pink ladder* representations, respectively. Two Mg^{2+} ions modeled from the bacterial structure are shown as *green spheres*. Residues forming the active site and the phosphate-binding pocket are shown as *sticks* and the latter are labeled. Phosphate group interacting with the active site and the P-binding pocket are indicated with *pink* and *light blue spheres*, respectively. (**b**) Close-up view of the active site (stereoview). The figure shows a superposition of the active sites of human RNase H1 (PDB ID:3K2P) and HIV-1 RNase H in complex with β-thujaplicinol and manganese ions. Protein residues forming the active site are shown as *sticks* (*orange* for HIV-1 and *green* for human) and a fragment of substrate RNA from human RNase H1 structure in *pink*. Calcium ions from human RNase H1 structure are shown as *green spheres* and manganese ions from HIV-1 structure as *gray spheres*. Metal ion coordination is shown as *green dashed lines*. *Small red sphere* represents the attacking nucleophilic water and the direction of the attack on the scissile phosphate is indicated with an *arrow*

two nucleotides on each side of the scissile phosphate form hydrogen bonds with RNase H which facilitates recognition of the RNA strand. The backbone of the DNA interacts with two elements. The first is the "phosphate-binding pocket," which forms a tight interaction with the phosphate group of DNA located two base pairs from the active site (Fig 8.4a). In most RNases H1 this motif comprises 3–4

residues, while in HIV-1 RNase H domain, there are only two residues: Thr473 and Lys476. The importance of the former is further underscored by the fact that its substitution in HIV-1 RT inhibits virus replication (Julias et al. 2002). The tight interaction between the phosphate-binding pocket and DNA results in deformation of the nucleic acid manifested by unusual torsion angles. This deformation also requires that the strand interacting with the pocket adopts B-form conformation with a narrow minor groove and B-form-like sugar puckers allowed only for DNA. This serves for the recognition of DNA as the non-cleaved strand. When the basic protrusion is present, it promotes additional DNA binding. A tight channel is formed between the core of the enzyme and the protrusion through which the DNA strand passes. In human RNase H, the channel contains an aromatic residue (Trp) which forms a stacking interaction with the ribose ring of the substrate. This interaction is efficient only when 2′-OH group is absent, thus further selecting for DNA. Equivalents of this Trp are not found in retroviral RNase H domains, i.e., perhaps this feature is unique to cellular enzymes.

Analogous to the mechanism of DNA synthesis, RNase H-mediated hydrolysis proceeds via two metal ion-assisted catalysis (Nowotny et al. 2005; Nowotny and Yang 2006), but the attacking nucleophile is water rather than the primer 3′-OH, resulting in hydrolysis and not phosphoryl transfer. The preferred catalytic metal ion is Mg^{2+}. Mn^{2+} can also support the reaction but for HIV-1 RNase H, Mn^{2+} induces nonspecific dsRNA cleavage termed RNase H* activity, which is considered nonphysiological (Ben-Artzi et al. 1992; Hostomsky et al. 1994). Ca^{2+} ions inhibit the RNase H activity (Nowotny and Yang 2006). Similar to the DNA polymerase active site, the metal ions are termed A and B, where A positions and activates the nucleophilic water and B stabilizes the transition state and the leaving group (Fig 8.4b). The reaction product contains 5′-phosphate and 3′-OH groups (Miller et al. 1973; Krug and Berger 1989).

The active site of RNases H is composed of carboxylate residues forming an -Asp-Glu-Asp-Asp- motif and coordinating the two metal ions (Fig 8.4b). The first three residues of this motif are absolutely conserved and their substitutions in HIV-1, Mo-MLV, and Ty3 RTs led to a complete loss of RNase H activity (Schatz et al. 1989; Mizrahi et al. 1990, 1994; Lener et al. 2002). The first Asp is located in the middle of the first β-strand of the central β-sheet, the second Asp, at the C-terminus of the fourth strand and Glu in the first α-helix.

The nucleic acid substrate contributes important coordination contacts for metal ions and only in the presence of the substrate is proper positioning and coordination of metal ions observed (Nowotny et al. 2005). Since, as mentioned above, catalytic complex structures are not available for RNases H from RTs, the configuration of the active site has been inferred from the structures of the cellular counterparts. From this comparison, an architecture can be proposed in which both metal ions are coordinated by the first carboxylate of the -Asp-Glu-Asp-Asp- motif which is the central element of the active site (Asp443 in HIV-1) and the non-bridging oxygen of the scissile phosphate (Fig 8.4b). In addition metal ion A is coordinated by the last carboxylate of the motif and coordinates two water molecules, one of which is the attacking nucleophile. Metal ion B is also coordinated by the O3′ the scissile

phosphate and Glu of the -Asp-Glu-Asp-Asp- motif. This residue also forms a hydrogen bond with 2′-OH group of the nucleotide on the 3′ side of the scissile phosphate, coupling RNA recognition to catalysis. This model is supported by crystal structures of complexes of HIV-1 RT with RNase H inhibitors interacting with the active site through coordination of metal ions. These inhibitors mimic the scissile phosphate and lead to proper positioning of the metal ions nearly identical to bona fide substrate complexes of cellular RNases H1 (Himmel et al. 2009).

In retroviral RNases H, but not in the domains from retrotransposons, an additional His (His539 in HIV-1 and His638 in Mo-MLV) is located in the proximity of the active site and has been shown to be important for catalysis. This residue located in a flexible loop before the C-terminal α-helix. The mobility of this residue is probably exploited by the enzyme to promote dissociation of the reaction product (Nowotny et al. 2007).

8.7 Substrate Binding and Coordination of DNA Polymerase and RNase H Activities

The details of substrate binding by retroviral RTs have been elucidated from crystal structures of HIV-1 RT in complex with nucleic acid (dsDNA and RNA/DNA) (Jacobo-Molina et al. 1993; Huang et al. 1998; Sarafianos et al. 2001) and recent structure of polymerase-connection fragment of monomeric XMRV RT in complex with RNA/DNA (Nowak et al. 2013). For HIV-1 RT the nucleic acid substrates span the entire length of the enzyme (Fig 8.3b) and the distance between the DNA polymerase and RNase H active sites is ~60 Å or 17–18 bp. The interactions between the substrate and DNA polymerase domain of HIV-1 and XMRV RTs can be divided into several segments. If an overhang is present in the template, it is stabilized by interactions with the fingers subdomain. The template nucleotide pairing with the incoming nucleotides is stabilized by a “pin”—an Arg from the fingers subdomain (Arg78 in HIV-1 and Arg116 in Mo-MLV)—to guide the nucleic acid. For an RNA template, after the “pin” there is a region of interactions between the protein and 2′-OH groups. This region is followed by a positively charged patch comprising Lys and/or Arg residues interacting with the nucleic acid backbone. An important element for substrate binding is an α-helix of the thumb subdomain inserted into the minor groove of the duplex and forming interactions predominantly with the primer strand. These interactions of the thumb subdomain are also conserved for RT from the *Saccharomyces cerevisiae* LTR-retrotransposon Ty3, as demonstrated by site-directed mutagenesis (Bibillo et al. 2005b).

Although interactions of substrate with polymerase domain are conserved between HIV-1 RT, XMRV RT, and likely Ty3 RT, especially around the active site, further towards the connection and RNase H domains, these are quite different. For example, HIV-1 RT contains in this region a unique and important element designated the “RNase H primer grip,” which comprises (i) Gly359, Ala360, and

His361 from the p66 connection subdomain (ii) Thr473, Asn474, Gln475, Lys476, Tyr501, and Ile505 from the RNase H domain of p66 and (iii) Lys395 and Glu396 from p51 subunit. This region is important for both the DNA polymerase and RNase H activities of HIV-1 (Arion et al. 2002; Julias et al. 2002, 2003; Rausch et al. 2002; McWilliams et al. 2006).

In many available crystal structures of HIV-1 RT, the nucleic acid substrate forms productive interactions with the active site of DNA polymerase domain but is bound by the RNase H domain without contacting its active site. This is also true for the complex between HIV-1 RT and the PPT-containing RNA/DNA hybrid (Sarafianos et al. 2001). The hybrid in this structure, however, has a conformation very similar to dsDNA and contains a region of anomalous base pairing, prominent among which is a G:T mispair. However, NMR analysis of HIV-1 RT bound to the PPT (Turner et al. 2008) and related studies with PPT variants (Le Grice et al., unpublished observations) together failed to confirm the presence of this mispair. It remains to be seen whether such a configuration will be confirmed for other RNA/DNA substrates. Nevertheless, the lack of interaction between the substrate and the active site of RNase H clearly does not allow for RNase H cleavage. This is in agreement with the biochemical data that show that the interaction of the substrate with the two active sites is mutually exclusive (e.g., see Delviks-Frankenberry et al. 2008). Recent crystal structures of HIV-1 RT in complex with RNA/DNA hybrids showed that a unique substrate conformation stabilized by interactions with the connection domain is required for the interaction with the RNase H active site. The duplex RNA/DNA in those structures is underwound in the middle with a widened major groove (Lapkouski et al. 2013). This changes the trajectory of the hybrid, so that the RNA strand moves closer to the RNase H active site and the 3′ end of the primer is removed from the polymerase active site precluding DNA polymerization.

The situation is very different for monomeric RTs which may not require a deformation of the substrate to allow RNase H cleavage. Instead, the RNase H is tethered to the connection domain via a flexible linker and very mobile, requiring organization on the substrate to catalyze hydrolysis (Nowak et al. 2013). The weak and transient interaction between the RNase H domain and the substrate that requires either substrate deformation (for dimeric HIV-1 RT) or RNase H domain organization (for monomeric XMRV RT) appears to emerge as a common feature of RTs. Its likely function is to allow for the regulation of the RNase H domain in cleavages that require a greater degree of precision—removal of tRNA primer and generation and removal of PPT primers.

Even though the DNA polymerase and RNase H activities are not simultaneous, the extensive interactions between the RNA/DNA substrate and the polymerase domain result in its strong influence on RNase H activity. It has been shown, for example, that substitutions in the DNA polymerase domain affect RNase H activity (Boyer et al. 1992a, b, 1994; Gao et al. 1998; Powell et al. 1999; Mandal et al. 2006). In fact, the isolated HIV-1 RNase H domain has very low activity (Hostomsky et al. 1991; Smith and Roth 1993) which can be restored by fusing it with artificial extensions, e.g., poly-His tags (Evans et al. 1991; Smith and Roth

1993), including the basic protrusion from cellular enzymes (Stahl et al. 1994; Keck and Marqusee 1995) or addition of p66 fragments N-terminal to the RNase H domain (Smith et al. 1994). Addition of the p51 subunit or the isolated connection domain has also been demonstrated to restore activity (Evans et al. 1991; Hostomsky et al. 1991; Smith and Roth 1993; Smith et al. 1994). In contrast to HIV-1 RNase H, the isolated domain of Mo-MLV RT retains some activity (Tanese and Goff 1988; Telesnitsky et al. 1992; Schultz and Champoux 1996; Zhan and Crouch 1997). This may be due to the fact that Mo-MLV RNase H contains a basic protrusion and its phosphate-binding pocket is comprised of four residues, which may promote stronger substrate binding, required for the organization of this domain on the RNA/DNA duplex.

The DNA polymerase domain also strongly affects the sites of RNase H cleavage. RNase H cleavage is defined as DNA 3′-end-directed, RNA 5′-end-directed, and internal (Fig. 8.5). The first occurs when the recessed 3′-end of the primer is positioned for extension at the DNA polymerase active site (Furfine and Reardon 1991b; Gopalakrishnan et al. 1992; Kati et al. 1992). For HIV-1 RT this results in RNase H cuts 15–20 bp from the primer 3′ terminus. For RTs from retrotransposons, Ty3 (Lener et al. 2002) and Ty1 (Wilhelm et al. 2000), the 3′-end-directed cleavages occur 13–14 bp from the primer terminus, most likely a consequence of the lack of a connection subdomain in retrotransposon RTs and a shorter distance between the two active sites.

3′-end-directed cleavages are further subdivided into polymerization independent, when the substrate is statically bound at the DNA polymerase active site, and polymerization dependent, which are simultaneous with DNA synthesis. For HIV-1 RT the RNase H activity is approximately sevenfold slower than polymerase activity (Kati et al. 1992), which results in incomplete degradation of the RNA strand. During processive DNA synthesis, 20 % of the RNA remained in fragments longer than 7 nt which can remain stably associated the DNA template.

5′-end-directed cleavages occur when recessed 5′-end of the RNA template is bound at the DNA polymerase active site (Fig 8.5) (Palaniappan et al. 1996; Wisniewski et al. 2000a, b; DeStefano et al. 2001). Primary and secondary cuts are observed, the former occurring 13–19 nt from the 5′-end of RNA and the latter 7–10 nt from the 5′-end and 5 nt from the 3′-end of the RNA (Wisniewski et al. 2000a, 2002). On long substrates RNase H can also perform internal cuts that do not depend of duplex ends (Schultz et al. 2004).

8.8 Conclusions and Perspectives

RTs are among the most extensively studied and best characterized enzymes, due to the clinical significance of HIV-1 RT. However, RTs are also very important tools that have allowed tremendous advancements of molecular biology, finding use in converting mRNA to complementary DNAs (cDNA) applied, among other things, in cloning of protein-coding regions of genes. RTs are intriguing enzymes able to

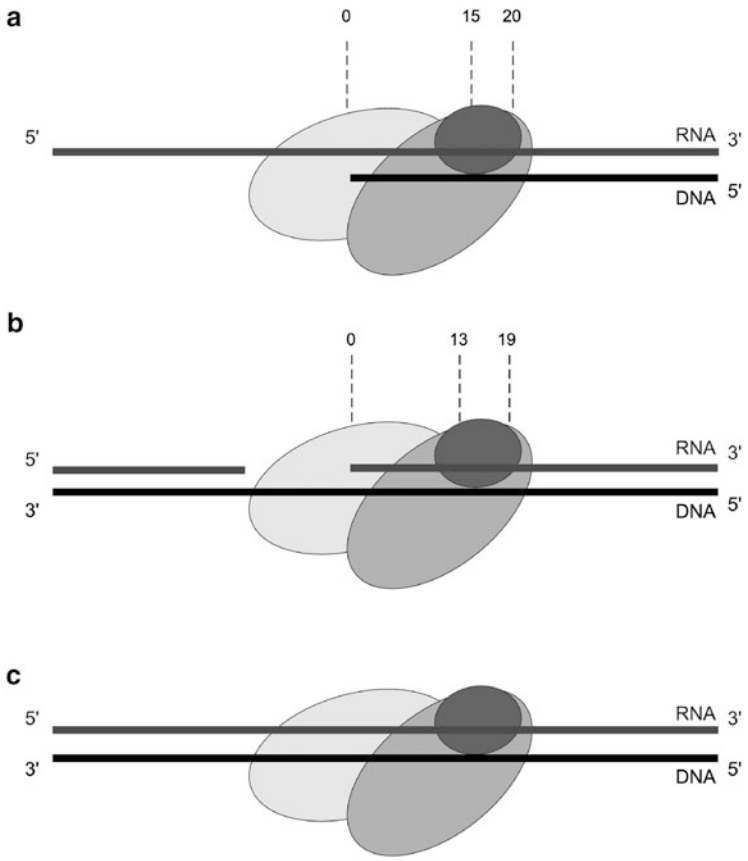

Fig. 8.5 Polymerase-guided RNase H cleavage modes. (**a**) 3′-DNA-directed cleavage. *Darker oval* represents the RNase H domain and *dashed lines* show the region in which the cleavages occur. (**b**) 5′-RNA-directed cleavage. (**c**) Internal cleavage

catalyze complicated multi-step reverse transcription reaction involving an elaborate protein-nucleic acid choreography.

As a final "chapter" of the HIV-1 RT journey, agents targeting this enzyme are showing promise as vaginal and rectal microbicides (Lewi et al. 2012). Prominent among these is tenofovir (TFV), shown in clinical trials to be safe and well tolerated in a study on HIV-negative women with a vaginal gel applied during 24 weeks. Repeated application of TFV intravaginal gel was well tolerated, produced low plasma levels, and, importantly, failed to select for resistance-conferring mutations, a continuing challenge of antiretroviral therapy. NNRTIs such as Dapivirine (DPV) have also displayed promising virucidal properties. When applied intravaginally, DPV is absorbed by the outer mucosal layers, while plasma concentrations reportedly remained low. Long-term constant DPV release has been obtained from a variety of intravaginal rings. However, HIV microbicide development still faces

formidable challenges, including conclusive demonstration of efficacy, selection of drug-resistant virus in clinical settings, cultural acceptability, and affordability. These issues notwithstanding, advances in HIV RT research over that last 25 years, ranging from expression of active recombinant enzyme for high throughput screening to the potential of inhibitors as vaginal and rectal microbicides in resource-limited settings, must be considered a bench-to-bedside success and a model for development of future antiviral agents.

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Chapter 9
Telomerase: A Eukaryotic DNA Polymerase Specialized in Telomeric Repeat Synthesis

Andrew F. Brown, Joshua D. Podlevsky, and Julian J.-L. Chen

Abstract Telomerase is an RNA-dependent DNA polymerase (reverse transcriptase) specialized in the synthesis of short DNA repeats onto chromosome ends, a function essential for chromosome stability and cellular immortality in eukaryotes. Unlike conventional polymerases, telomerase is a ribonucleoprotein (RNP) enzyme, minimally composed of the catalytic telomerase reverse transcriptase (TERT) and the telomerase RNA (TR) subunit. While the TERT catalytic core is well conserved and shares several motifs with conventional reverse transcriptases, the TR subunit is highly divergent and has evolved species-specific structural elements essential for telomerase RNP assembly and biogenesis. Telomerase is unique among polymerases, capable of producing a DNA product vastly longer than the RNA template. This unique polymerization reaction relies on repeatedly regenerating and reusing the short TR template during DNA synthesis, producing the characteristic repetitive telomeric DNA sequence. Processive telomeric repeat synthesis is dependent on "template translocation" for template regeneration, a complex mechanism that is only partially understood. Correspondingly, telomerase-specific domains within telomerase TERT-TR core function cooperatively with telomerase accessory proteins to coordinate template translocation during processive telomeric DNA repeat synthesis. Telomerase is thus a fascinating polymerase, singular in function and unrivaled in complexity.

Keywords Telomerase • Telomere • Reverse transcriptase • Template translocation • Repeat addition processivity

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Abbreviations

CAB	Cajal body localization
CP2	Ciliate counterpart
CTE	C-terminal extension
IFD	Insertion in fingers domain
LINE-1	Long interspersed element-1
PLE	Penelope-like element
RID1	RNA-interacting domain 1
RNP	Ribonucleoprotein
RT	Reverse transcriptase
scaRNA	Small Cajal body RNA
snoRNA	Small nucleolar RNA
TASC	Telomere adaptor sub-complex
TBE	Template boundary element
TEN	Telomerase essential N-terminal
TERT	Telomerase reverse transcriptase
TR	Telomerase RNA
TRBD	Telomerase RNA-binding domain
TWJ	Three-way junction
VSR	Vertebrate-specific region

9.1 Introduction

Eukaryotic cells evolved linear chromosomes with special end caps, termed telomeres, for distinction from double-stranded DNA breaks. These telomere-capped ends prevent DNA fusions that often occur after double-stranded DNA breaks and lead to chromosomal rearrangements (Muller 1938; McClintock 1941). Linear chromosomes also inherit an "end replication problem." Conventional DNA polymerases cannot initiate DNA synthesis de novo, requiring an RNA primer for extension, and are thus unable to fully replicate the ends of linear DNA (Watson 1972; Olovnikov 1973). Incomplete replication of chromosome ends results in the progressive loss of terminal DNA with each cell division, endangering genome integrity. The elusive nature of the telomere cap began to be understood by the discovery of repetitive $(TTGGGG)_n$ sequences in *Tetrahymena* telomeric DNA by Blackburn in 1978 (Blackburn and Gall 1978). Shortly after, a linear DNA with terminally flanked TTGGGG repeats was not only found to be stably maintained in budding yeast cells, but was even extended with yeast telomeric DNA repeats (Szostak and Blackburn 1982). This finding predicted the presence of a telomeric DNA-extending enzyme. This enzyme, responsible for synthesis of these unusual telomeric DNA repeats, was later discovered and termed "telomerase" (Greider and Blackburn 1985).

Telomerase is an RNA-dependent DNA polymerase that contains an intrinsic and essential RNA component, thus functioning as a ribonucleoprotein (RNP) enzyme (Greider and Blackburn 1987). The telomerase RNP core is composed of the telomerase reverse transcriptase (TERT) and the integral telomerase RNA (TR). The TR provides the template for DNA synthesis by TERT as well as essential structural domains for enzymatic activity. In addition to the catalytic core, the telomerase holoenzyme consists of a variety of telomerase-associated proteins. These accessory proteins are dispensable for enzymatic activity, while crucial for telomerase biogenesis, localization, and regulation (Fu and Collins 2003; Venteicher and Artandi 2009; Egan and Collins 2010; Kiss et al. 2010). The biogenesis pathway of telomerase RNA holoenzyme has been extensively reviewed elsewhere (Egan and Collins 2012a) and will only be discussed briefly in this chapter.

Distinct from all known DNA polymerases, telomerase synthesizes a DNA product that is vastly larger than the RNA template. The telomeric DNA product is composed of repetitive copies of a simple DNA sequence, TTGGGG in *Tetrahymena* and TTAGGG in human. Identification of the *Tetrahymena thermophila* TR revealed a template sequence complementary for 1.5 copies of telomeric sequence (Greider and Blackburn 1989). This implied a "template translocation" mechanism where the half repeat of the template sequence serves as a realignment site for repositioning the DNA primer on the RNA template after each repeat synthesis (Fig. 9.1). Such a mechanism would explain the processive synthesis of long stretches of telomeric DNA repeats from a short RNA template (Greider 1991). Similar to conventional reverse transcriptase (RT) reactions, the telomerase reaction is initiated by the DNA primer annealing to the RNA template, and DNA synthesis terminates upon reaching the end of template (Fig. 9.1). Remarkably, telomerase regenerates and reuses the same RNA template for successive telomeric DNA repeat synthesis. However, the detailed mechanism underlying telomerase repeat addition processivity and template translocation is only beginning to emerge in the last few years, impeded by the complex interactions of multiple domains within TERT, TR, and accessory proteins.

9.2 Telomerase Reverse Transcriptase

The catalytic TERT protein is well conserved among most known species. It comprises four structural domains: the telomerase essential N-terminal (TEN) domain, the telomerase RNA-binding domain (TRBD), the RT domain, and the C-terminal extension (CTE) (Fig. 9.2a). The RT domain contains motifs that are conserved in all RTs and constitutes the active site for RNA-dependent DNA polymerization (Lingner et al. 1997b). The other three domains are telomerase specific and function cooperatively with TR and telomerase accessory proteins for processive telomeric repeat addition.

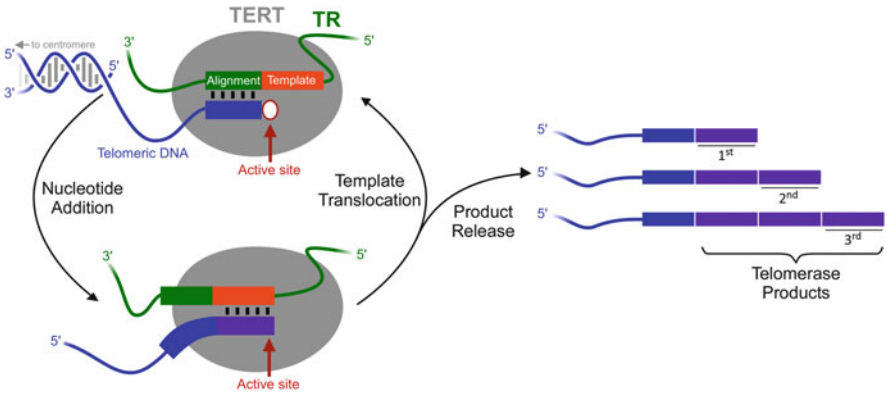

Fig. 9.1 The unique telomerase catalytic cycle. Telomerase functions as a conventional reverse transcriptase by synthesizing single-stranded DNA onto the ends of a telomeric DNA primer (*blue*) from an intrinsic RNA template (*orange*), *nucleotide addition*. Apart from conventional polymerases, upon reaching the end of the template, telomerase has the capacity to regenerate the template by a complex mechanism of *template translocation*. Following this, an additional round of *nucleotide addition* then proceeds generating longer telomerase products (*violet*). Unsuccessful *template translocation* terminates the synthesis of additional telomeric repeats by *product release*

The TEN domain of TERT has two important functions: binding the TR subunit and the single-stranded telomeric DNA (Fig. 9.2b). Binding the single-stranded telomeric DNA by the TEN domain is vital for enhancing processive repeat synthesis. TEN contains a high-affinity DNA "anchor" site for specifically binding single-stranded telomeric DNA (Jacobs et al. 2006; Lue and Li 2007; Romi et al. 2007; Sealey et al. 2010; Finger and Bryan 2008). This binding restrains the telomeric DNA within close proximity of the active site, delaying product release which in turn increases processive telomeric repeat synthesis (Wyatt et al. 2010). A conserved leucine residue outside the DNA anchor site was found to also enhance processive telomeric repeat synthesis (Zaug et al. 2008; Eckert and Collins 2012). This residue was first identified in *T. thermophila*, with putatively homologous residues found within yeast and human TEN domains. While mutating this residue decreased processive telomeric repeat synthesis, this mutation failed to decrease telomeric DNA retention by the DNA anchor site. Instead, this leucine is believed to function as a molecular switch. However, the mechanism is poorly understood at this time. Outside the DNA anchor site and the important leucine residue, TEN contains a low-affinity RNA-binding domain for binding the TR pseudoknot (Lai et al. 2001; Moriarty et al. 2004). However, the mechanistic significance of this TERT-pseudoknot interaction is also poorly understood. Interestingly, while TEN contains elements that enhance processive telomeric repeat synthesis and a low-affinity RNA-binding site, this domain is not essential for catalytic activity (Eckert and Collins 2012). Certain insect species, including *Tribolium castaneum*, have a truncated TERT N-terminus, seeming to lack the TEN domain entirely (Fig. 9.2) (Gillis et al. 2008).

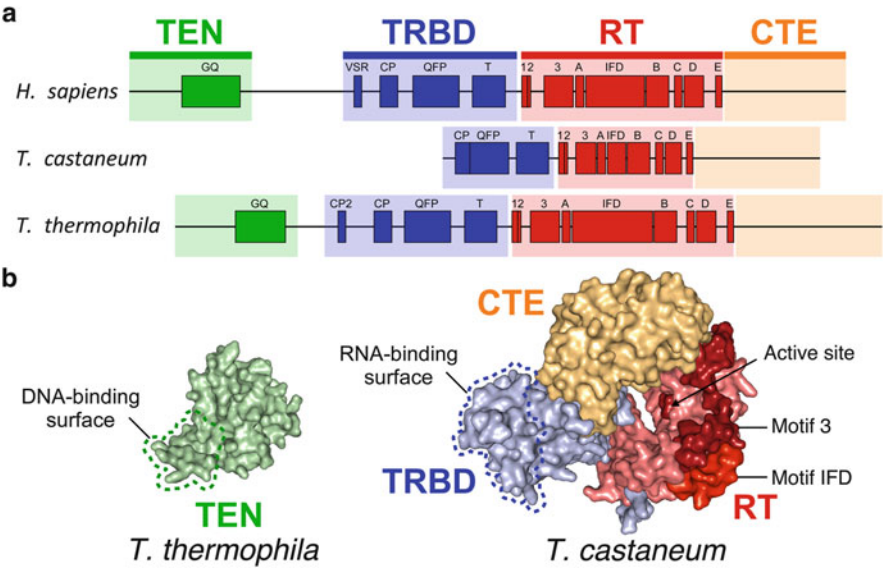

Fig. 9.2 Structural organization of the catalytic TERT protein. (**a**) TERT is composed of four structural domains: telomerase essential N-terminal (TEN, *green*) domain, telomerase RNA-binding domain (TRBD, *blue*), reverse transcriptase (RT, *red*), and the C-terminal extension (CTE, *orange*). The TEN and TRBD are telomerase-specific, essential for *template translocation*, and not found among conventional polymerases. Important motifs are colored similarly in the encompassing domains. (**b**) The crystallized *Tetrahymena thermophila* TEN and *Tribolium castaneum* TRBD crystal fragments present important nucleotide-binding surfaces. The *Tribolium castaneum* TERT protein forms a ring structure unseen in other polymerases. The TERT active site, motif 3 (*dark red*) and motif IFD (*red-orange*) in the RT domain are denoted

Unlike the TEN domain, TRBD is more conserved and found in all known TERT proteins to date (Podlevsky et al. 2008). This domain contains a high-affinity RNA interacting domain essential for RNP assembly (Moriarty et al. 2004). The highly helical TRBD structure comprises three universal motifs: CP, QFP, and T (Fig. 9.2a) (Rouda and Skordalakes 2007; Gillis et al. 2008). Separate from RNA binding, motif T has been implicated in processive telomeric repeat synthesis through proposed interactions between motif T and the RNA backbone of the TR template (Drosopoulos and Prasad 2010; Mitchell et al. 2010). In addition to the universally conserved motifs, TRBD has been shown to contain a vertebrate-specific region (VSR) and a ciliate counterpart (CP2). VSR and CP2 both bind the TR, and CP2 is involved with delineating the template boundary within the ciliate TR (Harley 2002; Moriarty et al. 2002).

The catalytic RT domain is centrally located within the primary sequence of the TERT protein (Fig. 9.2). As a reverse transcriptase, TERT contains all the hallmark motifs of DNA polymerases: 1, 2, and A through E. TERT is a right-handed polymerase containing the "fingers" and "palm" domains similar to conventional RTs. The TERT fingers bind incoming nucleotides and positions the RNA template

(Wyatt et al. 2010; Gillis et al. 2008; Bosoy and Lue 2001), while the TERT palm forms the catalytic site for DNA polymerization. Within the palm, motifs A and C contain a triad of invariant aspartic acids for conventional two-metal nucleotide synthesis. The loss of any of these three aspartic acids completely abolishes telomerase enzymatic activity in vitro and results in telomere shortening in vivo (Weinrich et al. 1997; Harrington et al. 1997; Bryan et al. 2000b; Counter et al. 1997; Nakayama et al. 1998; Wyatt et al. 2010).

In addition to the catalytic aspartic acids, flanking amino acids in motif C have been shown to play a role in repeat addition rate and processivity as well as nucleotide addition fidelity, though the effects appear to be species specific (Peng et al. 2001; Drosopoulos and Prasad 2007; Bryan et al. 2000a). TERT also contains an invariant lysine residue in motif D which is believed to function as an acid, activating the pyrophosphate generated from nucleotide addition. The loss of this lysine in TERT severely reduces telomerase activity (Miller et al. 2000; Bryan et al. 2000b; Sekaran et al. 2010). Motif E, in the TERT palm domain, functions as a primer grip for positioning the DNA primer (Peng et al. 2001; Wyatt et al. 2007). This function is further supported by the *Tribolium castaneum* TERT crystal structure, in which a coiled loop is proximal to the end of the DNA primer (Mitchell et al. 2010).

Although the TERT RT domain is well conserved among RTs, several telomerase-specific motifs have evolved within this domain for telomerase-specific functions. A large insertion found in the fingers domain, aptly termed the "insertion in fingers domain" (IFD), influences processive telomeric repeat synthesis in vitro and telomere maintenance in vivo (Lue et al. 2003). In addition to IFD, another telomerase-specific motif, motif 3—so named for its location immediately following motif 2 (Fig. 9.2a)—is directly involved in repeat addition processivity (Xie et al. 2010). Interestingly, alanine screening revealed that specific residues within motif 3 could alter the rate of telomeric repeat synthesis independent of altering the processivity of telomeric repeat synthesis. The helix-coil-helix structure of motif 3 lies atop the active site and appears to be in close contact with the RNA/DNA duplex (Gillis et al. 2008; Mitchell et al. 2010).

The C-terminal extension (CTE), comprising the C-terminus of TERT, contains little-to-no sequence homology to conventional RTs. However, the overall structure and function of CTE is similar to the "thumb" domain of retroviral RTs, specifically the HIV1 RT (Nakamura et al. 1997; Gillis et al. 2008). The TERT CTE affects telomeric DNA binding, telomerase activity, and processive telomeric repeat synthesis (Hossain et al. 2002; Huard 2003). The crystal structure of *Tribolium castaneum* TERT revealed interactions between CTE and TRBD (Fig. 9.2b) (Gillis et al. 2008). These interactions shape the global architecture of the TERT protein, forming a ring structure instead of the commonly seen horseshoe shape of conventional RTs. It has been proposed that CTE may interact with TR when bound to TRBD (Bley et al. 2011). Thus the TR could function as a brace to strengthen the CTE-TRBD interactions and maintain the TERT structure.

9.3 Telomerase RNA

Unique among RTs, telomerase contains an integral RNA component. The RNA associated with conventional RTs provides merely a template for reverse transcription. In contrast, TR is a multifaceted RNA with unique structural elements crucial for telomerase enzymatic activity in addition to providing the template for nucleotide addition. TR is remarkably divergent in size, sequence, and structure with distinct structural domains and motifs emerging along separate phylogenetic lineages (Fig. 9.3a–d). The inherent differentiation in TR is associated with a plethora of species-specific TR-binding proteins (Podlevsky and Chen 2012). However, despite this immense variation, two structural elements outside of the template are universal to all known TRs: the pseudoknot and a stem-loop moiety (Fig. 9.3a–d) (Chen et al. 2000, 2002; Chen and Greider 2004; Lin et al. 2004; Brown et al. 2007; Qi et al. 2012a). These two ubiquitous TR elements are sufficient for reconstituting telomerase activity in vitro when added to TERT either as a truncated TR or excised as two physically separated RNA fragments and combined in trans (Tesmer et al. 1999; Mitchell and Collins 2000; Qi et al. 2012a).

The pseudoknot element from human TR was found to contain a triple helix formed from Hoogsteen base pairings (Theimer et al. 2005). Additional TR pseudoknots from other species have either been shown, or predicted, to contain a similar triple helix (Qiao and Cech 2008; Shefer et al. 2007). While the pseudoknot—and apparently the triple helix—is well conserved and essential for telomerase activity, the precise function of either structure is not well known (Ly et al. 2003; Chen and Greider 2005; Qiao and Cech 2008). While the template is distant from the pseudoknot in the primary sequence, it is located proximal to the pseudoknot within the secondary structure (Fig. 9.3a–d). From this, the pseudoknot has been postulated to function for template positioning or retention of the template in proximity to the TERT active site. Furthermore, NMR structures revealed human TR has a sharp kink located between the pseudoknot and the template (Zhang et al. 2010, 2011). This kink is believed to facilitate proper positioning of the template and may be ubiquitous to TR.

In addition to the pseudoknot structure, the other universal TR element is a stem-loop moiety located downstream of the template-pseudoknot region (Fig. 9.3a–d). Discovered independently within three major phylogenetic groups, this element has been separately termed: CR4/5 in vertebrates (composed of P6 and P6.1), three-way junction (TWJ) in yeasts, and helix IV in ciliates (Brown et al. 2007; Blackburn and Collins 2011; Chen et al. 2000, 2002; Qi et al. 2012a). The vertebrate CR4/5 and yeast TWJ are both a junction of three stems, two of which are capped by an apical loop (Fig. 9.3b, c). It was found that the vertebrate CR4/5 was necessary for telomerase activity in vitro and telomere maintenance in vivo. Among the three stems in the vertebrate CR4/5, P6.1—composed of a 4 bp stem together with a 5 nt loop—is highly conserved and essential for telomerase activity (Chen et al. 2002). Mutations either disrupting the stem or altering the conserved residues in the loop abolished telomerase activity. Recent cross-linking studies mapped the essential

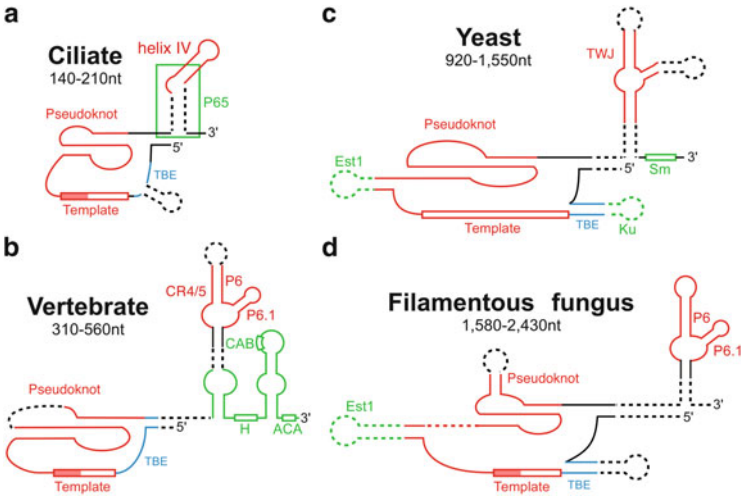

Fig. 9.3 The conserved core of the highly divergent TR. (**a**) Ciliate TRs include the smallest TR identified to date. The binding site for p65 (*green box*) is important for ciliate telomerase activity and RNP assembly. (**b**) Vertebrate TRs contain a CR4/5 domain (*red*) composed of P6 and P6.1. The 3′-proximal H/ACA domain with a CAB box in the apical loop (*green*) is crucial for TR biogenesis and telomerase localization. (**c**) Yeast TRs contain a three-way-junction element (TWJ, *red*) which is functionally dissimilar from vertebrate TRs. Additionally, there are Est1, Ku, and Sm protein-binding sites (*green*) important for telomerase activity in vivo. (**d**) Filamentous fungal TRs contain the largest known TR found and include a CR4/5-like domain (*red*) composed of P6 and P6.1 and an Est1-binding site (*green*). Two major structural elements are common to all known TRs: a template-proximal pseudoknot (*red*) and template-distal three-helical junction [P6/P6.1 and TWJ (*red*)]. Ciliates appear to have a homologous structure, helix IV (*red*). Additionally, there is a template boundary element (TBE, *blue*) which is functionally similar yet structurally divergent

vertebrate P6/6.1 stem-loop element onto the TRBD surface with single-residue resolution (Bley et al. 2011). The close proximity of the P6.1 cross-linking site with the CTE suggests the P6.1 stem-loop could interact with CTE and regulate the CTE-TRBD interactions and telomerase RNP assembly.

Yeast TWJ lacks the vertebrate conserved P6.1 loop and is dispensable for telomerase function in vitro (Brown et al. 2007; Zappulla et al. 2005). However, recent studies of fungal TRs from the fission yeast *Schizosaccharomyces pombe* and Pezizomycotina, such as the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*, identified a vertebrate-like P6.1 in this region (Fig. 9.3d). The filamentous fungal P6 and P6.1 were also found to be essential for telomerase activity in vitro (Qi et al. 2012a). While ciliates do not have a three-way junction, there is a helix moiety—helix IV—which is weakly bound by TERT and is necessary for telomerase activity (Mason et al. 2003; Lai et al. 2003). The ciliate helix IV has been postulated to be functionally analogous to P6.1 which is conserved in both vertebrates and filamentous fungi (Blackburn and Collins 2011).

Since the TR is vastly larger than the template sequence, a physical boundary is needed to define where reverse transcription must terminate. This template boundary element (TBE) prevents reverse transcription into the 5′ flanking region and the synthesis of non-telomeric DNA sequences. The mechanisms and structural elements of TBE appear to be as divergent as the rest of TR (Fig. 9.3a–d). In ciliates, the 5′ TBE includes conserved sequences at the base of the template adjacent helix II (Autexier and Greider 1995; Lai et al. 2002). The ciliate TBE also serves as a high-affinity TERT binding site, suggesting that the TBE is defined by TERT binding which blocks DNA synthesis from this region of the RNA (Fig. 9.3a). In budding yeast, the TBE is a stable helix located 0–2 nt 5′ of the template (Tzfati et al. 2000; Seto et al. 2003). This helix provides a physical barrier by limiting single-stranded RNA in this region that could function as template (Fig. 9.3c). In fission yeasts, the TBE partially overlaps with the template. This duality in function for specific nucleotides in the TR is responsible for the telomeric repeat heterogeneity found in *S. pombe* telomeres (Box et al. 2008). Filamentous fungal TRs appear to have a similar TBE structure to yeast, composed of a template adjacent helix (Qi et al. 2012a). This suggests that the filamentous fungal TBE is functionally similar to the yeast TBE as well (Fig. 9.3d). In human and most vertebrate TRs, the TBE is located 6–8 nt 5′ of the template and is also composed of a helical structure (Fig. 9.3b). The vertebrate TBE restrains the template in the active site during reverse transcription, rather than steric hindrance by protein–RNA or RNA–RNA interactions as seen in ciliates and yeasts (Moriarty et al. 2005; Chen and Greider 2003b). Surprisingly in vertebrates, certain rodent TRs lack this template-proximal helix and have only two remaining residues 5′ of the template (Hinkley et al. 1998). In these rodents, the lack of any significant sequence 5′ of template serves functionally as TBE (Chen and Greider 2003b).

Outside the universal template-pseudoknot and distal stem-loop moiety domains are species-specific TR structural elements that are essential for biogenesis, localization, and accumulation (Fig. 9.3a–d). Within vertebrate TR, the 3′ end contains an H/ACA domain (Fig. 9.3b). The H/ACA domain is composed of two stem-loops separated by a box H motif and followed by a box ACA motif, a structure also conserved in small nucleolar (sno) and small Cajal body (sca) RNAs (Kiss et al. 2010; Li 2008; Mitchell et al. 1999; Vulliamy et al. 2006). In accordance with the conservation of this moiety with sno- and scaRNAs, two copies of a protein complex composed of dyskerin, NOP10, NHP2, and GAR1 bind to each stem of the H/ACA domain for 3′-end processing and RNA accumulation (Egan and Collins 2012b; Mitchell et al. 1999). Additionally, the terminal loop of the H/ACA domain contains a Cajal body localization (CAB box) motif important for proper RNA localization and a biogenesis promoting (BIO box) motif (Reichow et al. 2007; Venteicher and Artandi 2009; Theimer et al. 2007). Furthermore, the 5′ end of the TR contains a guanosine-rich track which is postulated to form a G-quadruplex structure (Lattmann et al. 2011; Sexton and Collins 2011). The HEXH box RNA helicase RHAU is a part of the active telomerase holoenzyme complex which increases TR accumulation and would resolve the putative G-quadruplex structure.

Fungal TRs are significantly larger than vertebrate TRs and appear to function as a flexible scaffold for binding a separate set of telomerase accessory proteins (Fig. 9.3) (Zappulla et al. 2005). Between the template and the pseudoknot is an extended helix which binds a telomerase accessory protein called ever shorter telomere protein 1 (Est1p), which is essential for telomere maintenance in vivo (Seto et al. 2002; Evans and Lundblad 2002). Also found within all known yeast TRs is an Sm protein-binding site (Fig. 9.3c), which is essential for TR 3′ end processing (Seto et al. 1999). Yeast TRs also contain a template adjacent extended helix, and in most yeast TRs harbor a binding site for the Ku heterodimer, yKu70 and yKu80 (Stellwagen et al. 2003; Fisher and Zakian 2005). *Kluyveromyces lactis* TR includes this extended template-proximal helix; however it lacks Ku heterodimer association (Kabaha et al. 2008). TR is a functional RNA component of telomerase. Rather than merely providing the template for reverse transcription—as is true for all other known RTs—this RNA is an integral component, essential for telomerase activity, with functions yet to be fully understood.

9.4 Evolutionary Aspects of Telomerase RNP

In the transition from circular to linear chromosomes, ancestral eukaryotes required a means to counterbalance progressive terminal DNA loss after chromosome replication. The solution that emerged presumably was to extend the 3′ ends of genomic DNA by a primitive reverse transcriptase, the precursor to TERT, which used a simple RNA as the template. Over time, this TERT precursor evolved specific RNA-binding domains for internalizing this simple RNA template that later became the integral TR component. While little remains of this ancestral TR, the discovery of TRs from several phylogenetic groups is slowly increasing our understanding of TR evolution (Qi et al. 2012a; Sandhu et al. 2013).

The overarching structural and functional conservation within the TRs from fungi and vertebrates suggests that the pseudoknot and TWJ were important features of a common ancestor TR before the branching of fungi and vertebrate lineages. Similar to the TWJ in vertebrates and fungi, stem-loop IV in ciliates is a binding site for TERT and is required for telomerase RNP assembly (Robart et al. 2010). It is unclear if the TWJ structure in yeasts and vertebrates emerged independently or evolved from an ancestral structure homologous to the ciliate stem-loop IV.

Unlike TR, TERT is highly conserved across all explored taxa, although many adaptations have specialized TERT for DNA repeat synthesis. Phylogenetic comparative analysis indicates that TERT shares sequence attributes with eukaryotic retrotransposon RTs, a large and diverse group of self-replicating genetic elements found throughout eukaryotic genomes. The similarities between TERT and retrotransposon RTs provide insight into the evolutionary origins of the telomerase RNP. While the majority of retrotransposons employ endonuclease activity prior to nucleotide synthesis, several retrotransposons—such as Penelope-like element

(PLE) and long interspersed element-1 (LINE-1)—are endonuclease independent and require a free DNA 3′ end for retrotransposition (Kopera et al. 2011). These observations suggest a common ancestral RT in early eukaryotes diverged and specialized into various retrotransposons, including the unique telomerase enzyme.

9.5 Telomerase Mechanism

The mechanism by which telomerase functions is astonishingly complex. In addition to catalyzing nucleotide addition—similar to other polymerases—telomerase evolved a novel mechanism for the processive synthesis of telomeric repeats. Conventional RTs bind pre-annealed RNA/DNA duplex as substrate. While the substrate for telomerase is the single-stranded telomeric DNA, once bound to telomerase, the 3′ end of the DNA base-pairs with the RNA template to form a DNA/RNA duplex which is then bound by the TERT catalytic core. Thus the substrate for the TERT catalytic core is similar to the substrate of conventional RTs, while the cellular substrate for telomerase is only single-stranded DNA at chromosome ends. The complex and highly regulated recruitment of telomerase to telomeres in vivo has been reviewed elsewhere (Nandakumar and Cech 2013) and is not discussed in detail here.

Telomerase employs an incredibly short region within its integral TR component as template, while conventional RTs employ vastly longer RNA templates for the synthesis of complementary DNA. Remarkably, from this short template, TERT is capable of synthesizing a vastly larger DNA product within a single uninterrupted reaction. This is accomplished by regeneration of the template through a template translocation mechanism that readies the template for an additional cycle of DNA polymerization. This repeated DNA synthesis produces the characteristic telomeric repeat sequence. While the telomerase catalytic cycle is complex and remains poorly understood, still much has been discerned in recent years—sufficient for a working model. In this model, the template translocation cycle is minimally composed of five distinct steps: (1) nucleotide addition, (2) duplex disassociation, (3) strand separation, (4) template realignment, and (5) duplex binding, which are discussed below in much detail.

9.5.1 Nucleotide Addition

Similar to all known RTs, telomerase catalyzes nucleotide addition onto the 3′-OH of a given DNA strand employing the RNA strand as a template (Fig. 9.4a, *step 1*). The mechanism for DNA polymerization of incoming nucleotides is similar to other RTs—a two-metal ion system involving three invariant aspartic acids in the TERT palm domain which coordinate two magnesium ions (Lingner et al. 1997a; Gillis et al. 2008). Free nucleotides are bound and positioned by the nucleotide-binding pocket between the fingers and palm domain in TERT (Gillis et al. 2008).

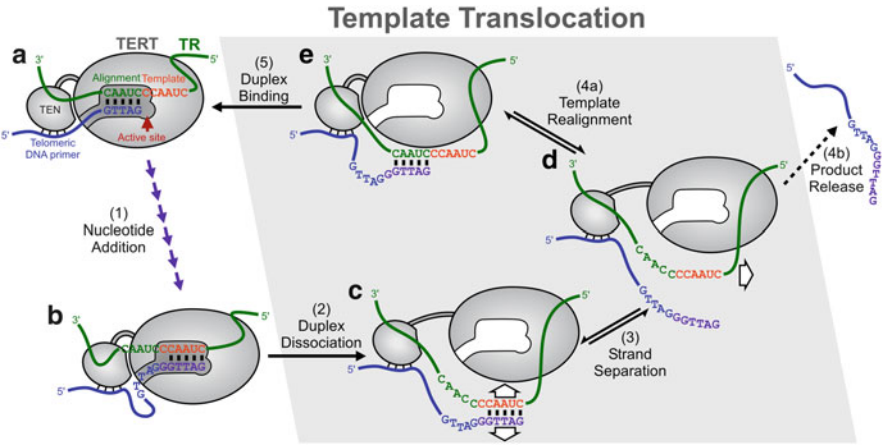

Fig. 9.4 A working model for the telomerase template translocation mechanism. (**a**) Schematic of human telomerase bound to a telomeric DNA primer (*blue*). The TR alignment region (*green*) is base-paired with the 3′-end of the telomeric DNA primer (*blue*) to form 5 base pairs adjacent to the active site (*red arrow*). The TR template (*orange*) is constrained by flanking sequences bound to the TERT protein, while the 5′ region of the telomeric DNA is bound to the TEN domain (*gray*). TERT (*gray*) catalyzes the addition of six deoxyribonucleotides (*violet arrows*) to the 3′ end of the DNA primer by reverse transcribing the TR template sequence (*step 1*). (**b**) After nucleotide addition, a new repeat (*violet*) is generated which retains only 5 base pairs. After reaching the end of the template, the duplex dissociates from the active site (*step 2*). (**c**) Outside the active site, the RNA/DNA duplex undergoes template translocation (*light gray box*) involving strand separation (*step 3*) and (**d**) template realignment (*step 4a*) to reform 5 base pairs. (**e**) Further nucleotide addition can proceed with the regenerated template. Unsuccessful realignment of the DNA primer to the RNA template eventually results in complete dissociation of the DNA product from the enzyme (*step 4b*). Strand separation and template realignment (*steps 3* and *4a*) are postulated as reversible, with multiple binding/separation steps possible

Upon base-pairing the telomeric DNA primer with the TR template, the RNA/DNA duplex is positioned within the active site, presumably by the cooperation of motif 3, IFD, and CTE which surround the central cavity in the *Tribolium castaneum* TERT crystal structure (Gillis et al. 2008; Mitchell et al. 2010; Xie et al. 2010).

After each nucleotide addition, the RNA/DNA duplex advances away from the catalytic site so as to position the 3′ end of the newly incorporated nucleotide adjacent to the catalytic site for further nucleotide addition. This "duplex translocation" during nucleotide addition is believed to be facilitated by both the flanking single-stranded DNA and RNA through a possible DNA secondary structure formation and/or the stretch and compression of the bound RNA (Jarstfer and Cech 2002; Berman et al. 2011). Weak interactions between the flanking single-stranded DNA and TERT surface could also promote duplex translocation during nucleotide addition.

During nucleotide addition, the active site-bound RNA/DNA duplex remains at a constant length. Evidence from both human and yeast telomerase reactions

indicates that the extended RNA/DNA duplex is maintained at 5–7 base pairs by the disruption of a base pair at the 5′ end of the DNA for each new base pair formed at the 3′ end (Qi et al. 2012b; Förstemann and Lingner 2005). Interestingly, in the *T. castaneum* TERT crystal structure, the central cavity of the protein is estimated to accommodate a duplex of 7–8 base pairs (Gillis et al. 2008). Moreover, the diameter of the central cavity decreases by 2–3 Å upon binding an RNA/DNA duplex, possibly an induced-fit conformational change (Mitchell et al. 2010). Disrupting base pairs at the 5′ end of the DNA also appears to be functional significant for duplex translocation through the active site during processive nucleotide addition. When supplied with pre-annealed five base-paired RNA/DNA duplex substrates, a template-free telomerase that has the template region deleted from TR can processively add multiple nucleotides to only duplexes with a 5′ single-stranded DNA overhang (Qi et al. 2012b). This suggests that TERT binds the unpaired single-stranded DNA for duplex translocation through the active site during nucleotide addition.

9.5.2 Duplex Dissociation

Upon reaching the end of the template, the RNA/DNA duplex must separate, realign, and re-anneal so that the 3′ end of the DNA is again at the start of the template for further nucleotide addition (Fig. 9.4b, *step 2*). Recent evidence suggests that RNA/DNA duplex dissociation from the active site is the first step of template translocation, occurring prior to strand separation and template realignment and duplex formation (Fig. 9.4c–e). In a pulse-chase assay, a five-base-pair RNA/DNA duplex significantly reduced telomerase repeat addition processivity (Qi et al. 2012b). Thus the active site is temporarily unoccupied during template translocation, supporting template translocation occurring outside the active site.

Separation of the duplex without dissociation from the active site seems less feasible. It would require a significant conformation change in the telomerase RNP to break the hydrogen bonds of the RNA/DNA duplex as well as the protein-nucleic acid contacts restraining the duplex within the active site. This is not consistent with the relatively minor changes observed in the crystal structures of *T. castaneum* TERT with and without an RNA/DNA duplex bound in the active site (Mitchell et al. 2010). Furthermore, the correlation between the repeat addition rate in telomerase mutants and the enzyme turnover rate with duplex substrates indicates that duplex dissociation is the rate-limiting step of template translocation (Qi et al. 2012b). This could explain the observation that changes in template sequence can drastically affect telomerase repeat addition rate, which cannot be explained by the thermodynamic stability of the RNA/DNA duplex (Drosopoulos et al. 2005). Under this model, changes in the dissociation rate of the duplexes with altered template sequences could be the cause of the changes in repeat addition rate.

9.5.3 Strand Separation

Once the RNA/DNA duplex dissociates from the active site, the duplex must dissociate into separate DNA and RNA strands for template realignment and re-annealing the RNA/DNA duplex so that the 3′-OH of the DNA is adjacent to the unoccupied template for further nucleotide addition (Fig. 9.4, *step 3*). The underlying mechanism for strand separation has yet to be determined. However, a 5–6-base-pair duplex—maintained during nucleotide addition—once released from the active site, could spontaneously separate in milliseconds at 37 °C based on thermodynamic predictions (Qi et al. 2012b).

Proceeding separation from the RNA template, the 3′ end of the DNA is no longer bound to the active site (Fig. 9.4d). To prevent complete DNA dissociation and the termination of repeat synthesis, the telomerase holoenzyme evolved several binding sites for DNA retention proximal to the active site, which greatly enhances repeat addition processivity (Fig. 9.4, *step 4a, 4b*). An upstream region of the DNA remains bound to TEN, even when the 3′ end of the DNA has dissociated from the active site (Fig. 9.2b) (Jacobs et al. 2006; Wyatt et al. 2007; Finger and Bryan 2008; Zaug et al. 2008). This DNA–protein interaction tethers the telomeric DNA to the TERT protein during template translocation so that only the DNA 3′ end dissociates from the active site for template realignment.

Additionally in human telomerase, the accessory proteins POT1 and TPP1 have been shown to greatly increase telomerase repeat addition processivity by decreasing DNA dissociation through synergetic interactions with single-stranded DNA and TERT (Wang et al. 2007; Latrick and Cech 2010). As part of the telomeric protein-nucleic acid complex, the POT1–TPP1 complex recruits telomerase to telomeric DNA in vivo in a cell cycle-dependent manner (Nandakumar et al. 2012; Sexton et al. 2012; Zhong et al. 2012; Zhang et al. 2013). While the DNA is constrained within proximity to the active site, the unbound 3′ end can re-anneal either with the template region, producing an un-extendable duplex, or with the alignment region ready for another repeat synthesis (Fig. 9.4, *step 4a*).

In ciliates, a functional equivalent to the POT1–TPP1 complex has been found, Teb1 and the three-subunit telomere adaptor sub-complex (TASC). The Teb1–TASC complex dramatically enhances *T. thermophila* telomerase repeat addition processivity (Min and Collins 2009, 2010). It has been postulated that there are multiple low-affinity DNA-binding sites on TERT and Teb1–TASC complex allowing single-stranded DNA to thread through the enzyme complex, while high-affinity binding sites on Teb1 are hypothesized to prevent secondary structure formation of the upstream DNA that could induce complete DNA dissociation from the enzyme (Min and Collins 2010; Collins 2011).

9.5.4 *Template Realignment*

Following strand separation, the DNA primer must re-anneal to the alignment region of the RNA template for further nucleotide addition (Fig. 9.4, *step 5*). However, the DNA could completely dissociate from the protein and terminate repeat addition (Fig. 9.4, *step 4b*), or the DNA could re-anneal and occupy the template, resulting in an unproductive template translocation event (Fig. 9.4d). The probability of successful template translocation over complete DNA dissociation defines the "translocation efficiency." Since the DNA must re-anneal to the alignment region for successful template translocation, the length of the alignment region of adjoining the template has been found to greatly influence repeat addition processivity (Chen and Greider 2003a). Steric forces from stretching and compressing the TR template-flanking linkers during nucleotide addition may assist in repositioning the DNA toward the alignment region (Berman et al. 2011).

9.5.5 *Duplex Binding*

After the DNA has re-annealed with the alignment region of the RNA template, the duplex is then ready to rebind to the TERT active site for another round of nucleotide addition (Fig. 9.4e). The newly formed duplex is incredibly short, at only 5 base pairs for the human telomerase reaction. This diminutive duplex is rather unstable at 37 °C. Rapid binding by the TERT active site is needed to stabilize the duplex with protein-nucleic acid contacts and result in a successful template translocation event (Fig. 9.4, *step 5*). Experiments with template-free telomerase and short duplex substrates show a strong correlation between TERT duplex binding affinity and the repeat addition processivity (Xie et al. 2010; Qi et al. 2012b). TERT mutations in motif 3, IFD, and CTE that reduced repeat addition processivity increased the Km for RNA/DNA duplex substrates and decreased template translocation efficiency (Qi et al. 2012b). This suggests that the TERT binding affinity for RNA/DNA duplex is a major determinant of telomerase repeat addition processivity.

9.6 Concluding Remarks

Over the past three decades, the importance of telomerase for telomere maintenance, cellular immortality, cancer, and human health has become clear. In humans, telomerase expression is tightly regulated: downregulated in healthy human somatic cells, reactivated in most cancer cells, and persistently expressed in germline and stem cells—with the loss or reduction of activity eventually bringing about age-related human diseases (Hiyama and Hiyama 2007). Germline and stem

cell defects which reduce telomerase activity result in a variety of human diseases, including dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis (Armanios 2009).

Telomerase has become a target for anticancer therapeutics as well as the promise of extended cellular replicative potential. Currently, a variety of telomerase-related cancer therapies have shown promise in preclinical studies, and a few have even moved into clinical trials (Agrawal et al. 2012). While telomerase inhibitors are of great interest, to suppress cancer cell progression, telomerase activators are also of growing interest, in the hope of delaying cellular and potentially even organismal aging. Despite detectable levels of telomerase activity, adult stem cells age with corresponding telomere shortening, telomere dysfunction, and limited cellular renewal capacities (Sahin and Depinho 2010). While telomerase-based therapeutics are still at inception, it has become increasingly clear that still greater knowledge of the inner workings of telomerase is necessary to design new, highly specific therapies that target individual aspects of the telomerase RNP. Further elucidation of telomerase mechanism will thus have direct and broad impacts on biomedical research and human health.

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Chapter 10
Bacteriophage RNA Polymerases

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Abstract Bacteriophage-encoded RNA polymerase (RNAP) was first discovered in T7 phage infected *Escherichia coli* cells. It was known that phage infection on host bacterial cells led to redirection of the host gene expression towards generation of progeny phage particles, but a previously uncharacterized "switching event" leading to the expression of late bacteriophage genes was first attributed to a phage-encoded RNAP. This phage RNAP could recognize promoters on the phage genome and express phage genes using a single-polypeptide polymerase of ~100 kDa molecular weight, which is ~4 times smaller than bacterial RNAPs. This was a substantial simplification from the previously known RNAPs from bacteria (5 subunits) and eukaryotes (more than 12 subunits); nonetheless, the single-unit T7 RNAP is able to recognize promoter DNA and unwind double-stranded (ds) DNA to form open complex, and after abortive initiation, it proceeds to processive RNA elongation. The simplicity of T7 phage RNAP made it an ideal model system to study the transcription mechanism and an ideal tool for protein expression system in bacterial cells. In this chapter, we will review the current state of knowledge of transcription mechanism in single-unit bacteriophage RNAPs from the two deeply studied T7 and the N4 phage RNAPs.

Keywords RNA polymerase • Transcription • Bacteriophage • T7 • N4

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10.1 T7 RNAP Structure: The Prototype of Single-Unit RNAP

The first crystal structure of RNAP was determined in 1993 from the single-unit bacteriophage T7 RNAP (Sousa et al. 1993). The domain organization of the T7 RNAP was found similar to the bacterial DNA polymerase (DNAP) I (Arnold et al. 1995), e.g., Klenow fragment (KF) of DNA Pol I (Fig. 10.1). The structure resembles the anatomy of a right hand comprising of palm, fingers, and thumb subdomains that are arranged around a DNA-binding cleft. In addition, an N-terminal domain (NTD) constitutes the front wall of the DNA-binding cleft, making the DNA-binding cleft deeper and narrower in RNAP, and also plays roles in promoter recognition and DNA unwinding for making transcription-competent open complex.

The palm subdomain (Fig. 10.1a) forms the base of the DNA-binding cleft with the fingers and the tall thumb subdomains forming either sidewalls of the channel. Invariant residues from motifs A, B, and C cluster around this catalytic cleft. Aspartate residues, conserved in all nucleic acid polymerases, bear the most important catalytic function of chelating two divalent metals (Mg^{2+}) at the active site. The catalytic metal A (Me_A) generates the nucleophile at the 3′-end RNA for the S_N2 nucleotidyl transfer reaction, and a nucleotide-binding metal (Me_B) stabilizes the charge distribution of the incoming nucleotide at the reaction transition state (Steitz et al. 1994; Sect. 10.3.3; Fig. 10.4).

10.1.1 Promoter Binding

The T7 promoter sequence is conserved from the −17 to +6 position with a highly AT-rich region centered around −17. The upstream duplex-form DNA from −17 to −5 binds to the NTD, and the DNA bases downstream are melted and a single-stranded template DNA is directed into the active site. T7 RNAP recognizes the promoter through three main interactions (Fig. 10.2a): (1) DNA bases are recognized by an antiparallel β-hairpin of the fingers, the specificity loop, from the major groove; (2) an AT-rich recognition motif in the NTD recognizes AT-rich (−17) region by inserting a flexible surface loop into the DNA minor groove; (3) the intercalating β-hairpin in the NTD melts the promoter DNA and marks the upstream edge of the transcription bubble. This precise location of the transcription bubble ensures correct positioning of the transcription start site DNA base at the active site. At this point, RNAP is ready to accept the two nucleotides that form base pairs with the +1 and +2 template DNA bases to initiate RNA synthesis.

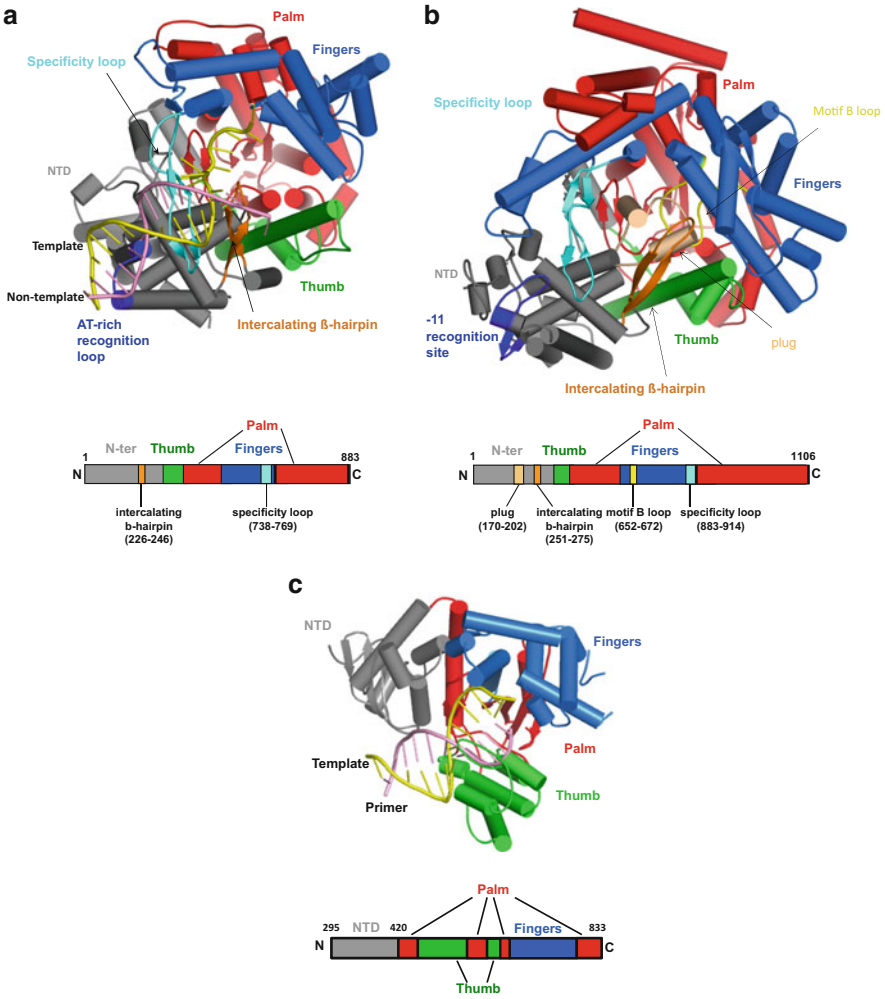

Fig. 10.1 Structures of single-unit DNA-dependent polymerases. Right hand-like organization domains in T7 RNAP complexed with promoter DNA (**a**), N4 mini-vRNAP (**b**), and KlenTaq DNAP I (**c**) are shown. Same orientation of the structures were obtained by superposing the palm domains. The palm (*red*), fingers (*blue*), thumb (*green*) domains, and NTD (*gray*) are shown as *cylinders* (α-helix) and *arrows* (β-strands). Double-stranded promoter DNA containing template (*yellow*) and non-template (*pink*) in T7 RNAP and the primer (*pink*) template (*yellow*) duplex in KlenTaq reach the active site cleft formed by three β-strands. Active site of N4 mini-vRNAP (**b**) is blocked by the plug (*wheat*) and the motif B loop (*yellow*). The primary structures of the polymerases are shown below each 3-D structure with the same color code

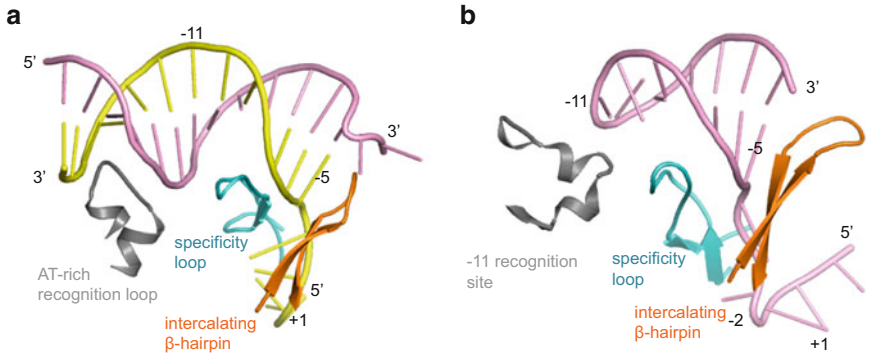

Fig. 10.2 Structural motifs for promoter recognition. (**a**) AT-rich recognition loop (*gray*), specificity loop (*cyan*), and intercalating hairpin (*orange*) in T7 RNAP binary complex. Template (*yellow*) and non-template (*pink*) are shown. (**b**) −11 recognition loop (*gray*), specificity loop (*cyan*), and intercalating hairpin (*orange*) in the N4 RNAP binary complex recognize the hairpin promoter (*pink*). The figure has been adapted from Gleghorn et al. (2008)

10.1.2 Transcript Initiation

The structure of T7 RNAP transcription initiation complex (Cheetham and Steitz 1999) showed how RNAP positions the template DNA bases at the active site at every subsequent step of NTP addition. In this structure, RNAP was bound to a 17-bp duplex promoter and a 3-mer RNA transcript base-paired with the single-stranded tailed template.

The presence of RNA in this polymerase structure offered an insight into the rNTP-specific RNA synthesis. In contrast to DNAP, a bulky glutamate "steric gate" near the active site is replaced to a glycine in RNAP that makes space for the 2′-OH of incoming NTP. Secondly, a carbonyl group of the active site amino acid residues hydrogen bonds with the 2′-OH of the 3′ primer end allowing only 3′-endo ribose conformation of the base. Further, the DNA-binding pocket is also complementary to the A-form DNA/RNA heteroduplex thus favoring the formation of DNA/RNA heteroduplex at the stage of RNA extension.

The promoter contacts are maintained, while the DNA/RNA heteroduplex accumulates in the active site, positioning the growing primer end at the active site. Accordingly, the −1 template base that stacked with the +1 template for proper positioning during *de novo* initiation assumes a flipped out conformation allowing transcript extension to RNA 3-mer stage. This observation served the first structural evidence for the "DNA scrunching" mechanism during initiation. The primer DNA scrunches into the active site through the initial synthesis phase until it chooses one of the two fates, abortive or productive transcription. During initiation, abortive transcripts are displaced from the template by collapsing the newly formed bubble due to small, weak DNA/RNA hybrids (Brieba and Sousa 2001). Alternatively, it could extend the DNA/RNA hybrid and scrunch it until it reaches the threshold

length of 8 nt, after which the entire complex undergoes a phenomenal conformational change into a processive elongation complex (Sect. 10.2).

10.2 Transcription Elongation

Two structures of T7 RNAP with 8-mer (Tahirov et al. 2002) and 11-mer (Yin and Steitz 2002) RNAs provide a holistic view of how the features of a transcription elongation complex emerge from the initiation complex after extensive reorganizations of the RNAP and DNA structures. A transcribing complex is committed to elongation when it has successfully accommodated numerous concurrent events including promoter release, partial collapse of the bubble, construction of an RNA exit channel, and peeling of the 5′ end of the nascent RNA from the template DNA. The threshold length of DNA/RNA hybrid determining this transition to elongation had been only predicted from biochemical observations, but in both the transcription elongation structures, the nascent RNA forms a heteroduplex with the template DNA of only 8 bp, upstream of which is peeled off the template and directed into a new formed exit channel.

10.2.1 Promoter Release and Processivity

The NTD being most closely involved in interaction with the promoter undergoes major rearrangement. On comparing the RNAP in the initiation and elongation stages, three subdomains in the NTD show independent rearrangement movements (Fig. 10.3). (1) A six-helix bundle called the promoter-binding domain (PBD) undergoes a massive rigid body rotation of 140° to a position previously occupied by the promoter, thereby destroying its promoter interaction sites. Along with PBD, the adjacent intercalating β-hairpin, an important motif for promoter melting in initiation, also becomes disordered in this promoter release event. (2) An α-helix (named C-helix) at the N-terminus of NTD nearly doubles in length by stacking of two smaller helices seen during initiation, forming part of the binding site for DNA/RNA hybrid. This helix protrudes into the region occupied by the PBD in initiation suggesting the concerted nature of the two motions. (3) Subdomain H undergoes extensive refolding into two antiparallel helices, paired with a large translation of 70 Å to the opposite side of the polymerase, forming the rim of the RNA exit channel on one side, and interacts with the non-template DNA from its opposite surface.

The formation of the RNA exit channel is the most important determinant of transcription processivity. Along with the subdomain H, two important motifs are involved in its formation including the thumb and the specificity loop. The interaction of the channel wall with RNA is only through salt bridges between the phosphate backbone and basic residues of specificity loop and subdomain H.

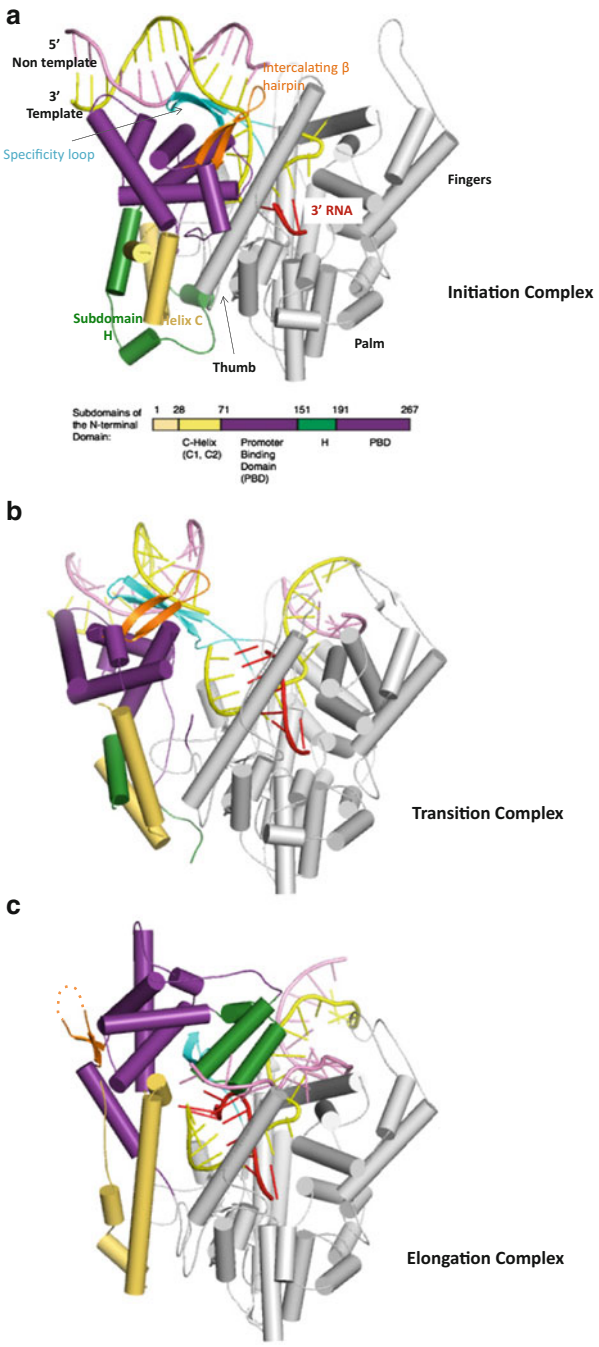

Fig. 10.3 Comparison of the structures of initiation complex (**a**), intermediate complex (**b**), and elongation complex (**c**). α-helices are represented by *cylinders* and β-sheets by *arrows*, while disordered regions are shown in *dotted lines*. The structures are similarly oriented by superposing the palm domains including the active site. Transition involves major conformational changes in the NTD colored as independently moving regions: promoter-binding domain (*purple*), intercalating hairpin (*orange*), helix C motif (*pale yellow*), and subdomain H (*green*). The CTD (*gray*) remains mostly unchanged except for movements in the long thumb helix and the specificity loop (*cyan*). The template strand is *yellow*, non-template is *pink*, and RNA is *red*. The downstream DNA is highly bent with respect to the upstream regions to help bubble formation (**c**). The primary structure of the NTD subdomains is also shown with the same color code (**a**). These figures have been adapted from Yin and Steitz (2002)

Processivity is also favored over abortive transcription due to the extensive interactions of the 7 bp DNA/RNA heteroduplex with its binding site.

Transcription processivity is coupled with the continuous downstream progress of the transcription bubble. At the onset of transcription, the bubble is generated from the unwinding of the downstream duplex DNA, approximately through 146° with respect to the upstream promoter. The template strand also plunges deep into the active site and comes out by bending about 80° at the upstream and downstream end of the bubble (Fig. 10.3c).

10.2.2 Transition to Elongation Complex

Transition from the initiation to elongation complexes should involve metastable complexes that not only form the basis of abortive cycling but also assume the conformation of an expanded active site to accumulate a growing DNA/RNA hybrid of about 8 nt length (Huang and Sousa 2000; Temiakov et al. 2000) while still bound to the promoter. Biochemical studies proposed that the transition was two steps (Bandwar et al. 2007; Guo et al. 2005; Ma et al. 2005), where RNA extended to about 8 nt followed by major refolding events accompanying synthesis of 9–14 nt (Tang et al. 2008). One of the intermediate structures with a 17-mer promoter DNA and 7-mer RNA transcript in a bubble showed the nature of transition between the vastly different initiation and elongation complexes (Durniak et al. 2008; Fig. 10.3b). This structure was captured using a mutant (P266L) in a loop connecting the polymerase NTD and C-terminal domains.

In the first stage of transition, NTD movements including PBD and helix C appear to have proceeded halfway, leading to the enlargement of the active site to accommodate the 7-bp DNA/RNA hybrid. Subdomain H of NTD, however, remains in its initiation orientation. The second stage of transition involves a final movement of the NTD, specificity loop, and subdomain H that loses promoter contact, completes the exit channel formation, and also forces the downstream duplex to its bent position.

10.2.3 Nucleotide Addition Cycle

In every nucleotide addition cycle, RNAP sieves through the pool of NTPs for the correct substrate through its intricate mechanism of nucleotide selection. The catalytic-competent nucleotide-binding N-site elicits the nucleotidyl transfer reaction between the RNA primer 3′-end base at P-site and the incoming NTP to extend the RNA through one base. The extended RNA then translocates upstream relative to the active site, opening the N-site for the next round of cycle. T7 RNAP conducts this harmonized process through fine regulation by elements mainly from the fingers and palm subdomain. Crystal structures of T7 RNAP complexes with the

DNA, RNA, and nucleotide provide great insights into its nucleotide addition cycle (Yin and Steitz 2004; Temiakov et al. 2004).

10.2.3.1 Substrate Selection in Pre-insertion Site

Structure of a ternary elongation complex with non-hydrolyzable ATP analog identified a "pre-insertion site" for a substrate binding prior to catalytically competent nucleotide binding at the "insertion" N-site (Temiakov et al. 2004; Fig. 10.4a). The pre-insertion site is linked to the "open" conformation of a conserved O-helix of the fingers. In this conformation, the templating base faces away from its accepting position, and the conserved tyrosine residue at the end of the O-helix meanwhile blocks the nucleotide insertion site. The tyrosine hydroxyl group interacts with the 2′-OH of the substrate, forming the primary discrimination of incoming rNTPs against dNTPs. The substrate is bound along the O-helix but is not Watson–Crick paired with the template DNA base, implying that the pre-insertion site is an early fidelity checkpoint.

10.2.3.2 Substrate Loading at the Catalytic Site

Substrate loading from the pre-insertion site to the catalytic insertion site is achieved by "closing of the fingers," wherein rotation of the O-helix is the most significant. Structure of a pre-catalytic substrate complex trapped with the use of a nonreactive nucleotide analog showed the nature of O-helix movement when substrate was loaded onto the catalytic insertion site (N-site) and thereby defined the closed conformation of the fingers (Yin and Steitz 2004). The O-helix rotates about a pivot point at its middle, causing the amino end of the helix to close onto the substrate triphosphate moiety, stabilized in this position through positive charge from lysine and arginine residues. Simultaneously, the opposite end of the O-helix, including the important tyrosine residue, moves away to make space for the base moiety of the incoming substrate. The base specific and ribose discriminating interactions of the pre-insertion site are maintained in the closed conformation to face a final round of fidelity check.

The incoming substrate alignment for catalysis, in the active site, is maintained not only by the O-helix residues but also by the accompanying nucleotide-binding metal Me_B, which is, in turn, positioned by chelating the conserved active site aspartates. Further, the octahedral coordination of catalytic metal ion Me_A maintains the critical alignment of the reactive groups, 3′ OH of primer terminus and 5′ αP of incoming substrate. The mechanism of nucleotidyl transfer reaction, which extends one RNA base and produces a pyrophosphate (PPi) by-product, will be described in Sect. 10.3.3.

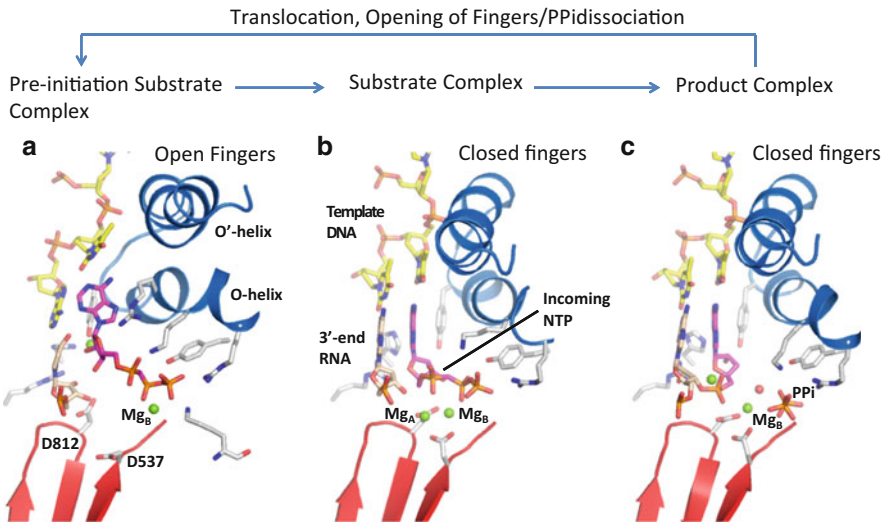

Fig. 10.4 Active site views during nucleotide addition cycle. Structures of the preinitiation complex (PDB: 1S07) (**a**), substrate complex (PDB: 1S76) (**b**), and product complex (PDB: 1S77) (**c**) are aligned similarly by superposing the palm. Important motifs (*ribbons*) and amino acid residues (*sticks*) are shown. Helix O and O′ from fingers (*blue*) and motifs A and C (*red*) from the palm provide amino acid side chains to bind the incoming nucleotide (*magenta* carbons), magnesium atoms (*green spheres*), or transcript primer end (*light pink* carbons). The template DNA is shown as *sticks* with *yellow* carbon atoms

10.2.3.3 Translocation

After the addition of a base to the transcript, the final step of the nucleotide addition cycle is the translocation of the DNA/RNA hybrid through one base distance such that the 3′-end RNA positions in the P-site. The structure of a post-catalytic, pre-translocated product complex, isolated right after phosphodiester bond formation but before dissociation of nascent PPi, showed that the phosphodiester bond does not cause any change in the RNAP or DNA/RNA hybrid (Fig. 10.4c). In the post-translocated state, the only difference lies in the dissociation of PPi along with the coordinated Me_B, which breaks the interactions with the O-helix residues and thus favors the open state. Moreover, the rNTP discriminator tyrosine at the O-helix moves towards the heteroduplex and stacks with the primer end base preventing backtracking of the hybrid, while opening the triphosphate site due to the pivoted helix motion. It is thus proposed that translocation of DNA/RNA hybrid in single-unit RNAP is coupled with PPi dissociation which leads to the opening of O-helix for the next round of nucleotide addition, often called the power-stroke mechanism of translocation (Jiang and Sheetz 1994).

10.3 N4 vRNAP: Factor-Dependent Single-Unit Polymerase

While the characterization of T7-related RNAP was progressing rapidly, the discovery of a unique phage-encoded, virion-encapsidated RNAP (vRNAP) isolated from a lytic coliphage N4 (Falco et al. 1977) added breadth to the studies of single-unit RNAPs. In contrast to other phage RNAPs, N4 vRNAP is encapsulated within the virion to being injected into the bacterial cell at the onset of infection. Upon injection of the N4 double-stranded DNA genome, host proteins DNA gyrase and single-stranded DNA-binding protein (EcoSSB) prepare the N4 vRNAP specific promoter comprising a DNA hairpin with a 5–7-base pair stem and 3-base loop (Glucksmann-Kuis et al. 1996; Haynes and Rothman-Denes 1985). This DNA hairpin promoter is a key to activate the co-injected vRNAP from transcription-inactive state. After transcription initiation, N4 vRNAP depends on EcoSSB for transcript elongation, which displaces nascent RNA transcripts from the template DNA for recycling (Davydova and Rothman-Denes 2003). Thus the part of NTD responsible for RNA separation in T7 transcription elongation (see Sect. 10.3.1) was absent in N4 vRNAP. EcoSSB could thus be termed as a transcription factor, and N4 vRNAP has been emerged as an important model for studying the structural basis of transcription activation as well as factor-dependent transcription of the single-unit RNAP.

Much larger than other phage RNAPs, the 320 kDa N4 vRNAP can be divided into three domains, and a central polymerase domain of 1100 amino acid (called mini-vRNAP) exhibits transcription initiation, elongation, and termination properties identical to full-length vRNAP (Kazmierczak et al. 2002). Sequence alignment classified the N4 mini-vRNAP as the most divergent member of the single-unit RNAP family. There is only small sequence similarity with other members except the catalytically important motifs A, B, C, and T/DxxGR.

10.3.1 Structure of N4 vRNAP

Studies of the structure and function of N4 mini-vRNAP advanced the scope of single-unit RNAP studies. Despite a lower sequence similarity, the N4 mini-vRNAP structure was highly similar to the T7 RNAP structure (Murakami et al. 2008) (Fig. 10.1b). It reiterated the modular organization of the right hand-like structure, with the same subdomains surrounding the DNA-binding cleft and the same structural motifs. However, the promoter recognition motifs in the fingers and NTD, although similar, had adapted interactions specific for the special hairpin N4 promoter (Sect. 10.3.2).

The two major differences between the N4 vRNAP and T7 RNAP structures are the presence of a plug module insertion in the NTD and a loop inserted in the middle of motif B (motif B loop) found in the N4 vRNAP. The plug and motif B

loop interact with the motifs A and C of palm that cover all catalytically essential amino acid residues of N4 vRNAP active site until the hairpin-form promoter DNA interacts and activates the polymerase.

10.3.2 Unique Hairpin Promoter DNA Recognition

The N4 vRNAP recognizes hairpin-form promoter DNA by using (1) −11 base recognition motif in the NTD, which recognizes the tip of hairpin loop, (2) specificity loop from the fingers that contact the hairpin from the major groove, and (3) intercalating β-hairpin to maintain the junction of the double- and single-stranded DNA (Fig. 10.2b). After binding the hairpin-form promoter DNA, the N4 vRNAP changes its conformation including a rigid body movement of the plug away from the active site and the motif B loop refolding outwards from the active site, which turns the residues on the O-helix for incoming NTP binding. The hairpin promoter-bound N4 vRNAP conformation is competent for transcription.

10.3.3 Nucleotidyl Transfer Reaction

The nucleotidyl transfer reaction by DNA and RNA polymerases follows a generalized mechanism of two-metal catalysis (Steitz et al. 1994), wherein the catalytic metal (Me_A) is known to work as a Lewis acid to enhance the nucleophilicity of the 3′ oxygen attacking group of the primer 3′ end, initiating the S_N2 reaction onto the 5′ αP of the incoming nucleotide. The nucleotide-binding metal (Me_B) is coordinated by the triphosphates of the incoming NTP, stabilizing the pentacovalent transition state.

In case of the transcription initiation, two NTP substrates bind to the empty P- and N-sites concurrently to form the first phosphodiester bond formation at the 5′-end RNA. The structural snapshots (Gleghorn et al. 2011), Raman crystallography (Chen et al. 2011), and time-resolved trigger-freeze crystallography (Basu and Murakami 2013) studies of ternary complexes of N4 mini-vRNAP during transcript initiation provide the most updated and complete knowledge of the reaction mechanism (Fig. 10.5). Two forms of pre-catalytic substrate complexes could be isolated; while both contained the two initiating nucleotides at the P- and N-sites, the catalytic metal (Me_A) was absent in one of them. This intermediate showed the important, final molecular rearrangements in the active site elicited by the critical Me_A binding to allow catalysis. In the absence of Me_A, the 3′ O from GTP (+1) and the 5′ αP of GTP (+2) were beyond reacting distance (4.1 Å) and were bought closer only with the binding of Me_A (Fig. 10.5b, c). This observation proposed that the catalytic metal binding, which is sensitive to its octahedral coordination requirements, serves as a final fidelity checkpoint where a fine misalignment of

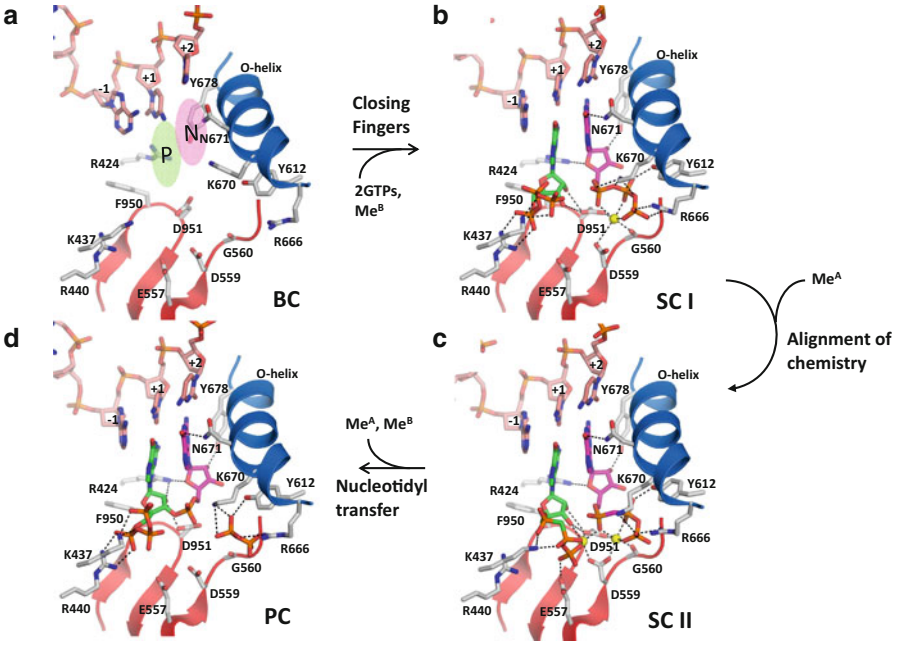

Fig. 10.5 Structures of active site, DNA and nucleotides during nucleotidyl transfer reaction. The main chains (*ribbon models*) of motifs A and C (*red*) and of the O-helix (*blue*) and the main and side chains (*stick models*) involved in nucleotide and metal binding in the promoter binary complex (**a**), substrate complex I (**b**), substrate complex II (**c**), and product complex (**d**). NTP binding P- and N-sites are indicated as *green* and *magenta circles* in **a**. DNA template (from −1 to +2, *pink*) and nucleotides at +1 (*green*) and +2 (*magenta*) positions are shown as *stick models*. Divalent metals are depicted by *yellow spheres*. Hydrogen bonds and salt bridges are depicted by *black dashed lines* (Gleghorn et al. 2011)

the 5′ αP of a mispaired incoming nucleotide disallows the Me_A binding and thereby prevents the catalysis.

Consistent with the steps in transcript elongation, the O-helix in the initiation complexes was in a closed state, providing its basic residues for stable interactions with the nucleotide at N-site. Additionally, the stability of the nucleotide binding at the P-site, an initiation-specific event, could be explained by electrostatic interactions of basic residues of the palm with the triphosphates and the partial base stacking with a purine at −1 template DNA position. Furthermore, the γP group of GTP (+1) participates in the Me_A octahedral coordination. Loss of affinity and catalytic activity with GDP (+1) compared with GTP (+1), led to the proposition of substrate-assisted catalysis for the first phosphodiester bond formation mediated by the γP group and Me_A binding.

A time course soak-trigger-freeze crystallographic study on this transcript initiation process provided the most direct real-time trace of the unperturbed events in the nucleotidyl transfer reaction (Basu and Murakami 2013). The observation of polymerase reaction in real time by high-resolution X-ray crystallography showed

that the nucleotide binding, the O-helix closure, and template DNA rearrangement were completed at early stages of reaction. Subsequently, the catalytic metal binding rearranges the reactive groups just prior to the phosphodiester bond formation. Owing to the sensitivity of Me_A for its octahedral coordination, its binding is subject to correct Watson–Crick pairing of the incoming nucleotide, and for the same reason it leaves the active site right after a phosphodiester bond formation, thereby also preventing the backward cleavage reaction.

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Chapter 11
Mitochondrial DNA and RNA Polymerases

Y. Whitney Yin

Abstract Mitochondria are vital to cellular activities, both cell growth and programmed death. The organelle contains its own DNA, which codes for a subset of genes oxidative phosphorylation electron transfer chain that couples oxidation–reduction reactions with ATP synthesis. Maintenance of mitochondrial DNA (mtDNA) requires a designated group of proteins that are nuclear encoded and transported to mitochondria. Both DNA and RNA polymerases have bacteriophage origins but have clearly deviated from their ancestors and have combined features of prokaryotic and eukaryotic enzymes. Mitochondrial DNA polymerase, Pol γ performs all DNA synthesis activities in replication and repair. Mammalian Pol γ holoenzyme consists of a catalytic subunit Pol γA and an accessory subunit Pol γB that regulates all activities of the catalytic subunit. Mitochondrial DNA replication and transcription are intimately related, as the mitochondrial transcription machinery provides transcripts for RNA primers for mtDNA replication as well as gene expression. Human mitochondrial DNA and RNA polymerases are adverse reaction targets for antiviral reagents against HIV and hepatitis viruses, and human Pol γ mutations have been implicated in multisystem clinical disorders. Studies of mitochondrial polymerases are therefore of immediate human health importance.

Keywords DNA polymerase gamma • mtDNA replication and repair • antiretroviral drug toxicity • Mitochondrial RNA transcription

Mitochondria are eukaryotic organelles enclosed by double membranes. Mitochondria are termed the "power plant" of cells, because they are the locations for metabolism of glucose, fatty acids, and some amino acids. The oxidation–reduction reactions generate protons that accumulate between the membranes; an electron transfer chain couples dissipation of this proton potential with synthesis

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of ATP. This important reaction, named oxidative phosphorylation (OX-PHOS), is critical in sustaining energy for many cellular activities. In addition, mitochondria are involved in signaling, cellular differentiation, cell death, as well as control of the cell cycle and cell growth (McBride et al. 2006).

Mitochondria share many features common with bacteria. They are elongated in shape, with dimension of 500–1,000 nm. The shape and number of mitochondria per cell vary widely by organism and cell type. Mitochondria are thought to have derived from endosymbiotic proteobacteria. Symbiosis resulted in the majority of mitochondrial genes migrating to the nucleus, so the size of mtDNA is only 1–5 % of free-living bacteria. For example, human mitochondrial DNA (mtDNA) is 16.9 kb, and yeast mtDNA is 80 kb. Regardless, all mtDNA code for a subset of OX-PHOS components, as well as tRNAs and rRNAs needed for mitochondrial protein synthesis. To assemble functional OX-PHOS electron transfer chain, gene expression in the nucleus and mitochondria has to be well coordinated. Proteins involved in mtDNA maintenance are exclusively nuclear coded. These proteins have a unique mitochondrial localization sequence (MLS) that is recognized by a specific translocation apparatus for their internalization into mitochondria.

11.1 Mitochondrial DNA Replication

Mitochondrial function is directly correlated with the integrity of its DNA. Unlike nuclear DNA (nDNA), human mitochondrial DNA (mtDNA) is in a relaxed circular form, free of histone proteins, and lacks introns. The DNA contains a 1 kb noncoding region, called the D-loop, containing a replication origin and two transcription promoters (Fig. 11.1). There are 10–1,000 copies of mtDNA per mitochondrion; their replication is not coordinated to the cell cycle. The precise control of mtDNA replication is not completely understood. The two strands of mtDNA are named H (heavy)-strand and L(light)-strand based on their density in poly(UG) CsCl gradients (Attardi and Attardi 1969). Replication on each strand initiates at a unique origin, O_H and O_L, and proceeds unidirectionally. O_H is located in the D-loop, whereas O_L is two-thirds of the way around the genome. The origin recognition proteins are unknown.

There are two competing models for mtDNA leading and lagging strand replication: a conventional mode where leading and lagging strand synthesis occur simultaneously (Aloni and Attardi 1971b; Yang et al. 2002; Yasukawa et al. 2005) and a displacement mode where synthesis initiating from the O_H origin displaces the parental H-strand to form a D-loop. Only when the newly synthesized H-strand DNA crosses a second origin (O_L) does initiation of L-strand synthesis occur. The nascent H and L strands are therefore extended asymmetrically (Clayton 1982; Xu and Clayton 1996). This model was recently modified to allow initiation of L-strand synthesis from a number of origins in addition to O_L (Brown et al. 2005). Supporting evidence for an asymmetrical synthesis mechanism includes that mtSSB can be found coating extensive region the human

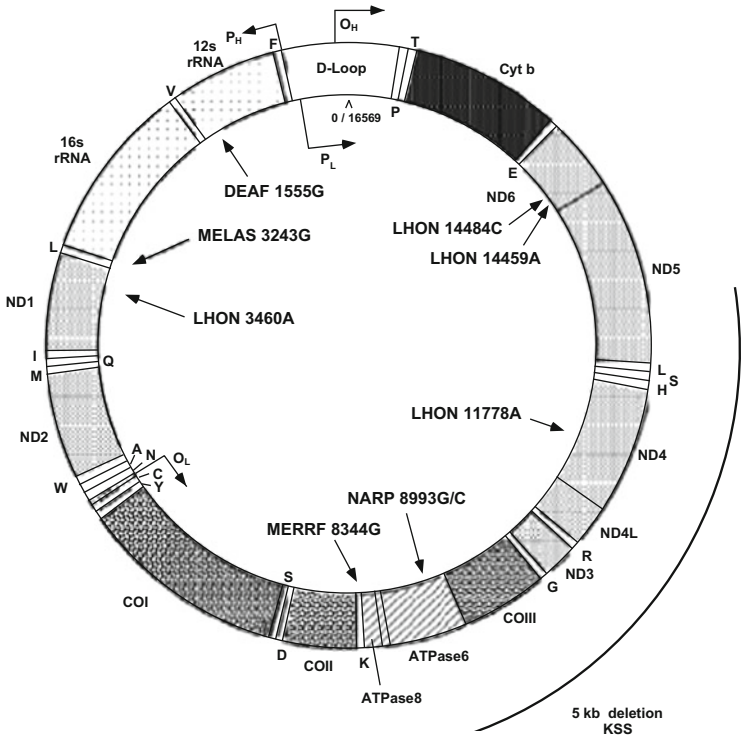

Fig. 11.1 Human mitochondrial DNA. It codes for 13 protein genes, 22 tRNAs, and 2 rRNA

mitochondrial genome and that O_L is important for DNA replication in vivo (Brown et al. 2005; Wanrooij et al. 2012) (Fig. 11.2).

No primase has been identified in mitochondria; maintenance of mtDNA replication therefore requires not only replisomal proteins but also topoisomerase and RNA polymerase with its transcription factors MTFA and MTFB in order to provide primers. The human mtDNA replisome consists of the helicase TWINKLE, DNA polymerase gamma, Pol γ, and a single-stranded DNA-binding protein (mtSSB). TWINKLE is hexameric and unwinds DNA in the 5′–3′ direction.

Human Pol γ is a two-subunit holoenzyme: a catalytic subunit Pol γA and a dimeric accessory subunit Pol γB. Pol γA possesses all enzymatic activities of the holoenzyme, including polymerase, 3′–5′ exonuclease for proofreading, and 5′ deoxyribose phosphate (5′-dRP) lyase activities. Pol γB has no enzymatic activity by itself, but upon association to form holoenzyme, it regulates all Pol γA activities. Holoenzyme exhibits markedly increased processivity of synthesis, reduced exonuclease activity, and elevated 5′-dRP lyase activity (Bogenhagen et al. 2001; Carrodeguas et al. 1999; Lim et al. 1999; Longley et al. 1998). From a crystallographic study, Pol γB can perform all accessory functions on a singly primed

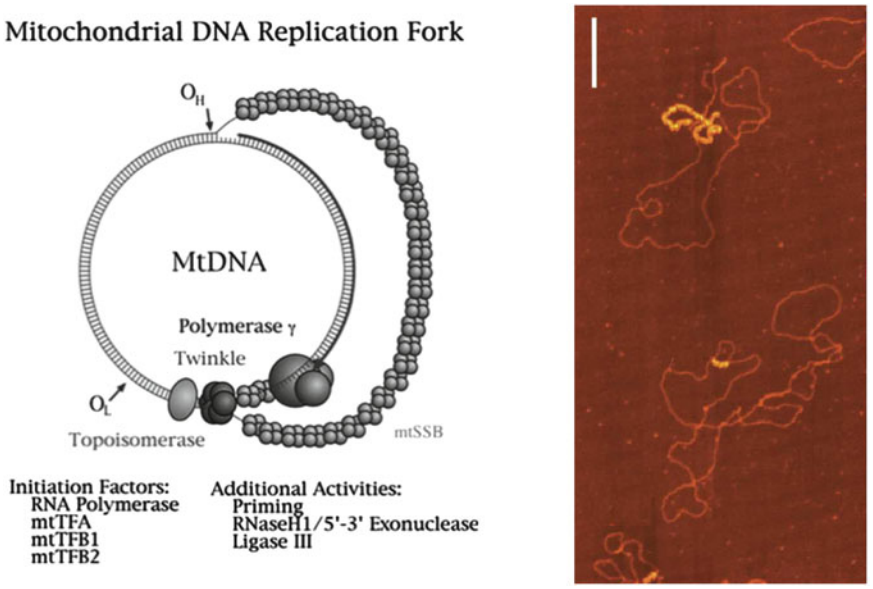

Fig. 11.2 (*Right*) Mitochondrial replisome components. The displaced strand is coated by mitochondrial single-stranded DNA-binding proteins (mtSSB). (*Left*) AFM images of two mtDNA molecules with single-stranded regions coated with SSB (Brown et al. 2005)

template without direct physical contact with DNA, acting entirely through interactions with Pol γA.

Mitochondrial DNA replication in lower eukaryotes is simpler. In fungi, there is no known DNA helicase. To compensate for the lack of helicase, mitochondrial DNAP must perform strand displacement DNA synthesis, i.e., unwind the downstream duplex concurrently with DNA synthesis. It has been shown that yeast (*Saccharomyces cerevisiae*) mtDNAP, Mip1, is indeed able to conduct strand displacement synthesis—an activity that is usually missing in purified replicases, which are normally associated with a helicase (Viikov et al. 2010).

Pol γA belongs to the polymerase A-family. Sequence comparisons with other family members aid identification of the exo and pol active sites and reveal ~400 aa spacer between the exo and pol domains. Although it has no counterpart in other family members, the importance of the spacer is manifested clinically: Pol γ containing mutations in this domain have been found in patients with several distinct mitochondrial diseases. The spacer domain presents a unique fold that can be further divided into two subdomains. The globular IP subdomain is located opposite the palm subdomain, and together they almost completely encircle the template. The IP subdomain thus potentially both enhances affinity for DNA and the intrinsic processivity of Pol γA. The second subdomain is AID, an elongated, flexible segment that extends away from the main body of the enzyme in order to interact with Pol γB (Fig. 11.3). The AID subdomain is likely to be disordered in the absence of Pol γB. The AID subdomain is amphipathic, with one surface being

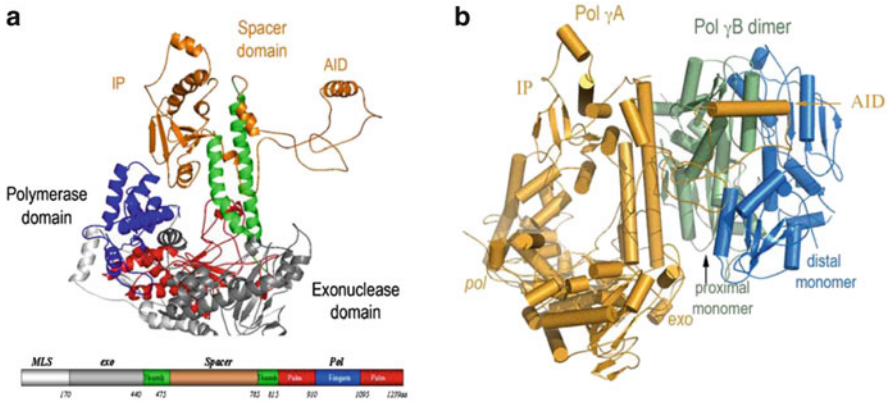

Fig. 11.3 Structure of human Pol γ. (**a**) Structure of Pol γA. The pol domain shows a canonical "right-hand" configuration with thumb (*green*), palm (*red*), and fingers (*blue*) subdomains and the exo domain (*gray*). The spacer domain (*orange*) presents a unique structure and is divided into two subdomains. Domains are shown in a linear form where the N-terminal domain contains residues 1–170; exo, 171–440; spacer, 476–785; and pol, 441–475 and 786–1239. (**b**) Structure of the heterotrimeric Pol γ holoenzyme containing one catalytic subunit Pol γA (*orange*) and the proximal (*green*) and distal (*blue*) monomers of Pol γB. Pol γA primarily interacts with the proximal monomer of the dimeric Pol γB (Lee et al. 2009)

hydrophobic and the opposite face highly positively charged (termed the K-tract, ^{496}KQKKAKKVKK^{505}).

In the trimeric holoenzyme, Pol γA asymmetrically interacts with the homodimeric Pol γB (Lee et al. 2009). This manner of subunit interaction raised the possibility that Pol γ could be a tetrameric enzyme with two Pol γA and a dimer Pol γB (A_2B_2). However, model building, using the twofold axis of Pol γB, indicates that the AID subdomains of two Pol γA monomers in a holoenzyme would result in steric clashes. Human Pol γ holoenzyme is therefore a trimer.

The structural arrangement of Pol γ suggests a mechanism explaining how Pol γB simultaneously affects exo and pol activities. When Pol γB binds to the Pol γA AID subdomain, it positions the positively charged K-tract to interact with upstream DNA, thereby increasing the affinity of holoenzyme for DNA by doubling the area of interaction. Secondly, the location of the primer 3′-OH determines the relative activities of pol and exo. Because of the mode of Pol γA binding to Pol γB, holoenzyme preferentially positions the 3′-OH in pol and away from exo, resulting in increased polymerase but reduced exonuclease activities. It should be noted that even though exonuclease activity is suppressed, after an incorrect nucleotide is incorporated, the synthesis reaction slows, providing the time necessary for the primer terminus to transfer to exo. The fidelity of holoenzyme is not therefore compromised by the interactions of Pol γB with Pol γA in forming holoenzyme.

Despite the strong circumstantial evidence of a bacterial origin of mitochondria, Pol γA shows homology to bacteriophage T7 DNA polymerase, especially in the palm subdomains that contains the catalytic active site; the rmsd of the subdomain

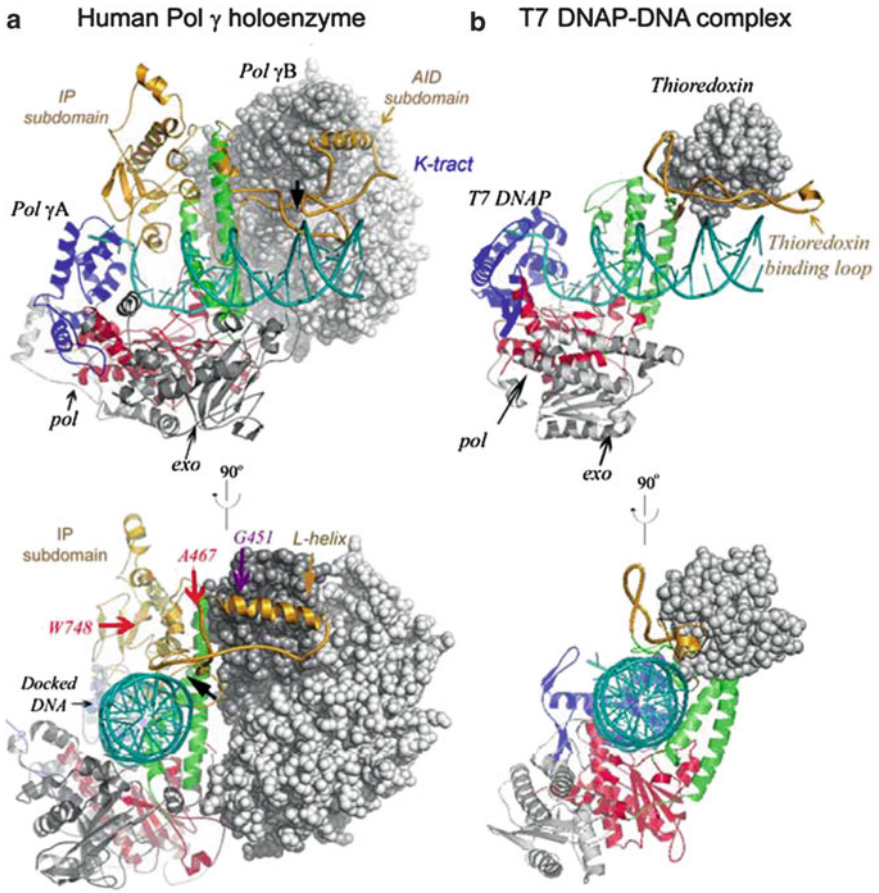

Fig. 11.4 Comparison of a Modeled Pol γ–DNA. (**a**) Modeled Pol γ–DNA complex containing Pol γA (shown in *ribbons*), Pol γB (*gray* CPK), and a docked DNA (*blue ribbons*) shows that IP and AID subdomains enhance DNA binding. Mutations and the region protected by DNA from proteolytic digestion (*black arrow*) are indicated. (**b**) Crystal structure of T7 DNAP–DNA complex containing gp5 (*ribbons*), thioredoxin (*gray* CPK), and a primer-template DNA (*blue ribbons*) (Lee et al. 2009)

between the two polymerases is merely 2.3 Å. A comparable structure to the AID subdomain is also seen, where the flexible thioredoxin-binding domain of T7 gp5 makes the only contact with the processivity factor and the upstream primer-template (Fig. 11.4).

Like the catalytic subunit of many replicases, human Pol γA exhibits limited processivity in synthesis, incorporating ~100 nt per binding event. In the presence of Pol γB, processivity of the holoenzyme increases to ~1,500 nt. Pol γB has a different mode of increasing processivity than other accessory proteins for replicases: it simultaneously enhances DNA binding and the reaction rate. In contrast, thioredoxin only increases the binding affinity of T7 gp5 for DNA.

Nonetheless, because thioredoxin and Pol γB are tightly associated with their catalytic subunit in forming a holoenzyme, they both function differently than loosely bound processivity factors, such as sliding clamps. The ring-shaped sliding clamp, after being loaded on the template, binds to polymerase and reduces its dissociation rate during synthesis. There are mutually beneficial interactions in forming the trimeric Pol γ holoenzyme: Pol γA enhances dimerization of Pol γB, and the interaction between Pol γA and Pol γB is enhanced by the presence of primer-template DNA (Lee et al. 2010b).

Despite an overall similarity, both Pol γA and Pol γB exhibit species-dependent structural variations: for the catalytic subunit, these are focused in the subdomain that interacts with Pol γB and in the C-terminus. In contrast to mammals, Drosophila and mosquitoes have a simpler, monomeric Pol γB, and fungal mtDNAPs are single polypeptides, with no known accessory subunit (Kaguni 2004). They also lack an AID subdomain, yet biochemical characterization of yeast Mip1 indicates the polymerase is still highly processive (Viikov et al. 2010). Relative to the human enzyme, fungal polymerases have C-terminal extensions. *S. cerevisiae* polymerase, Mip1, has the longest known extension: 279 aa, which has been hypothesized to function as an intrinsic processivity factor that provides the same function as Pol γB. Interestingly, deleting 175 aa of the 279 aa extension yields an enzyme with higher processivity than wild type; further deletion (216 aa) results in reduced processivity but increased 3′–5′ exonuclease activity. Deletion of the entire extension abrogates all polymerase activity, but the enzyme retains higher 3′–5′ exonuclease activity than wild type (Viikov et al. 2012).

Pol γB also displays species-dependent variation: mammalian Pol γB is a homodimer of two 55 kDa subunits. It structurally resembles class II aminoacyl-tRNA synthetases, however, the residues for catalytic activity are not conserved, so that Pol γB lacks synthetase activity (Carrodeguas et al. 2001). Nevertheless, the overall tRNA synthetase fold led to examination of Pol γB binding to tRNA-like or stem-loop DNA. This is significant because the single-stranded O_L may also adopt a stem-loop structure. Indeed, Pol γB has high affinity for double-stranded DNAs longer than 45 bp (Carrodeguas et al. 2002). DNA binding by Pol γB is associated with two positively charged regions, termed RK and RKK. Alanine substitutions abolished DNA-binding activity. The substitutions have no effect on holoenzyme Pol γ DNA synthesis on single-stranded templates. This result agrees well the structural studies, where Pol γB appears to enhance DNA binding indirectly. However, when tested on duplex templates using the entire replisome, i.e., in the presence of TWINKLE and SSB, both the RK and RKK mutants result in defective DNA synthesis (Farge et al. 2007). This suggests that the RK and RKK regions only function in synthesis at a replication fork, either by directly contacting the branched DNA or perhaps other components of the replisome. Pol γB therefore appears to perform an additional, as yet unknown, function during replication on duplex templates.

In the trimeric human Pol γ holoenzyme, there are extensive hydrophobic interactions between the AID and thumb subdomains of Pol γA and one Pol γB monomer. In contrast, very limited interactions with the distal Pol γB are observed,

with only one salt bridge formed between Pol γA and the distal Pol γB monomer. This asymmetrical provides a structural explanation for the insect monomeric Pol γB can be functional. The limited interaction with the distal Pol γB is of importance in humans, as a mutation that disrupts the only Pol γA and Pol γB distal monomer interaction has severe consequences (Ferrari et al. 2005). Biochemical studies indicate that the mutants abolished the function of Pol γB without physical dissociation of the holoenzyme. The Pol γA–distal Pol γB monomer contact appears to regulate both pol and exo activity; disruption of this interaction elevates exonuclease activity while decreases DNA polymerization (Lee et al. 2010a).

11.2 Pol γ in Mitochondrial DNA Repair

The unique mitochondria environments make mtDNA extremely vulnerable to oxidative damage. Radicals escaping from the oxidative phosphorylation electron transfer chain and reactive oxygen species generated from redox reactions are particularly damaging to DNA. Exogenous radiation sources and the lack of histones in mitochondria further exacerbate mtDNA damage. Consequently, mtDNA suffers much higher levels of oxidative DNA damage than genomic DNA (Bohr et al. 2002; Richter et al. 1988; Wiseman and Halliwell 1996). Oxidative stress plays a large role not only in tumor pathologies but in other diseases of aging, such as Alzheimer's, Parkinson's, and Huntington's diseases (Shoffner et al. 1993; Wang et al. 2005; Yang et al. 2008).

Base excision repair (BER) is the main mechanism for repairing damage to mtDNA. Single-nucleotide BER (SN-BER) begins with damaged base excision by a glycosylase, AP-endonuclease cleavage to generate a 3′-OH, removal of the 5′-deoxyribose by the lyase activity of Pol γ, followed by gap-filling synthesis, and then nick sealing by DNA ligase (Bohr et al. 2002; Hegde et al. 2010; Liu and Demple 2010). However, the 5′-dRP lyase activity of Pol γ is weaker than that of the nuclear repair DNAP Pol β (Longley et al. 1998; Pinz and Bogenhagen 2006), and Pol γ cannot efficiently recognize single-nucleotide gaps (He and Yin, unpublished data). In principle, the latter problem could be overcome by another BER protein actively recruiting Pol γ to the gap, but it is possible that in mitochondria most repair is biased towards the long-patch BER (LP-BER) pathway. Indeed, if the 5′-ribose is oxidatively damaged, often resulting in a deoxyribonolactone, the 5′-dRP lyase activity of Pol γ cannot function to provide a downstream 5′-phosphate for ligation. In this situation, LP-BER must be activated to remove the damaged sugar together with some downstream DNA. The resulting gap is then efficiently filled-in by Pol γ.

The key difference between SN-BER and LP-BER is that the repair extends beyond the lesion site. After excision of the damaged nucleotide, it is replaced along with the strand downstream from the lesion with a nascent DNA. This fashion of DNA synthesis, called strand displacement, requires coordination of Pol γ and a nuclease to synthesize and cleave the displaced strand. The mechanism of removal

of downstream DNA has not been resolved, in particular, which nucleases participate in mitochondrial LP-BER. Nucleases known to be active in mitochondria include DNA2, a 5′–3′ helicase and exonuclease; EXOG, a 5′ exonuclease; and FEN1, a flap DNA nuclease (Liu et al. 2008; Tann et al. 2011; Zheng et al. 2008). Both DNA2 and EXOG can be co-immunoprecipitated with Pol γ, suggesting they either directly or indirectly interact. However, both DNA2 and EXOG removed exactly 2-nt from a 5′-flap even though the flap was much longer. FEN1 can remove the entire flap, but as yet there is no evidence of an interaction with Pol γ.

Additionally, Pol γ is able to perform strand displacement synthesis when its 3′–5′ exonuclease activity is abolished, even when the downstream DNA contains a 5′ oxidized ribose (He and Yin, unpublished data). This observation suggests yet another possibility for LP-BER that is suppression of Pol γ exo activity. Whether a major pathway exists or these enzymes can all be involved in redundant pathways requires future studies.

11.3 Pol γ as an Adverse Reaction Target for Antiviral Drugs

The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) ushered in a new era of antiviral treatment for human immunodeficiency virus (HIV) and hepatitis virus infections. NRTIs are substrate mimics for DNAPs, but they lack a 3′-OH and therefore function as chain terminators that stop viral replication. The success of highly active antiretroviral therapy (HAARP), which includes inhibitors for HIV reverse transcriptase and protease, also brought attention to the problem of drug toxicity. This is of special importance with long-term usage as the life span of patients extends. Management of toxicity has been ameliorated with varying therapeutic regimes but remains a major challenge. Clinical manifestations of NRTI toxicity are often multisystem, including muscular, cardiovascular, and neurological system dysfunctions, i.e., the systems relying on high-energy output from mitochondria. Histological examinations coupled to electron microscopy show swollen, misshapen mitochondria that have decreased numbers of cristae and non-membrane-bound fat droplets of various sizes, indicative of decreased mitochondrial function for fatty acid β-oxidation (Lewis et al. 2003). The causes of mitochondrial toxicity include the active transport of NRTIs into the organelle and inhibition of major adverse target, human Pol γ.

Studies of isolated human DNA polymerases, Pol α, Pol β, Pol γ and Pol ε showed that Pol γ is most susceptible to inhibition by NRTIs (Hart et al. 1992). This may be attributed to biochemical and structural similarities of Pol γ with the viral enzyme. The structural basis for Pol γ susceptibility is thought to reside in the pol active site and the mechanism for discriminating (or lack thereof) against nucleotide analogs.

The reason for inhibition of Pol γ by drugs designed against both HIV and HBV polymerases may be related to the fact that both viral proteins are reverse transcriptases (RT) that use DNA or RNA templates. Pol γ also possesses RT activity, although synthesis is less processive on RNA templates. This activity is relevant to the presence of RNA in human mtDNA, which is estimated to contain 1–3 % ribonucleotides. The RNA has been hypothesized to be due to incompletely removed primers. Persistence of RNA requires an RT activity of Pol γ to replicate over the RNA segments. The inhibition of Pol γ by NRTIs suggests structural/functional similarities between the human and viral proteins.

Toxicities of NRTIs can be correlated with the kinetics of incorporation and exonucleolytic removal by the mitochondrial DNA polymerase (Fig. 11.5) (Lee and Johnson 2006), supporting the idea that Pol γ is the adverse reaction target. Nonetheless, some NRTIs have high efficacy and low toxicity, suggesting differences exist between the viral and human enzymes (Table 11.1). Kinetic analyses show that Pol γ could not distinguish substrate dNTP from NRTIs, thereby causing cytotoxicity. These NRTIs either have high affinity to Pol γ (Low K_d) that is either comparable to higher substrates, or efficiently incorporated (high k_{pol}). For example, HIV RT inhibitors dideoxy cytosine (ddC or zalcitabine) and 3′-fluoro-3′-deoxythymidine (FLP) (Fig. 11.5) bind to Pol γ with affinity equal or higher than native nucleotides, which could explain their high toxicities. However, the drug toxicity is complicated, because subtle change in NRTI structures can alter their effect on Pol γ drastically. For example, Pol γ discriminates FLP only 8.3-fold from the native nucleotide dTTP; however, it can discriminate a structurally related NRTI, 2′3-didehydro-2′3′-dideoxythymidine (Ed4T), from dTTP greater than 12,000-fold (Sohl et al. 2012). Another NRTI, (−)-2′,3′-dideoxy-3′-thiacytidine [(−)SddC or 3TC] is a potent low-toxic HIV RT inhibitor, but its D(+) isomer, (+) SddC that has the natural nucleoside configuration, is less potent but more toxic (Feng and Anderson 1999a; Johnson et al. 2001). To completely understand drug toxicity will require combinatory studies of enzyme kinetics and computational and structural biology.

Structural comparison of human Pol γ and HIV RT structures reveal differences between the two enzymes that may be exploited in future drug design. For example, the distinct subunit interactions result in substrate DNA being bound in the active site of Pol γ at an angle of 45° to that in HIV RT (Fig. 11.6). More importantly, while the catalytic aspartates of the HIV RT p66 subunit and Pol γA have a similar spatial arrangement, the incoming nucleotide-binding sites formed between the palm and fingers subdomains are structurally distinct, being α-helical in Pol γ but β-sheet in HIV RT.

NRTIs have also been most widely used therapy for HBV. About 350 million people worldwide are living with hepatitis infection (WHO 2003). Hepatitis can be caused by hepatitis virus types A, B, C, D, or E. Of all viral causes of human hepatitis, hepatitis B virus (HBV) is of global importance, because of the sheer number of viral carriers; persistent HBV infection can lead to chronic hepatic insufficiency, cirrhosis, and liver cancer. About one million people die each year

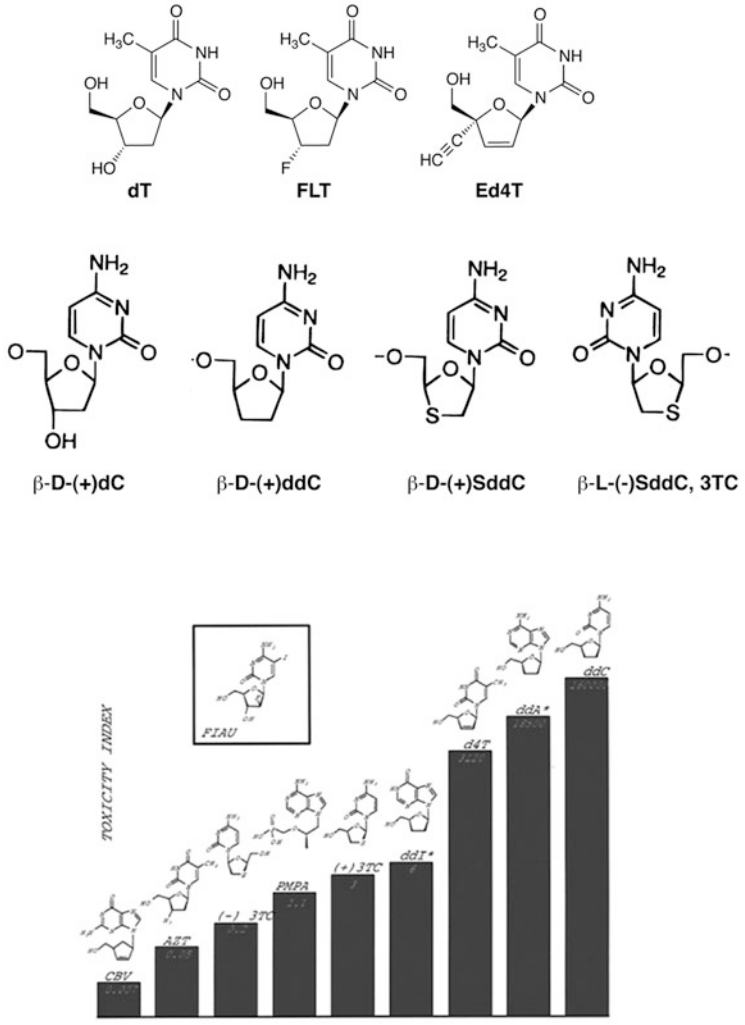

Fig. 11.5 NRTIs drug toxicity correlates with human Pol γ inhibition and structures of selective NRTIs (Lee and Johnson 2007)

of these complications. In the United States, complications from chronic HBV infection count for 5–10 % of cirrhosis and liver transplant (McKenzie et al. 1995).

Clinical treatment of HBV includes administration of Adenine arabinoside (vidarabine), acyclovir, didanosine, zidovudine, and ribavirin (Fried et al. 1992; Garcia et al. 1987; Katsuragi et al. 1997). Unfortunately these drugs are ineffective or too toxic for prolonged use. A clinical trial used the second-generation nucleoside analog fialuridine (1-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)-5-iodouracil, or FIAU) as a treatment for chronic hepatitis B virus infection (McKenzie et al. 1995).

Table 11.1 Comparison of efficiency of human Pol γ and HIV RT incorporation of nucleoside analogs NRTIs that are FDA approved and under development

	Discrimination		
dNTP analog	Human Pol γ	HIV RT	Reference
ddC-TP	2.9	10	Feng and Anderson (1999b), Feng et al. (2001), Ray et al. (2003), Ray and Anderson (2001)
ddA-TP	4.0	5	Johnson et al. (2001)
d4T-TP	7.4	0.56	Vaccaro et al. (2000), Johnson et al. (2001)
KP1212-TP	26	14	Murakami et al. (2005)
FLT-TP	35	4.2	Sohl et al. (2012)[a]
(−)-3TC-TP	2,900	40	Feng and Anderson (1999a), Feng et al. (2001), Ray et al. (2003)
EFdA-TP	4,300	N.D.	Sohl et al. (2011)
Ed4T-TP	6,200	0.51	Sohl et al. (2012)
PMPApp	11,400	6.1	Johnson et al. (2001)
AZT-TP	37,000	2.7	Vaccaro and Anderson (1998), Johnson et al. (2001)
(−)-FTC-TP	290,000	16	Feng et al. (2004)
CBV-TP	900,000	34	Johnson et al. (2001)

N.D. no data, *KP1212* 5-aza-5,6-dihydro-2′-deoxycytidine, *3TC* lamivudine, *PMPApp* tenofovir diphosphate, *FTC* emtricitabine, *CBV* carbovir

[a]Steady-state studies indicated an efficiency of 0.5 (Michailidis et al. 2009)

While nontoxic in animal studies, humans treated with FIAU developed severe hepatotoxicity, with progressive lactic acidosis, and liver failure, even after discontinuation of the drug. Several patients also had pancreatitis, neuropathy, or myopathy. Of the seven patients with severe hepatotoxicity, five died and two survived after a liver transplant.

Extensive investigations revealed that toxicity was caused by FIAU inhibition of human Pol γ. Toxicity predominantly affected organs and tissues that have a slow turnover of cells and a major dependence on mitochondrial function. Triphosphate forms of FIAU and its metabolites, FMAU (1-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)-5-methyluracil) and FAU (1-(2-deoxy-2-fluoro-b-D-arabinofuranosyl)-5-uracil) compete with dTTP for incorporation by human Pol γ. FIAUTP, FMAUTP, and FAUTP can be incorporated into singly primed DNA opposite to adenosine effectively without affecting chain elongation. However, multiple FIAUTP incorporation in adjacent positions dramatically impaired chain elongation by Pol γ (Colacino 1996; Lewis et al. 1996). Consequently, mtDNA content became severely decreased in patients.

11.4 Pol γ Mutations Are Implicated in Clinical Disorders

Mitochondrial diseases are devastating disorders for which there is no cure or proven treatment. About 1 in 4,000 individuals is at risk of developing a mitochondrial disease sometime in their lifetime. Half of those affected are children who

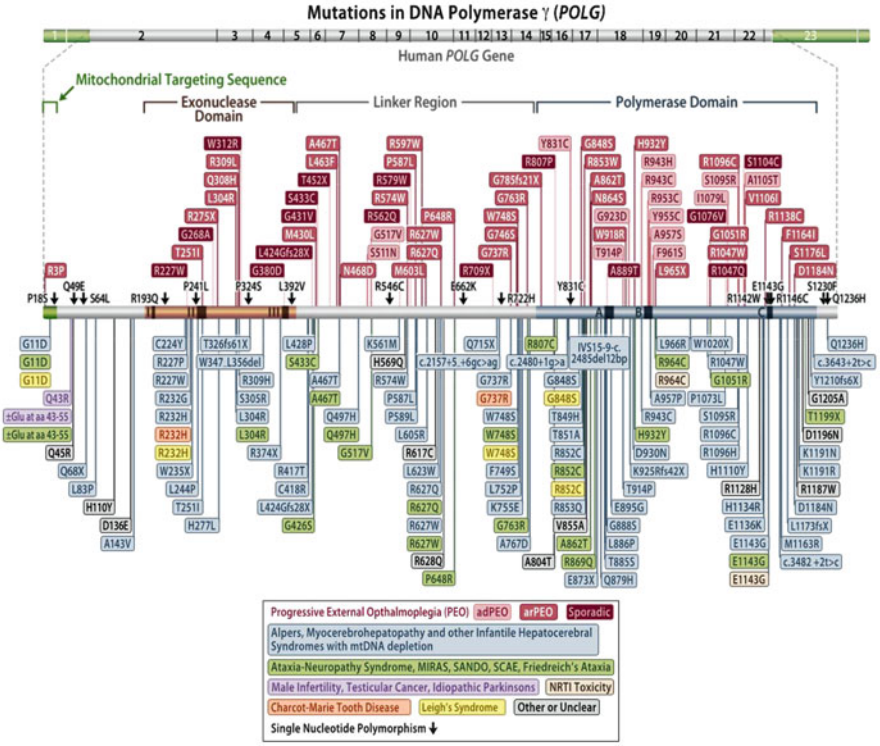

Fig. 11.6 Mutations on human Pol γA associated with diseases (Chan and Copeland 2009)

show symptoms before age 5, and approximately 80 % of them will die before age 20. The mortality rate is roughly that of cancer (Longley et al. 2005).

More than 90 Pol γ mutations have been reported associated with multisystem diseases, spanning cardiovascular, neurological, and muscular systems (Chan and Copeland 2009). Progressive external ophthalmoplegia (PEO) is a late onset mitochondrial disease, with bilateral ptosis and progressive weakening of the external eye muscle, proximal muscle weakness, and wasting as well as exercise intolerance. Skeletal muscles of PEO patients present red ragged fibers and lowered activity of respiratory chain enzymes. Muscle biopsies show that mtDNA contains multiple large-scale deletions (Zeviani et al. 1989). To date, with the exception of one mutation, the autosomal dominant *POLG* mutations responsible for PEO development have been mapped to the pol domain of Pol γ (Fig. 11.6).

Alpers syndrome is a rare but severe, heritable, autosomal recessive disease that afflicts young children. Within the first few years of life, patients develop seizures, cortical blindness, deafness, liver failure, and eventual death. Many Pol γ mutations have been found in Alpers patients. Among them, the Pol γA A467T substitution is most common, accounting for 36 % of all mutations. The mutation is located in the Pol γ thumb subdomain, which interacts directly with the template DNA and guides

the primer terminus between exo and pol active sites. The substitution therefore influences the relative activities of both activities; holoenzyme containing Pol γA A467T has only 4 % polymerase activity and a reduced k_{cat} and $K_{m(dNTP)}$ (Chan et al. 2005).

Two other Alpers substitutions, R943H and Y955C, retain less than 1 % of wild-type polymerase activity and display a severe decreased processivity (Graziewicz et al. 2004). The mutations are located on a positively charge helix—O helix—that, based on structural homology modeling with T7DNAP, is critical for dNTP binding. R943 is predicted to interact with the triphosphate moiety of the dNTP, where Y955 facilitates translocation by serving as a pawl to prevent DNA duplex backtracking. The R943H substitution reduces the enzyme's affinity for dNTP, whereas Y955C allows excessive backtracking, which could therefore both reduce the DNA synthesis rate and increase the frequency of insertions or deletions in mtDNA. Using a mouse transgenic model in which Y955C *POLG* was targeted to the heart, the mouse developed cardiomyopathy, loss of mtDNA, an enlarged heart, and increased levels of 8-oxo-deoxyguanine (8oxoG) in its mtDNA (Lewis et al. 2007). Collectively, these phenotypes suggest that patients harboring the Y955C mutation may suffer elevated oxidative damage and should benefit from antioxidant therapy.

Substitutions A957P, A957S, R1096C, and R1096H are also found in patients suffering from aggressive Alpers syndrome to mild PEO. Kinetics analyses of these mutants indicate that A957P is most defective, the deficiency of other point mutants following the order of R1096H > R1096C > A957S, an order that matches well with the severity of clinical symptoms (Sohl et al. 2013).

Substitution reducing Pol γ fidelity will increase mtDNA mutation, which have been associated with numerous other clinical multisystem human disorders, including mitochondrial encephalopathy, lactate acidosis, and stroke-like syndrome (MELAS); cardiovascular, skeletal muscular, endocrinology (Type II diabetes); and neurodegenerative disorders (Alpers syndrome, Parkinson-like, and Alzheimer). Accumulated mutation on mtDNA is correlated with natural process of aging. Because reactive oxygen species (ROS) generated inside the cell will lead, with time, to increasing amounts of oxidative damage to various cell components. Impaired respiratory chain function causes degenerative diseases and accelerates aging. Transgenetic mice harboring exonuclease-deficient Pol γA shows elevated mutation rate; the animals show premature aging phenotype and have significantly reduced life span (Trifunovic et al. 2005).

11.5 Mitochondrial RNA Transcription

11.5.1 Mitochondrial Gene Structure

Human mitochondrial DNA encodes 13 genes that are components of the oxidative phosphorylation electron transport chain, 2 rRNAs (12S and 14S), and 22 tRNAs required for mitochondrial translation. These genes are distributed asymmetrically

on both strands of dsDNA: the H-strand encodes the 2 rRNAs, 14 tRNAs, and 12 mRNAs and the L-strand encodes 8 tRNAs and 1 mRNA. As both strands of human mitochondrial DNA code for essential genes, transcription of both strands is necessary. There are two strong promoters, HSP1 and LSP, located in the D-loop, that drive transcription on the H- and L-strand, respectively (Montoya et al. 1982, 1983) (Fig. 11.1). Another promoter, HSP2, located downstream from HSP1 between rRNA and $tRNA^{phe}$, is a weak H-strand promoter (Litonin et al. 2010; Lodeiro et al. 2012). A transcription terminator between 12S rRNA and $tRNA^{leu}$, mediated by a termination factor (mTERF), is an important regulator for the quantity of rRNA and tRNA (Fernandez-Silva et al. 1997; Kruse et al. 1989).

The mitochondrial transcription machinery not only generates mRNAs, tRNAs and rRNAs, but is also an integral component of DNA replication. Mitochondria lack a specialized primase and the RNA polymerase (mtRNAP) must generate RNA primers for DNA synthesis. This suggests that the mitochondrial transcription system has nonspecific as well as promoter-specific initiation abilities. On the H-strand, the HSP promoter is close to the replication origin O_H, so transcripts can also serve as a replication primer. Significantly, transcription from HSP1 pauses or terminates at a conserved sequence (CSBII) near O_H (Pham et al. 2006), which may facilitate primer transfer to DNAP. If replication of mtDNA follows the canonical symmetrical synthesis and the L-strand is replicated as a lagging strand, RNA primers must be produced by mtRNAP in a sequence-independent manner. Indeed, mtRNAP transcribes on a promoter-free, single-stranded template to yield 20–50 nt RNA (Wanrooij et al. 2008).

In contrast to the typically increased complexity in gene structure in higher eukaryotes, yeast mtDNA appears more complicated than its human counterpart. Unlike the circular dsDNA of human mtDNA, that of yeast is a mixture of linear and circular DNA. Although yeast mtDNA codes for the same set of genes, 80 % of the genome codes for type II introns (Tabak et al. 1984), making it five times the size. Interestingly, these group II introns exhibit an extraordinary mobility mechanism in which the excised intron RNA reverse splices directly into a DNA target site and is then reverse transcribed by the intron-encoded protein (Lambowitz and Zimmerly 2004).

11.5.2 Mitochondrial RNA Polymerase and Transcription Factors

All components of mitochondrial transcription machinery are nuclear encoded and are transported into mitochondria via their mitochondrial localization sequences. Early mitochondrial transcription studies were conducted in yeast, which consists of a RNA polymerase, Rpo41, and a single transcription factor, Mtf1 (Greenleaf et al. 1986; Riemen and Michaelis 1993). Rpo41 displays a clear bacteriophage origin, as Rpo41 is homologous to T7 RNAP (Masters et al. 1987), but has an

N-terminal domain that is absent in the phage enzyme. However, unlike T7 RNAP, Rpo41 has lost independent transcription initiation activity and is functional only in the presence of the transcription factor Mtf1. Mitochondrial transcription therefore presents a unique opportunity to understand the evolution of complex transcription machinery. Mtf1 was originally thought to be a σ factor-like protein but, surprisingly, a crystal structure of Mtf1 revealed that Mtf1 is similar to bacterial ribosomal rRNA dimethyltransferase, an enzyme that confers kasugamycin resistance by modifying an adenosine residues in a rRNA stem loop (Seidel-Rogol et al. 2003). Interestingly, even though the yeast Rpo41 is larger than T7 RNAP, the minimal yeast mitochondrial promoters is only 9 bp, about half of a T7 promoter (Marczynski et al. 1989; Tracy and Stern 1995).

Human mitochondrial transcription activity was first detected using partially purified mitochondrial extracts, establishing that mitochondria have a transcription system independent of the nucleus (Aloni and Attardi 1971a, 1972; Pica-Mattoccia and Attardi 1971). However, the purified RNAP showed no activity, which led to the discovery of its transcription factors. The first transcription factor was named TFAM (Fisher and Clayton 1988), a member of the HMG (high-mobility group) family that includes many transcription factors, including LEF-1, TCF-1, and SRY (Rubio-Cosials et al. 2011). Mitochondrial DNA lacks the protection of histones; however, in certain cells, TFAM is so abundant so that it can coat the entire mtDNA (Takamatsu et al. 2002). In additional to its role in transcription, TFAM is also important for mtDNA maintenance, as mutations affecting TFAM reduce mtDNA content.

A common feature of HMG proteins is their high affinity for, and their ability to bend, DNA. An HMG-box domain bends dsDNA by about 90°, forming a superhelical protein–DNA complex. The crystal structure of TFAM complexed to the LSP promoter reveals how this small 25 kDa protein generates a large footprint. The two HMG-box domains of TFAM bind to dsDNA almost symmetrically, forcing the DNA into a 180° U-turn (Rubio-Cosials and Sola 2013). This extreme bending is reminiscent of the pre-initiation transcription complex of TBP to TATA box-containing DNA region (Nikolov et al. 1996) or the bacterial integration host factor (IHF) and HU or "DNABII" family of nucleoid-associated proteins—e.g., Hbb (Mouw and Rice 2007). The two HMG boxes of TFAM make sequence-specific interaction with DNA, suggesting its contribution to promoter specificity.

However, in the presence of TFAM, mtRNAP did not achieve maximal transcriptional activity in vitro. Further studies revealed two homologous proteins, TFB1M and TFB2M, both of which share sequence similarity with the yeast mitochondrial transcription factor, Mtf1, and were predicted by bioinformatics to be methyltransferases (Falkenberg et al. 2002; Seidel-Rogol et al. 2003).

TFB1M complements a *ksg* bacterial mutant, indicating that the human TFB1M can methylate the ksg stem loop in bacterial rRNA, confirming its methyltransferase activity (Seidel-Rogol et al. 2003). However, TFB1M was later shown functioning in translation, and only TFB2M is a transcription factor (Matsushima et al. 2005), interacting with mtRNAP in the absence of any promoter. The minimal human transcription system in vitro consists of an mtRNAP (POLRM)

and TFB2M. TFAM differentially affects transcription from HSP and LSP. In the minimal transcription system, HSP1 is a stronger promoter than LSP. However, in the presence of TFAM, equal numbers of transcripts are obtained from the two promoters. At equimolar concentration of TFAM and template, full activation of transcription from LSP is achieved without changing transcription levels from HSP1. When TFAM is in molar excess, transcription from LSP is substantially inhibited, while HSP1 remains active (Lodeiro et al. 2012). These observations support the hypothesis that a TFAM-regulated, two-component transcription system is operative in human mitochondria. The transcription initiation complex is structured that only mtRNAP and TFMA directly contact the promoter DNA, whereas TFBM2 is sandwiched between mtRNAP and TFAM, making only protein–protein interactions (McCulloch and Shadel 2003).

Like yeast Rpo41, human mtRNAP is homologous to T7 RNAP. The similarity allowed an accurate delineation of domains. The various functions of T7 RNAP are neatly divided into two domains: an N-terminal domain (NTD) for promoter specificity and a C-terminal domain for catalysis. Human mtRNAP exhibits 30 and 50 % sequence similarity with the phage NTD and CTD domains; however, as in yeast, the human enzyme also contains an N-terminal extension (Fig. 11.7). Interestingly, an N-terminal-truncated mtRNAP exists naturally in humans as a product of alternative splicing. The truncated enzyme lacks the N-terminal 262 amino acid extension but still functions as an RNAP (Kravchenko et al. 2005) and is confined to the nucleus. Transcription of certain mRNAs in humans and rodents is mediated by this single polypeptide nuclear enzyme.

11.5.3 Mitochondrial Transcription Reaction and Regulation

General mitochondrial transcription follows the same scheme for all promoter-specific transcription:

$$\underset{\text{promoter melting}}{\mathrm{E}+\mathrm{PDc}\leftrightarrow\mathrm{E}\bullet 2\mathrm{PDc}\leftrightarrow\mathrm{E}\bullet\mathrm{PDo}}\xrightarrow{\mathrm{NTP}}\underset{\text{initiation}}{\mathrm{E}\bullet\mathrm{D}\bullet R_2}\xrightarrow{\mathrm{NTP_s}}\underset{\text{elongation}}{\rightarrow\rightarrow\mathrm{E}\bullet\mathrm{D}\bullet R_n}$$

As the N-terminal domain (NTD) of T7 RNAP is responsible for promoter recognition, the Rpo41 NTD was tested for promoter specificity. Rpo41 alone is incapable of transcribing on duplex DNA, and promoter-specific transcription is only seen after Rpo41 associates with Mtf1, suggesting that Mtf1 is responsible for promoter unwinding. The first step of transcription is unwinding the double-stranded promoter; many RNAPs bend DNA ~90° to facilitate the process. However, fluorescence energy transfer (FRET) studies show that Rpo41 by itself bends not only promoter DNA by ~52° but also non-promoter DNA to the same extent (Tang et al. 2010). This level of bending is insufficient for T7 RNAP to open its promoter (Tang and Patel 2006). Remarkably, the addition of Mtf1 to Rpo41

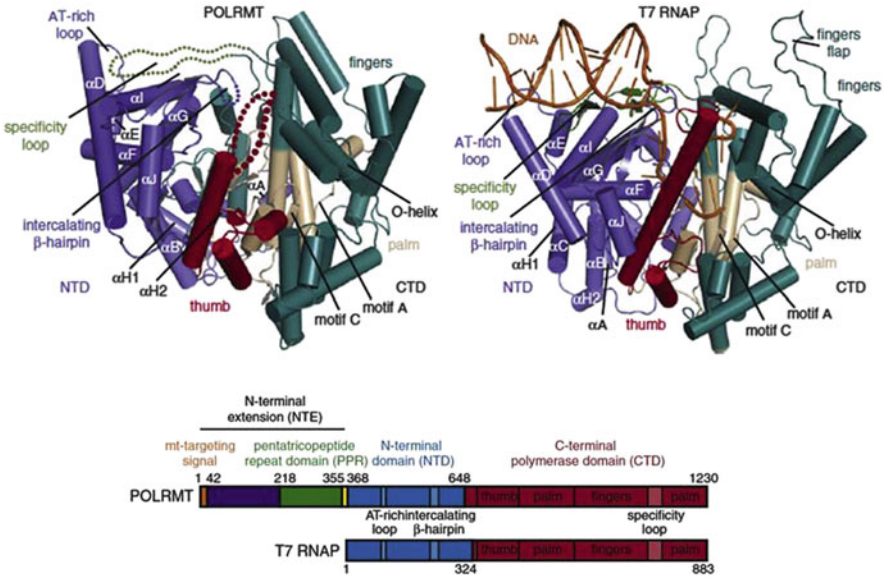

Fig. 11.7 Structural and sequence comparison of human mitochondrial RNA polymerase and T7 RNAP (Ringel et al. 2011)

increases bending ~90°, and the increase is specific for promoter DNA. It appears that Rpo41–Mtf1 catalyzes promoter-specific initiation by an induced-fit mechanism, where the two proteins jointly bend and melt the promoter DNA. This suggests that either both proteins bind the promoter or Mtf1 changes the conformation of Rpo41 to expose additional promoter-binding sites.

Rpo41 displays strong activity on a pre-unwound (bubble) promoter, indicating it contains full catalytic activity, and its inability to transcribe on a closed promoter is therefore due to its inability to separate the DNA strands (Matsunaga and Jaehning 2004). Removal of the N-terminal extension of Rpo41 does not result in Mtf1-independent activity; rather, Rpo41 shows even greater dependency on Mtf1 for activity. This observation, together with that Mtf1 does not bind to DNA, implies that Mtf1 induces significant conformational change on Rpo41 upon formation of holoenzyme on a promoter.

A crystal structure of human mtRNAP provides a clear molecular basis for the unique mechanism of mitochondrial transcription. The NTD of the mtRNAP resembles T7 RNAP, but the N-terminal extension consists of nine α-helices, four of which comprise two pentatricopeptide repeat (PPR) motifs found in both plant and mitochondrial proteins (Delannoy et al. 2007; Lightowlers and Chrzanowska-Lightowlers 2008). The PPR motif appears to be involved in RNA processing and editing. The structure explains the necessity of transcription factors for mtRNAP, as a region in human mtRNAP, equivalent to the promoter recognition motif of T7 RNAP (the AT-rich loop), instead of binding to promoter, is seen to interact with TFAM. Whether promoter interaction is mediated by TFAM has to wait for the crystal mtRNA–TFAM complex structure.

A modeled mtRNAP initiation complex was constructed by docking DNA from a T7 RNAP-promoter complex after superimposing the two active site domains (Cheetham and Steitz 1999; Ringel et al. 2011). Although tentative, the model initiation complex illustrates how promoter recognition may be achieved. Biochemical analyses indicate that the specificity loop confers promoter specificity, interacting with positions -6 and -11 of the promoter (Nayak et al. 2009). The mtRNAP transcription initiation model supports the idea that the specificity loop of mtRNAP, albeit shorter than that of T7 RNAP, could recognize the same region of the promoter. The AT-rich recognition loop is situated in a similar location as T7 RNAP, but it is sequestered by the N-terminal extension and thus does not contribute to promoter binding. In fact, the mtRNAP AT-rich loop appears to have lost promoter-binding activity, because deletion of the extension does not restore factor-independent, promoter-specific transcription (Chang and Yin, unpublished data). It is puzzling why mtRNAP possess the same promoter recognition elements as T7 RNAP but lacks intrinsic promoter specificity. Perhaps substitution of residues in critical regions causes subtle structural changes so that certain elements are less than optimally situated for promoter binding. However, the structure of mtRNAP allows testable hypotheses regarding factor-dependent transcription initiation: TFB2M may reposition the misaligned intercalating hairpin to allow promoter melting or it may directly melt the promoter.

Combined structural and biochemical studies describe the transcription complex on the 38 bp LSP promoter: TFAM binds 23 bp from −38 to −15 at LSP and that the mtRNAP–TFB2M complex covers the region immediately downstream, from bp −14 to +10, with TFB2M putatively positioned close to TFAM (Fig. 11.7) (Dairaghi et al. 1995; Gaspari et al. 2004; Litonin et al. 2010).

Mitochondrial gene expression is regulated by cellular ATP levels. Regulation is best understood in yeast: cells grown in glucose have high levels of ATP, which suppresses transcription. Conversely, cells grown anaerobically produce less ATP and exhibit elevated transcription (Amiott and Jaehning 2006). The increase in transcription is mainly due to increased synthesis rates, as opposed to an increase in mtDNA copy number or elevated Rpo41 and Mtf1 levels. Moreover, different yeast promoters respond to changes in ATP levels to varying degrees. Rpo41–Mtf1 itself acts as the in vivo ATP sensor that couples RNA abundance to respiration (Amiott and Jaehning 2006). In addition, exogenous factors, such as NRTIs, inhibit human mtRNAP activity (Arnold et al. 2012), thus directly contributing to drug toxicity.

Mitochondrial function requires control of mtDNA content, and both are intimately related to human health. Oxidative mtDNA damage has been associated with aging; mutations on mtDNA are implicated in cancers and multisystem clinical disorders. Mitochondrial polymerases, as adverse reaction targets for nucleoside based inhibitors, are important for antiviral drug design. Although much progress has been made in the past decades, a complete understanding of mitochondrial structure and function requires substantial support for further research.

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Chapter 12
Eukaryotic RNA Polymerase II

David A. Bushnell and Roger D. Kornberg

Abstract Structures of yeast RNA polymerase II, alone and in the act of transcription with associated DNA and RNA, have been determined to near-atomic resolution. The structures illuminate the basis for the fidelity of transcription, for translocation on the DNA template, and for release of the product RNA. Structures of an RNA polymerase II-general transcription factor complex have led to a model for a closed transcription initiation complex and have suggested a possible basis for promoter escape.

Keywords Yeast • RNA polymerase II structure • X-ray crystallography • Trigger loop • Nucleotide addition • TFIIB

Structural studies of cellular RNA polymerases, both bacterial and eukaryotic, began in the 1980s, with the formation of 2D crystals on lipid layers, electron microscopy, and 3D reconstruction (Darst et al. 1988). This approach is rapid, requires very little material, and can reveal even the slightest degree of ordering. It resulted in low resolution structures of Escherichia coli RNA polymerase, yeast RNA polymerase II (pol II), and a yeast pol II transcribing complex (Darst et al. 1989; Poglitsch et al. 1999; Fu et al. 1999). These studies had three implications for further work: the first was the possibility of crystallization of cellular RNA polymerases, alone and in the act of transcription (crystallization of such large molecules had rarely been attempted, and the size and complexity of these RNA polymerases placed them beyond reach of X-ray structure determination at the time); the studies also revealed that a substoichiometric level of two pol II subunits, Rpb4 and Rpb7, caused heterogeneity that was an impediment to crystallization; and finally, with the removal of Rpb4 and Rpb7 by genetic means, large, well-ordered 2D crystals were obtained

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that showed a tendency towards epitaxial growth (Darst et al. 1991a), leading to the formation of large single crystals for X-ray diffraction analysis.

12.1 Structure of Pol II

A 5 Å electron density map of pol II was obtained by X-ray diffraction with the use of tungsten (W_{18}), tantalum (Ta_6), and iridium (Ir_2) cluster compounds for phase determination (Fu et al. 1999). Close agreement of the map with the results from 2D crystallography (Darst et al. 1991b) provided key validation of the 5 Å phase set. These phases could be used to locate individual heavy atoms in derivative crystals at high resolution. There were two major obstacles. First, the crystals were sensitive to oxidation, resulting in a loss of diffraction to high resolution. The problem was overcome by the growth and maintenance of the crystals in an inert atmosphere or by the use of a high concentration of reductant, such as dithiothreitol. Secondly, the crystals were polymorphic, varying by as much as 10 Å along one unit cell direction. This problem was solved by dehydration of the crystals, resulting in shrinkage of the unit cell volume by 5 % and consistent diffraction to about 3 Å resolution.

Structure determination was finally made possible by developments at synchrotrons, especially increased photon flux and more rapid detectors. For these reasons, the structures of pol II and bacterial RNA polymerase, and also those of ribosomal subunits that had been crystallized long before, were all solved at about the same time. Photon physicists were the unsung heroes of the work. For our studies of pol II, we owe a special debt to the beamline scientists at the Stanford Synchrotron Radiation Lightsource, who gave us extraordinary access during a period of intensive search for heavy atom derivatives.

The structure of ten-subunit pol II (lacking Rpb4 and Rpb7) was solved at 2.8 Å resolution (Fig. 12.1a) (Cramer et al. 2001). The structure reveals the two largest subunits, Rpb1 and Rpb2 on either side of a central cleft, with a magnesium ion marking the location of the active center. The remaining subunits are arrayed around the periphery, with Rpb5 and Rpb9 at the front end or leading edge of the enzyme, on opposite sides of the cleft, and Rpb3, 10, 11, and 12 forming a cluster on the backside of the enzyme.

12.2 Structure of Pol II Transcribing Complex

The 2.8 Å structure was used to solve that of an actively transcribing enzyme by molecular replacement (Gnatt et al. 2001). The transcribing complex was formed by the initiation of transcription on a DNA template with a "tail" of twelve residues at one 3′-end. Initiation on a tailed template avoids the requirement for Rpb4, Rpb7, and a large set of general transcription factors. Transcription on the tailed template was stalled after the synthesis of a 14-residue transcript by the omission of one of the four nucleoside triphosphates (NTPs). The reaction mixture was passed over a

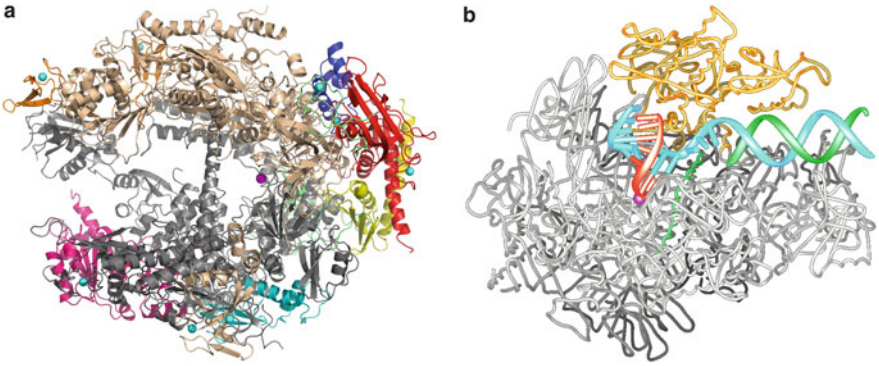

Fig. 12.1 (**a**) Ribbon diagram of 10-subunit yeast RNA pol II. Each subunit is shown in a different color. A *pink sphere* represents a Mg^{2+} ion, marking the location of the active center. *Cyan spheres* represent structural zinc atoms (Cramer et al. 2001). (**b**) Chain traces of polypeptides and nucleic acids in a pol II transcribing complex, formed by initiation of transcription on a tailed template. Protein is *gray* except for the "bridge helix" extending across the central cleft (*green*) and the clamp domain (*gold*). Template DNA strand is *blue*, nontemplate strand is *green*, and RNA is *red*. The view is obtained from (**a**) by a 90° rotation about a horizontal axis (upward rotation) and a 180° rotation about a vertical axis (flip from left to right). Some protein has been removed from the front to expose the nucleic acids in the central cleft. The direction of transcription is to the right; DNA enters from the right (downstream) and translocates during transcription to the left (upstream) (Gnatt et al. 2001)

heparin column, to separate inactive enzyme (which bound to the column, due to interaction of heparin with the empty DNA-binding cleft) from actively transcribing complexes (which flowed through the column, because template DNA filled the cleft). This step was crucial for obtaining homogeneous transcribing complexes for crystallization.

The first transcribing complex structure, at 3.1 Å resolution, differs from that of pol II alone by a major conformational change. A massive protein element, formed by parts of two subunits, swings up to 30 Å over the DNA and RNA in the course of forming a transcribing complex (Fig. 12.1b). This mobile element, referred to as a "clamp," appears to hold the DNA and RNA in place and may be important for the processivity of transcription. The structure reveals the course of nucleic acids through the transcribing complex. Duplex DNA enters the cleft between Rpb5 and Rpb9 and remains in duplex form over a length of 15 bp. The DNA unwinds three residues before the active site, after which there is a bend of about 90° in the template single strand, in consequence of which the next base is flipped and points down towards the floor of the cleft for readout in transcription. This base is paired with that of the ribonucleotide just added to the growing RNA strand. There are eight more DNA–RNA hybrid base pairs in the structure, but no evidence of the corresponding nontemplate strand nor of the duplex DNA beyond the unwound region ("transcription bubble"), due to motion or disorder. The DNA–RNA hybrid interacts with a "wall" of protein density that prevents straight passage of nucleic acids through the cleft.

Because the ribonucleotide just added to the RNA chain is still in the active center, in the nucleotide addition or "A" site, paired with the coding base in the DNA, this structure is referred to as a "pre-translocation complex." The next structure of a transcribing complex was obtained by direct binding of pol II to a nucleic acid "scaffold," consisting of a DNA–RNA hybrid and downstream duplex DNA, rather than by initiation and arrest of RNA synthesis on a tailed template (Westover et al. 2004a). This simplified approach was based on biochemical studies demonstrating the efficient elongation of RNA chains on such scaffolds (Kireeva et al. 2000). The structure represents a "post-translocation complex," in which the nucleic acids have advanced by one residue along the pol II surface, bringing the ribonucleotide just added to the RNA chain to the next position of the DNA–RNA hybrid and leaving the "A" site open for binding NTP. This structure, and others based upon it, reveals the following structural correlates of transcription (Fig. 12.2a, b).

12.2.1 Magnesium Ions and NTP Entry Site

A single metal ion is identified in the active center of pol II. This ion is presumed to be Mg^{2+}, and its location could be confirmed by substitution with Mn^{2+} or Pb^{2+} and anomalous difference diffraction (Cramer et al. 2000, 2001). Upon soaking with NTP and difference diffraction analysis, a second metal ion is observed. In the case of NTP matched with the coding base in the DNA template and with the use of a chain terminator on the RNA strand to prevent nucleotide addition, the NTP is seen to occupy the A site. In the case of NTPs not matched with the coding base, binding with a metal ion is also observed, but with the NTP in an adjacent location, referred to as the entry or "E" site (Westover et al. 2004b). The occurrence of such a second NTP-binding site was anticipated by biochemical studies and may serve to increase NTP concentration in the active center and thereby to facilitate transcription (Sosunov et al. 2003). The series of post-translocation complex structures, with no bound NTP, with NTP in the E site, and with NTP in the A site, along with the pre-translocation complex, recapitulate a round of the transcription cycle (Fig. 12.2b).

12.2.2 Trigger Loop and the Fidelity of Transcription

The first structures of post-translocation complexes with NTP in the A site revealed only interactions of the NTP with DNA, RNA, and Mg^{2+}. Improved structures disclosed additional interactions with a protein element termed the "trigger loop" (Wang et al. 2006). In the absence of NTP, the trigger loop is located some 30 Å from the A site and is partially disordered (Fig. 12.3a). In the presence of NTP, the trigger loop swings into position for contact with the nucleotide base, through a

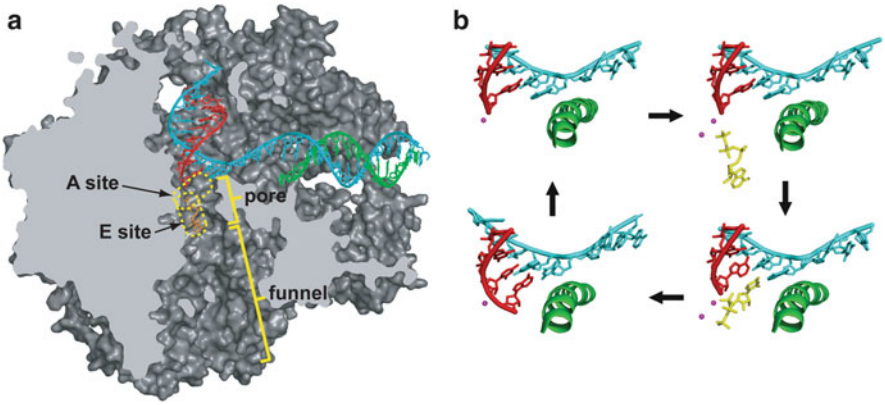

Fig. 12.2 (**a**) Pol II transcribing complex, identical to Fig. 12.1b except shown as a surface representation rather than chain traces. *Solid light gray* areas are cut surfaces where protein was removed to expose the nucleic acids in the central cleft. Also are indicated the locations of the NTP entry (E) and addition (A) sites, as well as the presumed pathway for passage of NTPs to the active center, through a funnel-shaped opening at the bottom of the polymerase, narrowing to a pore beneath the active center (Westover et al. 2004b). (**b**) A transcription cycle, from structures of transcribing complexes formed by initiation on a tailed template (pre-translocation complex, *lower left*) or through binding a nucleic acid scaffold alone (post-translocation complex, *upper left*), soaked with unmatched NTP (E site occupied by NTP, *lower right*) or soaked with matched NTP (A site occupied by NTP, *upper right*). The structures are shown as in Fig. 12.1b, with all protein removed except for the bridge helix (*green*) and with nontemplate DNA strand removed, leaving only the template (*blue*) and RNA strands (*red*) and the magnesium ion (*pink sphere*) in the immediate vicinity of the active center (Gnatt et al. 2001; Westover et al. 2004a, b)

leucine residue, and for contact with the β-phosphate, through a histidine residue (His1085). The imidazole side chain of the histidine can trigger phosphodiester bond formation by promoting the flow of electrons from the 3′-hydroxyl group of the RNA chain terminus to the α-phosphate of the NTP to the pyrophosphate leaving group (Fig. 12.3b), a mechanism common to nucleic acid polymerases (Castro et al. 2009). The imidazole side chain serves as a protein donor to the pyrophosphate leaving group. The alignment of the leucine and histidine residues with the NTP must be precise for this mechanism. A mismatched NTP will be misaligned, resulting in failure of the mechanism. Even a correctly matched dNTP will likely be misaligned, due to the difference in diameter of DNA–DNA and DNA–RNA double helices. Biochemical studies have confirmed a role of the trigger in discrimination between NTP and dNTP (Kaplan et al. 2008; Zhang et al. 2009). Deletion of the trigger loop in the bacterial system leads to a reduction of about 60,000-fold in the rate of RNA chain elongation (Yuzenkova et al. 2010). The trigger loop couples nucleotide specificity to catalysis (Kaplan et al. 2008, 2012; Zhang et al. 2009; Yuzenkova et al. 2010; Vassylyev et al. 2007; Temiakov et al. 2005).

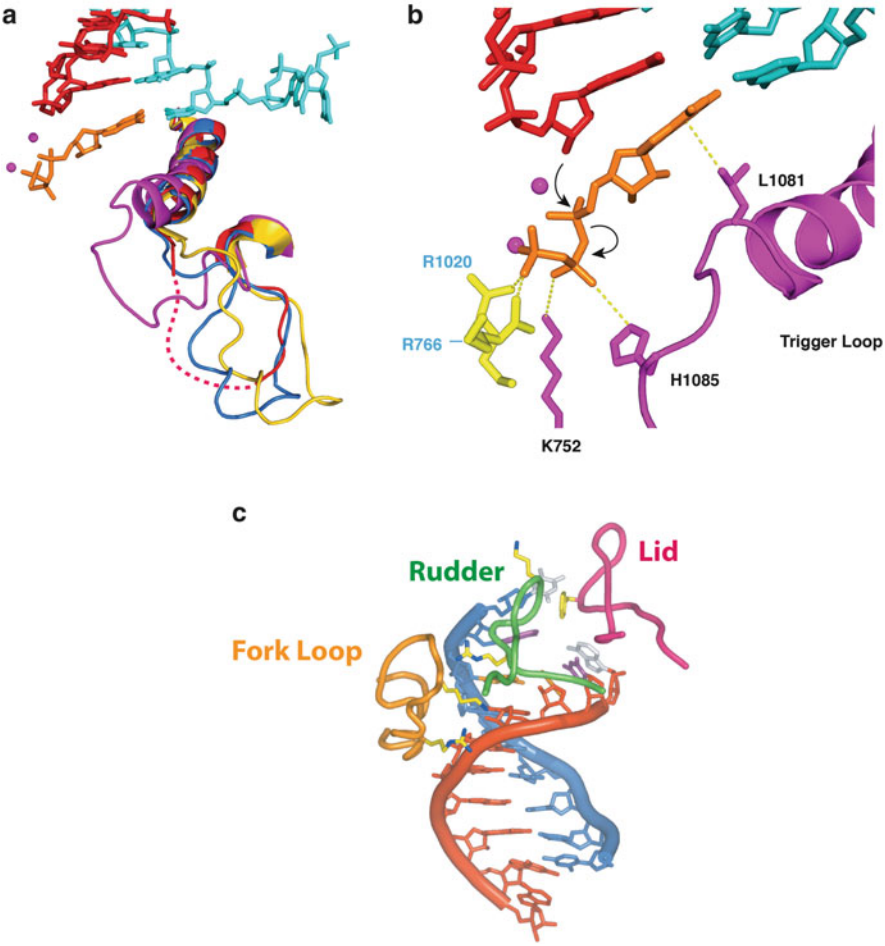

Fig. 12.3 (**a**) The trigger loop swings beneath NTP in the A site. View as in Fig. 12.2b, with multiple states of the trigger loop from various crystal structures in the absence of NTP in *red* (partially disordered), *blue*, and *yellow*. Only in the presence of NTP (*gold*) is the trigger loop (*purple*) directly beneath the A site, in position to contact the NTP (Wang et al. 2006). (**b**) Interactions of the trigger loop with NTP in the A site. Same as (**a**) with trigger loop in *purple*, with interactions indicated by *dashed yellow lines*, and the flow of electrons for phosphodiester bond formation and phosphoanhydride bond breakage indicated by *black arrows* (Wang et al. 2006). (**c**) Disruption of DNA–RNA hybrid in structure of pol II transcribing complex in post-translocation state. Same as *upper left panel* of Fig. 12.2a, except showing the DNA–RNA hybrid upstream of the A site, with protein loops in *green* (rudder), *purple* (lid), and *gold* (fork loop). A tyrosine side chain of the lid that appears to act as a wedge between DNA and RNA, and lysine and arginine side chains that interact with phosphates are shown in *yellow sticks* (Westover et al. 2004a)

12.2.3 Bridge Helix

An α-helix, spanning the cleft between Rpb1 and Rpb2 and therefore termed the "bridge helix," lies at the downstream edge of the transcription bubble in a transcribing complex (Fig. 12.1b). Alanine and threonine residues of the bridge helix interact with the coding base in the template DNA strand. The structure of bacterial RNA polymerase also contains a bridge helix, but in a partially unwound or bent state that would clash with the template DNA in the eukaryotic transcribing complex (Zhang et al. 1999). It was proposed that alternation of the bridge helix between straight and bent states underlies the translocation step in transcription (Gnatt et al. 2001). Biochemical studies have provided support for this idea and have further suggested a coupling of conformational changes of the bridge helix and trigger loop (Temiakov et al. 2005; Epshtein et al. 2002; Tuske et al. 2005).

12.2.4 Rudder, Lid, and Fork Loop

In contrast to the transcribing complex formed by initiation on a tailed template, in which a uniform DNA–RNA hybrid of nine base pairs is observed, the complex formed on a nucleic acid scaffold reveals disruption of the upstream end of the hybrid (Fig. 12.3c) (Westover et al. 2004a). The first 6 bp from the downstream end of the hybrid are coplanar with an appropriate separation for hydrogen bonding of the bases. The seventh, eight, and ninth base pairs are progressively noncoplanar, with an increasing separation of the bases. Protein loops termed "rudder" and "lid" interact with the separated DNA and RNA strands. A "fork loop" interacts through lysine side chains with the phosphates at positions 6 and 7 to stabilize the hybrid helix and prevent the separation of strands from extending downstream towards the active center. In this way, the enzyme achieves the disruption of the very stable DNA–RNA hybrid required for release of the RNA into solution.

12.2.5 Rpb4 and Rpb7

During logarithmic growth of yeast, the ratio of subunits Rpb4 and Rpb7 to other subunits of pol II is about 1:5, whereas during stress or in stationary phase, the ratio rises to near 1:1 (Kolodziej et al. 1990; Choder and Young 1993). Although Rpb4 and Rpb7 were originally removed by the deletion of the gene for Rpb4, to obtain homogeneous enzyme for crystallization, 12-subunit pol II containing Rpb4 and Rpb7 was later obtained in homogeneous form as well, either by purification of the enzyme with the use of an affinity tag on Rpb4 or by addition to the enzyme of Rpb4 and Rpb7 expressed in bacteria (Armache et al. 2003; Bushnell and Kornberg 2003). The structure of the 12-subunit enzyme at about 4 Å resolution reveals a

heterodimer of Rpb4 and Rpb7 adjacent to the clamp, along a possible path of RNA exit from the enzyme. The heterodimer encloses part of the path, forming a tunnel large enough for RNA to pass through.

12.2.6 α-Amanitin

The death cap mushroom *Amanita phalloides* produces a specific inhibitor of pol II, α-amanitin (Wieland and Faulstich 1991). Upon soaking of pol II crystals with α-amanitin and difference diffraction analysis, the bound inhibitor could be seen beneath the bridge helix making contact primarily with Rpb1 (Bushnell et al. 2002). With improved computational methods, the inhibitor could be seen to contact the trigger loop and in particular His1085, the residue important for the role of the trigger loop in catalysis. Mutation of His1085 renders pol II resistant to inhibition by α-amanitin (Kaplan et al. 2008).

12.2.7 Pol II–TFIIB Complex

Biochemical studies have shown that general transcription factor TFIIB interacts with a promoter DNA–TFIID complex and with pol II (Buratowski and Zhou 1993; Ha et al. 1993). It has therefore been thought that TFIIB brings promoter DNA to the pol II surface. This idea was borne out by structure determination of a pol II–TFIIB cocrystal. The structure was solved at 4.5 Å by molecular replacement (Bushnell et al. 2004). A zinc anomalous difference map contained an extra peak beyond the eight normally found for pol II. The additional peak was located adjacent to the wall and the clamp domains of pol II, associated with a region of the pol II surface termed the "dock" domain. Electron density adjacent to the dock domain matched the NMR structure of the human IIB zinc ribbon (Fig. 12.4a). There was additional electron density between the wall and clamp domains, extending almost to the active center. A loop of polypeptide modeled into this density, termed the "B-finger," would appear to occupy the same location as the DNA–RNA hybrid in a transcribing complex. Superimposing the B-finger and DNA–RNA hybrid reveals no interference with the template DNA and no interference with RNA up to position 5 from the active center, but a steric clash with RNA at positions 6 and beyond (Fig. 12.4b). Consistent with this picture, biochemical studies show not only compatibility but a stabilizing effect of TFIIB on transcribing complexes containing RNA of five residues. Competition between TFIIB and RNA of length six residues or more could play a role in displacement of TFIIB, release of promoter DNA, and consequent "promoter escape."

The recent pol II–TFIIB cocrystal structure, obtained under different solution conditions from the original structure, reveals additional regions of TFIIB, including one of two C-terminal cyclin domains and a linker between the N- and

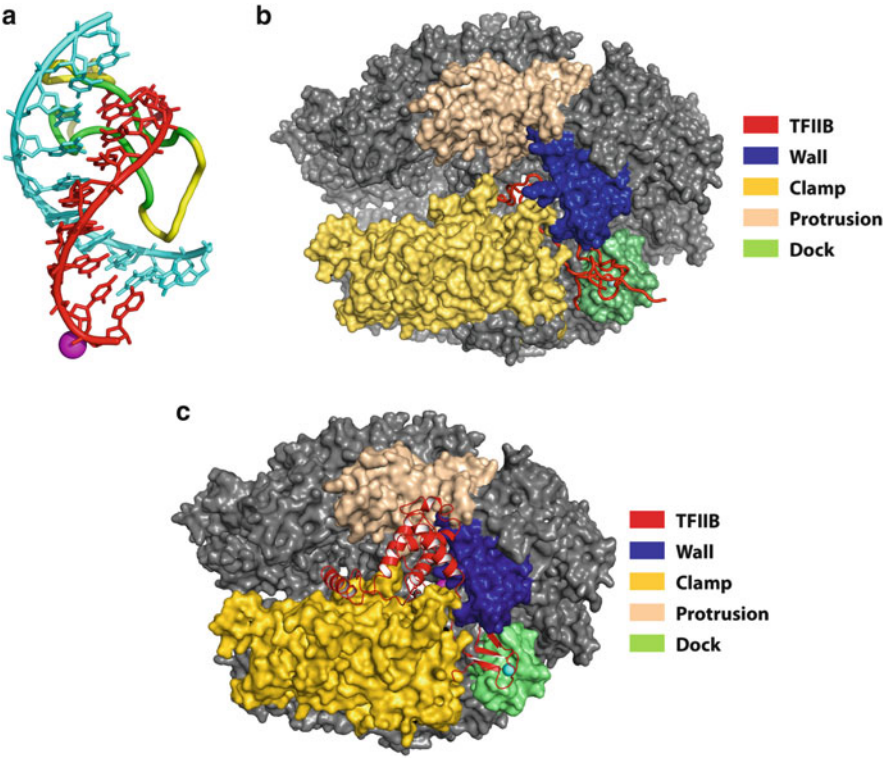

Fig. 12.4 (**a**) B-finger from pol II–TFIIB cocrystal structure superimposed on DNA–RNA hybrid from transcribing complex structure. View as in Fig. 12.3c, with B-finger in *yellow* and *green* (to indicate conserved regions) (Bushnell et al. 2004). (**b**) Pol II–TFIIB cocrystal structure showing the zinc ribbon domain and B-finger of TFIIB (*red chain trace*). View as in Fig. 12.1a except as surface representation. Dock, clamp, and wall domains of pol II are indicated, as well as "protrusion," a prominent feature of subunit Rpb2 (Bushnell et al. 2004). (**c**) Pol II–TFIIB cocrystal structure showing the linker and first cyclin domains of TFIIB (*red ribbon*). Otherwise the same as (**b**) (Liu et al. 2010)

C-terminal domains (Fig. 12.4c) (Kostrewa et al. 2009; Liu et al. 2010). The structure of a TFIIB cyclin domain in a complex with TATA-binding protein (TBP) and TATA box DNA, determined long ago by others, could be docked to the cocrystal structure to produce a model of a "closed" promoter complex (Fig. 12.5). Unwinding the promoter DNA to create an "open" complex, followed synthesis of RNA up to five residues, would cause a switch from the conformation represented by the recent cocrystal structure (Fig. 12.4c) to that of the original one (Fig. 12.4a). Contact between pol II and TFIIB would be disrupted in two stages: first, interaction of a short transcript with the B-finger and release of the cyclin domain and, second, ejection of the B-finger by a longer transcript to complete promoter escape.

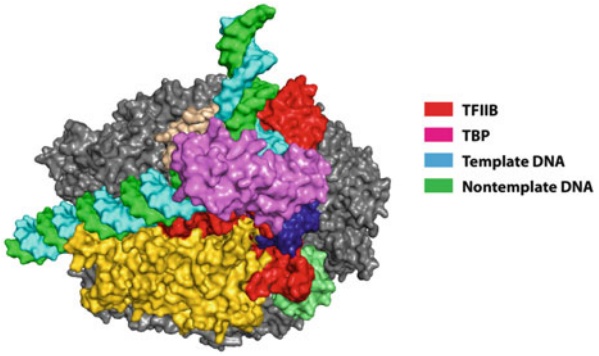

Fig. 12.5 Model of closed promoter complex, produced by docking X-ray structure of TFIIB–TBP (*pink*)–TATA box complex to structure of pol II–TFIIB complex, followed by extension of the ends of the TATA box DNA fragment with straight B-form DNA (*magenta* and *green*) (Liu et al. 2010)

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Chapter 13
Plant Multisubunit RNA Polymerases IV and V

Thomas S. Ream, Jeremy R. Haag, and Craig S. Pikaard

Abstract Plants are unique among eukaryotes in having five nuclear multisubunit RNA polymerases. These include RNA polymerases I, II, and III (Pols I, II, and III), which are ubiquitous among eukaryotes, plus two plant-specific RNA polymerases, Pol IV and Pol V, that are 12-subunit enzymes that evolved as specialized forms of Pol II. Pols IV and V are nonessential for viability but play important roles in RNA-mediated gene silencing pathways that tame transposons, defend against invading viruses, mediate cross talk among alleles, and influence development. Numerous amino acids that are invariant in the catalytic subunits of Pols I, II, and III are substituted, or even absent, in Pols IV or V, implying that Pols IV and V have fewer functional constraints on their evolution than other multisubunit RNA polymerases. In vitro, Pol IV and Pol V can extend an RNA primer hybridized to a DNA template, but the templates transcribed by Pol IV and Pol V in vivo are unclear. Likewise, the boundaries of Pol IV and Pol V transcription units and the characteristics of their primary transcripts remain undefined. In this chapter, the state of our understanding of Pol IV and Pol V subunit compositions and functions is discussed.

Keywords Pol IV • Pol V • *Arabidopsis* • Noncoding RNA • RNA silencing • siRNA • Heterochromatin • RNA-directed DNA methylation • Transcription

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13.1 Introduction and Overview

All known eukaryotes require nuclear DNA-dependent RNA polymerases I, II, and III (Pols I, II, and III) for cell viability (see Chap. 12). In all but trypanosomes, Pol I specializes in the transcription of a single gene sequence, repeated in hundreds of copies, that encodes a primary transcript that is then processed to form the three largest RNAs of ribosomes, the protein synthetic machines of the cell. Pol II transcribes thousands of genes, including the genes that encode pre-messenger RNAs (mRNAs), pre-microRNAs (miRNAs), and a variety of small structural and regulatory RNAs. Pol III transcribes hundreds of genes, including those encoding pre-tRNAs, 5S ribosomal RNA, short interspersed nuclear elements (SINEs), and a collection of other relatively short (<400 nt) catalytic or regulatory RNAs.

Given the expectation of three, and only three, nuclear multisubunit RNA polymerases in plants, as in all other known eukaryotes, completion of the *Arabidopsis thaliana* genome sequence in 2000 (The Arabidopsis Genome Initiative 2000) revealed a surprise that plants have catalytic subunits for two additional nuclear RNA polymerases, now known as Pol IV and Pol V (Fig. 13.1a, b). The two genes encoding the largest subunits of nuclear Pols IV and V, *NRPD1* and *NRPE1*, respectively, are quite different in sequence compared to other multisubunit RNA polymerase largest subunits but have recognizable similarity to their conserved domains, including core sequences of the catalytic site (Fig. 13.1a, c) (Herr et al. 2005; Haag et al. 2009; Onodera et al. 2005; Pontier et al. 2005). Multiple intron–exon positions in the first half of *NRPD1* and *NRPE1* are identical to their positions in *NRPB1*, the gene encoding the largest subunit of Pol II in *Arabidopsis* (Luo and Hall 2007). However, the C-terminal domains (CTDs) of the NRPB1, NRPD1, and NRPE1 proteins are unrelated, with the heptad repeats typical of the Pol II largest subunit CTD missing. A domain related to the *DEFECTIVE CHLOROPLASTS AND LEAVES* gene implicated in chloroplast rRNA processing (Bellaoui et al. 2003) is present in the CTDs of both NRPD1 and NRPE1 (Fig. 13.1a). The CTD of the Pol V largest subunit, NRPE1, also contains a region composed of ten imperfect copies of a 16 amino acid sequence and a domain of repeating glutamine and serine amino acids, neither of which are present in the Pol II largest subunit, NRPB1 (Fig. 13.1a, e). Collectively, these observations suggest that duplication of the Pol II *NRPB1* gene, combined with rearrangements involving unrelated genes, gave rise to the *NRPD1* gene in an ancestor common to modern day algae and land plants (Luo and Hall 2007). Subsequent duplication of the *NRPD1* gene, occurring prior to the divergence of moss from vascular plants, is then thought to have given rise to the ancestral Pol V largest subunit gene (Luo and Hall 2007; Tucker et al. 2011).

In addition to *NRPD1* and *NRPE1* genes, the *Arabidopsis* genome sequence revealed two nearly identical genes (one of which is a nonfunctional pseudogene) encoding an atypical second-largest subunit, NRP(D/E)2 (Fig. 13.1b). The NRP (D/E)2 amino acid sequence is more similar to the NRPB2 subunit of Pol II than to

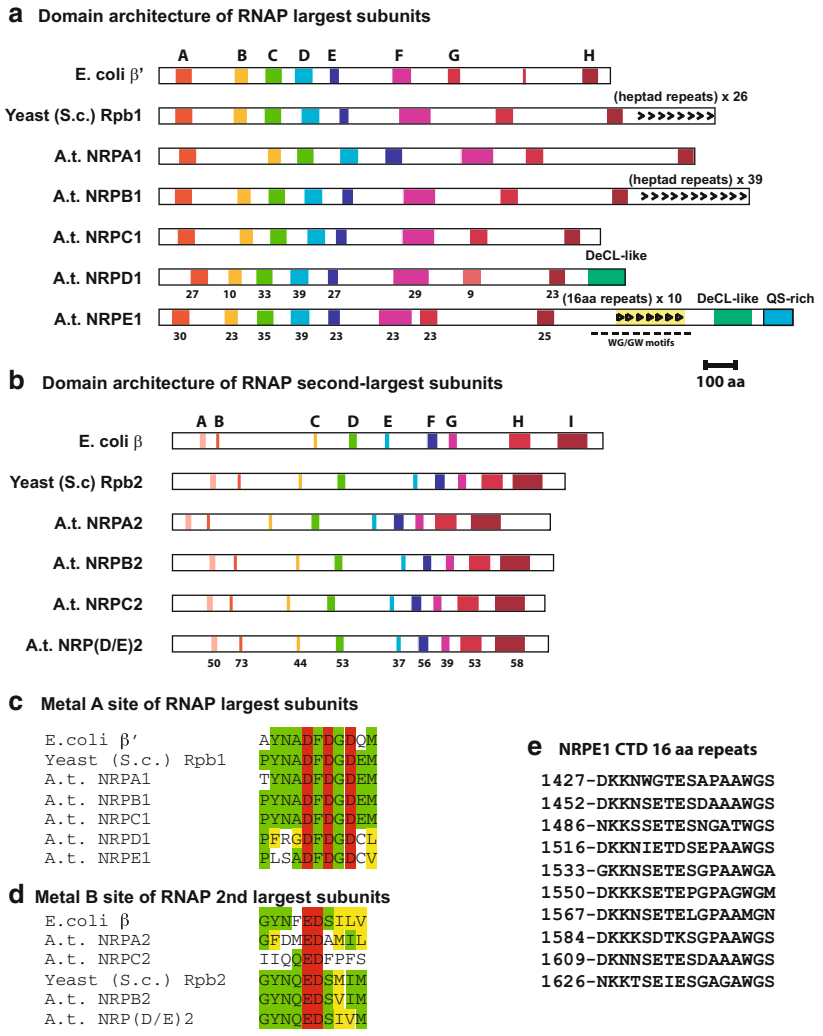

Fig. 13.1 Comparison of the catalytic subunits *Arabidopsis* nuclear RNA polymerases to those of *E. coli* RNA polymerase and yeast Pol II. (**a**) Diagrams for the largest subunits, with conserved domains A–H shown in different colors. Heptad repeats in the Pol II CTDs refer to tandemly arrayed seven amino acid sequences that are conserved in sequence, but not number, throughout eukaryotes. The DeCL-like domain of the Pol IV and Pol V subunit CTDs shows sequence similarity to the DEFECTIVE CHLOROPLASTS AND LEAVES protein in *Arabidopsis*. The QS rich domain is composed primarily of alternating glutamines and serine amino acids. The number below each of the Pol IV or Pol V domains shows the percent identity of that domain to the corresponding domain of the *Arabidopsis* Pol II subunit. (**b**) Diagrams for the second-largest subunits, with conserved domains A–I highlighted in different colors. The number below each of the Pol IV or Pol V domains is the percent identity between those domains and the corresponding domain of the *Arabidopsis* Pol II subunit. (**c**) Sequences surrounding the invariant aspartates of the magnesium-binding Metal A sites within conserved domain D of the largest subunits shown in panel **a**. (**d**) Sequences surrounding the invariant aspartate-glutamate duo of the magnesium-binding Metal B sites within the second-largest

the equivalent subunits of Pols I and III (Onodera et al. 2005), including sequences in the vicinity of the catalytic site (Fig. 13.1d). Genetic evidence indicated that *NRP(D/E)2* encodes the second subunit of both Pol IV and Pol V (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005).

The first insights into the functions of Pols IV and V (initially known as Pol IVa and Pol IVb) were revealed in 2005, based on molecular phenotypes of *nrpd1*, *nrpe1*, and *nrp(d/e)2* mutants (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005). These studies showed that Pols IV and V play nonredundant roles in a transcriptional silencing process known as RNA-directed DNA methylation (RdDM) that is important for silencing transposable elements, endogenous repeats, and transgenes (Zhang and Zhu 2011; Lahmy et al. 2010; Matzke et al. 2009). Roles for Pols IV and V in additional RNA-mediated silencing pathways have since been revealed. These include inter-allelic interactions responsible for the epigenetic phenomenon paramutation, pathogen resistance, and the spread of RNA silencing signals from cell to cell and via the vascular system (for reviews see Haag and Pikaard 2011; Molnar et al. 2011; Erhard and Hollick 2011; Arteaga-Vazquez and Chandler 2010; Brosnan and Voinnet 2011).

Subunit compositions of affinity-purified *Arabidopsis* Pols II, IV, and V, determined in 2009, showed that Pols IV and V are 12-subunit enzymes (Ream et al. 2009) that include subunits shared by Pol II (Ream et al. 2009; Huang et al. 2009; Lahmy et al. 2009; He et al. 2009a) and unique subunits that arose via stepwise duplication and sub-functionalization of Pol II subunit genes (Tucker et al. 2011; Ream et al. 2009) (Fig. 13.2). However, it remains unclear whether Pol IV and Pol V transcription is initiated in a Pol II-like fashion, making use of general transcription factors, activators, or coactivators that recognize promoters or enhancer sequences. Likewise, few details are known concerning Pol IV and Pol V activities as enzymes, including their templates in vivo, their modes of recruitment to specific loci, their transcription initiation or termination sites, the sizes of their primary transcripts, their processivity, or their fidelity.

Multiple subunits that are common to Pols II, IV, and V are encoded by two or more genes (Ream et al. 2009; Law et al. 2011), and one case study shows that alternative subunits can generate functionally distinct polymerase subtypes (Tan et al. 2012). New insights into the roles of the different subunits of Pols IV and V, and by inference, Pol II, may come from such studies, aided by the fact that null mutations eliminating Pol IV- or Pol V-specific subunits are not lethal. The recent demonstration that Pols IV and V can carry out RNA-primed transcription of DNA templates in vitro (Haag et al. 2012) also provides a starting point for learning more about their enzymatic capabilities and, in conjunction with genetic studies, the roles of catalytic versus non-catalytic subunits. These issues of general relevance to multisubunit RNA polymerase functions are explored in more detail in the subsequent sections of this chapter.

Fig. 13.1 (continued) subunits shown in panel **a**. (**e**) Sequence of the ten 16aa tandem repeats in the CTD of NRPE1. Numbers refer to amino acid positions within the full-length protein

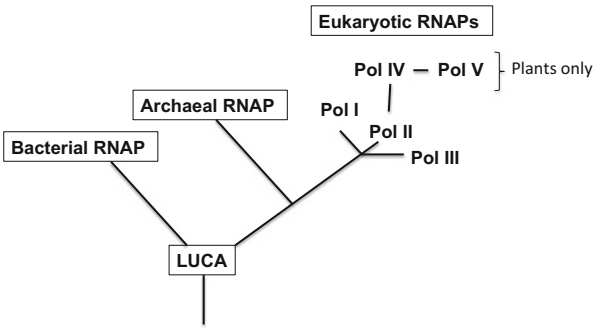

Fig. 13.2 Evolution of multisubunit RNA polymerases. LUCA refers to the last universal common ancestor of extant bacteria, archaea, and eukaryotes. The three essential polymerases of eukaryotes, Pols I, II, and III, must have existed in the common ancestor of all eukaryotes to explain their ubiquity

13.2 Pols IV and V Are Nonessential for Viability but Important for Development

In plants, as in all eukaryotes, Pols I, II, and III are essential. Heterozygous plants that have one wild-type allele and one null mutant allele for Pol I, II, or III catalytic subunits are viable, producing haploid gametophytes that are 50 % normal and 50 % mutant. Haploid female gametophytes that carry the mutant allele arrest early in development, failing to complete the three post-meiotic rounds of mitosis that are needed to form a mature eight-celled gametophyte that includes an egg cell. Thus, no transmission of the mutant alleles takes place through the female (Onodera et al. 2008). In contrast, haploid three-celled male gametophytes (pollen) bearing null alleles for essential Pol I, II, or III subunits are still able to complete development thanks to parentally supplied polymerase subunits (or holoenzymes) that persist from the post-meiotic pollen mother cell into the mature pollen. This parental contribution of Pols I, II, and III is sufficient to allow pollen germination upon landing on the stigma of a receptive female floral organ. There is also just enough parental Pol I, II, or III to allow for the growth of short pollen tubes and fertilization of the nearest (wild-type) ovules but not ovules deeper in the flower. In this way, some paternal transmission of mutant alleles to the next generation can take place, albeit at a low frequency (Onodera et al. 2008).

In contrast to null alleles for essential Pol I, II, or III subunits, null alleles of Pol IV or Pol V catalytic subunit genes (*nrpd1*, *nrpe1*, or *nrp(d/e)2*) are both male and female transmissible and homozygous mutants are viable and fertile. In *Arabidopsis thaliana*, the developmental consequences of being a Pol IV or Pol V mutant are subtle, at least under laboratory or greenhouse conditions; plants are normal in appearance but slower to flower, especially under short-day (long night) conditions. However, in maize, Pol IV null mutants display a number of developmental abnormalities, including altered cell fate in vegetative organs, stem outgrowths,

and partial feminization of male floral organs, in addition to late flowering (Erhard et al. 2009; Parkinson et al. 2007). Collectively, these observations show that although Pols IV and V are nonessential for viability, they are nonetheless important for development. The differing severities of Pol IV mutant phenotypes in maize and *Arabidopsis* may be related to the different transposon contents of their genomes, with ~85 % of the maize genome composed of transposons, as opposed to ~15 % in *Arabidopsis*. Failure to tame transposons through Pol IV- and Pol V-mediated transcriptional silencing may lead to misregulation of adjacent genes.

13.3 Roles of Pols IV and V in RNA Silencing

In a wide variety of eukaryotes, small RNAs known as short-interfering RNA (siRNAs) or piRNAs direct the transcriptional silencing of homologous genes by bringing about DNA methylation and/or repressive histone modifications (Ishizu et al. 2012; Chen 2012; He et al. 2011; Law and Jacobsen 2010). In plants, this transcriptional silencing function is carried out by a specific class of siRNAs that are 24 nt in length and produced by DCL3, one of four Dicer endonucleases encoded by the *Arabidopsis thaliana* genome. The 24 nt siRNAs guide DNA methylation and heterochromatin formation to homologous loci, mostly retrotransposons or other repetitive nuclear elements, resulting in transcriptional silencing if promoters are methylated. This process, known as RdDM (Matzke et al. 2009; Zhang and Zhu 2011), is the process for which Pol IV and Pol V functions are best understood (Haag and Pikaard 2011). An abbreviated version of the RdDM process, emphasizing the steps involving Pols IV and V, is shown in Fig. 13.3. More complicated representations of the pathway involving additional activities can be found in recent reviews (Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011).

RdDM has two major phases: the first involved in the biogenesis of 24 nt siRNAs and the second encompassing siRNA-programmed chromatin modifications. Pol IV is required in the first phase, and Pol V is critical for the second phase (Fig. 13.3). Mutations that knock out the catalytic subunits of Pol IV (*nrpd1* or *nrp(d/e)2*) essentially eliminate 24 nt siRNAs, as do mutations eliminating RDR2, one of six RNA-dependent RNA polymerases in *Arabidopsis*. These data provided early genetic evidence that Pol IV and RDR2 collaborate in the production of double-stranded RNA (dsRNA) precursors. Immunolocalization experiments showed that Pol IV colocalizes with repetitive loci that give rise to abundant 24 nt siRNAs and that RDR2 becomes mislocalized in *pol IV* mutants; in contrast, Pol IV localization is unaffected in *rdr2* mutants (Pontes et al. 2006). These observations suggested that Pol IV acts upstream of RDR2. More recently, affinity-purified Pol IV complexes analyzed by mass spectrometry were found to contain RDR2, with reciprocal immunoprecipitation experiments confirming that Pol IV and RDR2 associate in vivo (Haag et al. 2012; Law et al. 2011). The Pol IV–RDR2 interaction is dispensable for Pol IV transcription in vitro but appears to be required for RDR2

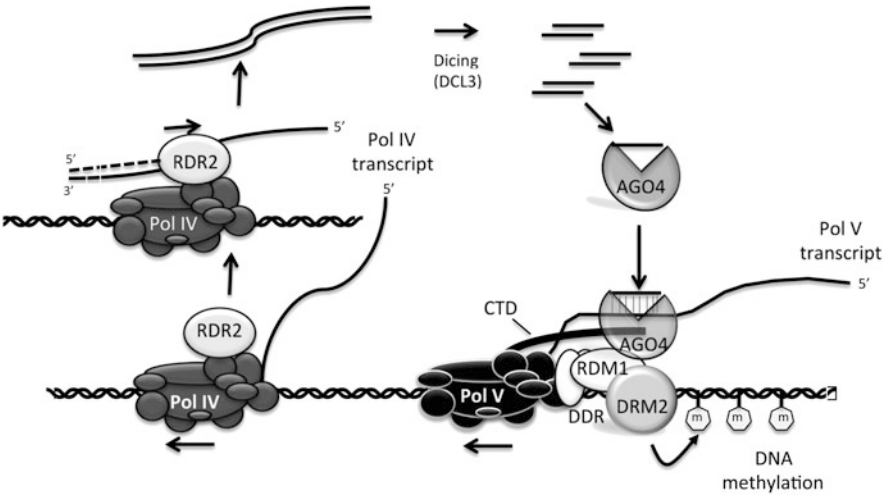

Fig. 13.3 Roles of Pols IV and V in RNA-directed DNA methylation in *Arabidopsis*. The diagram shows a simplified version of the pathway, focused on the roles of Pols IV and V. Double-stranded RNAs made by the Pol IV–RDR2 enzyme complex are diced by DCL3 and loaded into ARGONAUTE 4 (AGO4). The AGO4–siRNA complex is then recruited to target sites through binding to Pol V transcripts, and by physical interactions between AGO4 and the CTD of the Pol V largest subunit. The DDR complex assists in Pol V transcription and may also mediate interactions between AGO4 and the de novo DNA methyltransferase, DRM2

activity (Haag et al. 2012). This Pol IV-dependence of RDR2 activity suggests a mechanism in which Pol IV and RDR2 activities are coupled, with single-stranded Pol IV transcripts handed off to RDR2 to be used as templates, thus producing and channeling dsRNAs into the RdDM pathway (Fig. 13.3).

dsRNA products produced via Pol IV and RDR2 are diced by DCL3, and resulting 24 nt single-stranded siRNAs are loaded into the Argonaute family protein, AGO4, or its closest relatives, AGO6 or AGO9 (Zheng et al. 2007; Zilberman et al. 2003; Qi et al. 2006; Havecker et al. 2010). dsRNA synthesis, dicing, and AGO loading may be spatially coordinated given that RDR2, DCL3, and AGO4 partially colocalize, along with siRNAs and Pol V, within a nucleolus-associated Cajal body (Li et al. 2006; Pontes et al. 2006). Pol V has been shown to physically interact with AGO4 via WG or GW amino acid motifs (known as "AGO hooks") present in the Pol V CTD (see Fig. 13.1a) (El-Shami et al. 2007). Taken together, these observations suggested that Pol V might be guided to its sites of action by associating with AGO4–siRNA complexes within the Cajal bodies and then using the siRNAs to guide the complex to complementary target sites through base-pairing interactions (Li et al. 2006; Pontes et al. 2006). However, subsequent studies have argued against this hypothesis by showing that Pol V transcription at target loci is unaffected in *pol IV*, *rdr2*, or *dcl3* mutants defective for 24 nt siRNA biogenesis and is also unaffected in *ago4* mutants (Wierzbicki et al. 2008). Instead, siRNA binding to Pol V transcripts appears to be what recruits AGO4 to target loci.

Key evidence is that AGO4 can be chemically cross-linked to Pol V transcripts and that AGO4 associates with target loci (as shown using chromatin immunoprecipitation, or ChIP) in a Pol V-dependent manner (Wierzbicki et al. 2009). Moreover, AGO4's association with chromatin is abrogated in Pol V mutants bearing point mutations in the NRPE1 active site (Wierzbicki et al. 2009). These point mutations abolish Pol V transcriptional activity in vitro (Haag et al. 2012) and all known biological functions attributable to Pol V in vivo (Haag et al. 2009). Collectively, these findings indicate that AGO4 is primarily recruited to Pol V-transcribed loci via siRNA-mediated interactions with Pol V transcripts. AGO4 interactions with the Pol V CTD may further stabilize the complex or mediate downstream events (El-Shami et al. 2007). The functional significance of the Cajal bodies containing RDR2, DCL3, AGO4, siRNAs, and Pol V is not clear, but roles in RNA processing, chromatin modification, transport, or storage are possibilities (Li et al. 2008; Pontes and Pikaard 2008).

Several activities identified in genetic screens for disrupted RdDM are important for Pol V activity. Among these are DRD1, a putative SWI2/SNF2-family chromatin remodeling ATPase (Kanno et al. 2004); DMS3, a protein related to the hinge domains of cohesins and condensins (Kanno et al. 2008); and RDM1, a protein that binds methylated single-stranded DNA in vitro (Gao et al. 2010). Wierzbicki et al. showed that DRD1 and DMS3 are required for the production of Pol V transcripts and for stable Pol V association with chromatin (Wierzbicki et al. 2008, 2009). Subsequent studies showed that DRD1 and DMS3 associate with RDM1 to form a so-called DDR complex, named for the first initials of the three proteins (Law et al. 2010). Mass spectrometric analyses also identified Pol V subunits in affinity-purified DDR samples, suggesting that DDR and Pol V can physically associate (Law et al. 2010). The RDM1 protein of the DDR complex has also been shown to colocalize with AGO4 and the de novo DNA methyltransferase DRM2 (Gao et al. 2010), suggesting that RDM1 may serve as a bridge for recruitment of DRM2 to AGO4-siRNA- and Pol V-engaged loci (Zhang and Zhu 2011).

An SPT5-LIKE, KOW-domain transcription factor, SPT5L/KTF1, is involved in the downstream phase of the RdDM pathway, such that *ktf1*/*spt5l* mutants display a reduction in DNA methylation and reduced levels of siRNAs, similar to the effects of mutating the Pol V-specific subunit, NRPE5 (Bies-Etheve et al. 2009; Huang et al. 2009; Ream et al. 2009). In yeast and humans, Spt5 interacts with Spt4 to form the DSIF elongation factor complex, which is involved in mRNA capping, Pol II elongation, and prevention of premature transcription termination (Yamaguchi et al. 2013). One might expect SPT5L/KTF1 to similarly act as a Pol V elongation factor, consistent with the identification of SPT5L/KTF1 peptides in affinity-purified Pol V (Huang et al. 2009). However, Pol V transcripts are not decreased in abundance in a *spt5l*/*ktf1* loss-of-function mutant, but actually increase slightly (He et al. 2009c). A similar increase in Pol V transcript abundance is observed in *ago4* mutants (Wierzbicki et al. 2009) and SPT5L/KTF1 and AGO4 interact, via WG/GW "AGO-hook" motifs within SPT5L/KTF1 (He et al. 2009c; Bies-Etheve et al. 2009). These and other data have led to an alternative hypothesis that SPT5L/

KTF1 acts downstream of Pol V transcription possibly by helping AGO4 associate with Pol V transcripts or associated chromatin (Rowley et al. 2011). The increased abundance of Pol V transcripts observed in *ago4* and *ktf1* mutants may be a consequence of decreased slicing of Pol V transcripts by AGO4.

13.4 Subunit Compositions of *Arabidopsis* RNA Polymerases II, IV, and V

13.4.1 Subunits Common to Pols II, IV, and V

Affinity purification of *Arabidopsis* Pols II, IV, and V, followed by trypsin digestion and analysis of their peptides by LC-MS/MS mass spectrometry, revealed that Pols IV and V are composed of twelve subunits that are identical or homologous to the 12 core subunits of Pol II (Ream et al. 2009). Seven of the twelve subunits present in *Arabidopsis* Pols II, IV, and V are encoded by the same genes: the subunits homologous to yeast Rpb3, Rpb6, Rpb8, Rpb9, Rpb10, Rpb11, and Rpb12.

For all of the Pol II, IV, or V common subunits, except the 11th subunit (NRP(B/D/E)11), there are two genes in *Arabidopsis*. Mass spec analyses have revealed that the different variants can often be detected within the purified polymerases, sometimes equally and sometimes with only one variant being the major form (Ream et al. 2009; Law et al. 2011). Due to the high degree of similarity among most of these alternative subunits (~90 %, or higher, identity), one might expect the proteins to be functionally equivalent. However, a recent analysis of single or double mutants disrupting the two alternative forms of the ninth subunit [NRP(B/D/E)9a and NRP(B/D/E)9b] that are utilized by Pols II, IV, or V has forced a reconsideration of this assumption. This study revealed that the alternative ninth subunit proteins (and/or their genes) are only partially redundant with respect to Pol II functions and are nonredundant for Pol V-specific functions despite being 92 % identical (Tan et al. 2012). These data suggest that different Pol II, IV, and V subtypes exist in plants, resulting from their assembly using alternative subunits in various permutations. Consistent with this hypothesis, maize has three genes potentially encoding alternative NRP(D/E)2 subunits for Pol IV and/or V. These genes are not completely redundant, given that mutations in only one of the three genes disrupt the inter-allelic gene silencing phenomenon known as paramutation (Stonaker et al. 2009; Sidorenko et al. 2009). However, the developmental phenotypes of these mutants are not as severe as for mutants defective for the Pol IV largest subunit, NRPD1. Given that largest and second-largest subunits are equally important for forming the polymerase catalytic center, these observations suggest that there must be partial redundancy among the three second subunit variants with respect to functions affecting development (Pikaard and Tucker 2009).

13.4.2 Pol V Makes Use of a Distinct 5th Subunit

Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12 are subunits common to Pols I, II, and III in yeast and humans; thus one might expect these subunits to be common to Pols I through V in *Arabidopsis*, as well. This is mostly true (as discussed above), but not entirely—although Pol IV uses the same fifth subunit as Pols I, II, and III, Pol V makes use of a distinct fifth subunit encoded by the *NRPE5* gene (Lahmy et al. 2009; Ream et al. 2009; Huang et al. 2009). Three additional Rpb5-like genes are also present in the *Arabidopsis* genome, at least one of which is used as an alternative minor subunit of Pol IV (Law et al. 2011). NRPE5 is distinguished from other eukaryotic Rpb5 subunits by the presence of an N-terminal extension, required for the protein's stability in vivo, and by divergence in its C-terminal assembly domain (Lahmy et al. 2009; Ream et al. 2009). In *nrpe5* mutants, normally silenced elements (such as retrotransposons) are derepressed as in *nrpe1* mutants, indicating that NRPE5 and the catalytic subunits are equally required for Pol V-dependent gene silencing (Huang et al. 2009; Lahmy et al. 2009; Ream et al. 2009; Douet et al. 2009). However, DNA methylation and siRNA abundance are less affected in *nrpe5* than *nrpe1* mutants (Ream et al. 2009; Lahmy et al. 2009), which is also the case for *nrp(d/e)4* mutants (He et al. 2009a). These results suggest that non-catalytic subunits of Pols IV and/or V may play roles in RNA silencing that are separable from the RNA synthesis function(s) of the catalytic subunits.

13.4.3 Arabidopsis *Pols II, IV, and V Have Unique Subunit 4/7 Sub-complexes*

In addition to their second-largest subunit, NRP(D/E)2, which is encoded by the same gene, Pol IV and Pol V have in common their fourth subunit, NRP(D/E)4, which is distinct from the NRPB4 subunit of Pol II (Ream et al. 2009). NRPD4 does not rescue a yeast *rpb4* mutation, whereas NRPB4 does (He et al. 2009a). Rpb4 and Rpb7 subunits interact, forming a dissociable sub-complex that in yeast has been shown to interact with the RNA transcripts of Pol II and regulate RNA processing, trafficking, and even translation (Choder 2004; Ujvari and Luse 2006; Runner et al. 2008; Harel-Sharvit et al. 2010). Interestingly, Pol II, Pol IV, and Pol V utilize distinct proteins as their major NRPB7, NPRD7, and NRPE7 subunits in *Arabidopsis* (Ream et al. 2009), with NRPE7 also serving as a minor form of the seventh subunit in Pol IV (Ream et al. 2009; Law et al. 2011). The *NRPD7* and *NRPE7* genes lack introns, suggesting that retrotransposition of an NRPB7 cDNA was the duplication event early in Pol IV/V evolution that gave rise to their seventh subunit genes (Tucker et al. 2011). The functional significance of having unique subunit-4/7 sub-complexes in Pols II, IV, and V is unclear but is likely to be important.

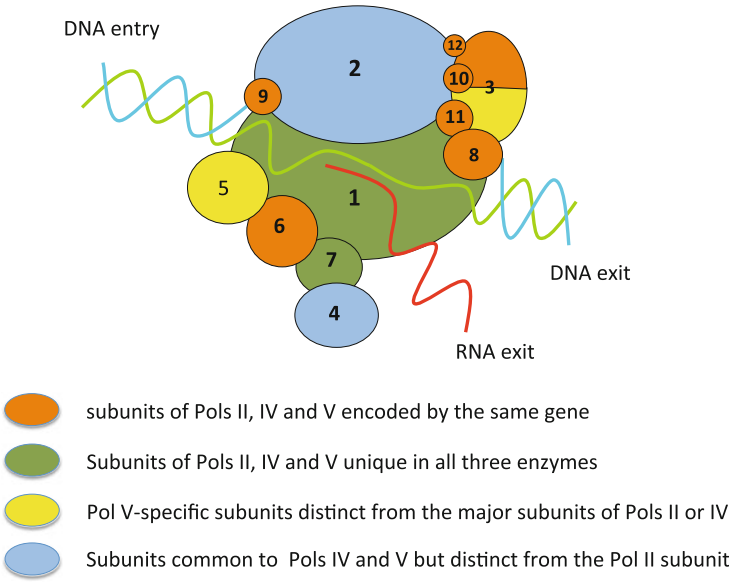

Fig. 13.4 Shared and unique subunits of *Arabidopsis* RNA polymerases II, IV, and V

To summarize, Pols II, IV, and V differ from one another in a subset of their subunits (Fig. 13.4). Pol IV differs from Pol II in four subunits (NRPD1, NRP(D/E)2, NRP(D/E)4, and NRPD7). Pol V differs from Pol II in five subunits (NRPE1, NRP(D/E)2, NRP(D/E)4, NRPE5, and NRPD7) and also makes nearly equal use of two alternative forms of the 3rd subunit, only one of which is primarily used by Pol II (or Pol IV). Pols IV and V differ in subunits 1, 5, and 7, and in the frequency of their use of the two alternative third subunits, with the major form of the Pol IV 3rd subunit being the same as for Pol II. Understanding how the different subunits contribute to the distinct functions of the three polymerases is a goal of ongoing research.

13.5 Pol V and Pol IV Largest Subunit CTD Functions Are Largely Unknown

As discussed previously, the Pol V largest subunit, NRPE1, has a long CTD that lacks the heptad repeats present in the Pol II CTD but has several unique domains, including a QS-rich domain, DeCL domain, and 10 imperfect tandem repeats of a 16 amino acid sequence (see Fig. 13.1a). Embedded within and adjacent to these 16 amino acid repeats are 18 WG/GW AGO-hook motifs implicated in interactions with AGO4 (El-Shami et al. 2007; He et al. 2009c). To date, this is the only function ascribed to the Pol V CTD. Unlike the Pol II largest subunit CTD, whose variable phosphorylation patterns confer regulatory meaning, nothing is known about

potential posttranslation modifications of the NRPD1 or NRPE1 CTDs or their regulatory significance.

13.6 Rules Governing Locus-Specific Transcription by Pols IV and V Are Unknown

It is not clear how Pols IV or V are recruited to the thousands of loci where Pol IV-dependent siRNAs are generated or where RdDM occurs. Recent studies identified genomic sites of Pol V occupancy using ChIP-seq (Wierzbicki et al. 2012; Zhong et al. 2012), but motif finding algorithms failed to identify specific DNA sequences, such as putative or known promoter elements or transcription factor binding sites, that could explain the occurrence of Pol V in these regions. Pol IV ChIP-seq studies have not yet been reported nor have Pol IV primary transcripts been identified, but sites of 24 nt siRNA production are signatures of Pol IV (see Fig. 13.3), and motif finding at these siRNA loci has also failed to identify consensus sequences thus far.

Understanding more about the DDR complex may tell us more about Pol V recruitment or transcriptional regulation. In mutants such as *drd1* or *dms3* that disrupt the DDR complex, Pol V does not stably associate with chromatin, as shown by ChIP, and Pol V transcripts are no longer produced (Wierzbicki et al. 2008, 2009; Zhong et al. 2012). Whether the DDR complex plays a role in Pol V recruitment and initiation, or Pol V elongation and processivity is not yet known, but ChIP analyses of the DDR complex may reveal whether DDR localizes at specific subregions within Pol V-associated loci, perhaps revealing potential start sites.

Only two proteins with a known connection to Pol II regulatory mechanisms have emerged thus far in genetic screens for defects in RdDM. These are the SPT5-like protein, KTF1 discussed previously, and an *Arabidopsis* homolog of yeast IWR1, a protein which facilitates the nuclear import of assembled Pol II from the cytoplasm to the nucleus (Czeko et al. 2011), that was identified in two independent genetic screens (Kanno et al. 2010; He et al. 2009b). Mutant plants (*dms4*/*rdm4*) defective for the IWR1 homolog display pleiotropic phenotypic abnormalities in addition to disrupted RdDM, consistent with impaired functions of Pol II in addition to Pols IV and V.

13.7 RNAs Synthesized by Pols IV and V In Vivo

To date, no RNA corresponding to a primary transcript of Pol IV has been cloned and sequenced or detected in vivo by RT-PCR or other molecular assays. Most likely, this is due to the fleeting existence of these transcripts before being

converted into dsRNA through the coupling of Pol IV and RDR2 activities, followed by dicing into siRNAs. As a result, nothing is known about Pol IV transcription start sites, termination sites (or regions), or potential transcript modifications, such as the possible addition of a 7-methylguanosine cap on the 5′ end. However, preliminary studies suggest that Pol V transcripts are not polyadenylated.

Unlike Pol IV transcripts, Pol V transcripts have been identified in vivo. Wierzbicki et al. examined genomic regions where no gene models existed and no significant transcription had been detected using DNA microarrays, yet where deep sequencing identified small RNAs that must have had precursors (Wierzbicki et al. 2008). This approach led to the identification of multiple loci where low-abundance transcripts detectable by RT-PCR are absent in *nrpe1* or *nrp(d/e)2* mutants, yet still detectable in *nrpd1* (Pol IV) mutants (Wierzbicki et al. 2008). ChIP analyses using anti-NRPE1 antibodies showed that Pol V physically associates with these loci. For several loci examined, transcripts appeared to initiate at multiple sites, based on 5′-RACE, were enriched among poly-A$^-$ RNA and had either 5′ caps or triphosphates—characteristic of newly initiated RNAs, as opposed to processed RNAs (Wierzbicki et al. 2008). Production of Pol V-dependent transcripts in vivo also requires the conserved aspartates of the NRPE1 Metal A site (see Fig. 13.1c), and these transcripts can be chemically cross-linked to NRPE1, allowing their immunoprecipitation using anti-NRPE1 antibodies. Collectively, these assays strongly suggested that these RNAs are Pol V transcripts.

13.8 Pol IV and Pol V Transcription In Vitro

RNA polymerase activity assays typically make use of sheared genomic DNA, or double-stranded DNA having 3′ overhangs, as a source of templates for promoter-independent transcription. Interestingly, Pols IV and V show no activity in such assays, unlike Pols I, II, or III (Onodera et al. 2005), thwarting early attempts by several laboratories, including ours, to detect Pol IV or Pol V biochemical activities. These negative results suggested that Pols IV and V might transcribe unconventional templates, or might even lack RNA polymerase activity altogether, consistent with the divergence, or absence, of more than 160 amino acids that are invariant in the catalytic subunits of Pols I, II, or III (Haag et al. 2009; Landick 2009). These substituted amino acids are clustered in the vicinity of the Metal A and Metal B sites of the catalytic center; in the bridge helix, trigger loop, cleft, and funnel domains of the largest subunits; and within the hybrid-binding domain of NRPD2 (Fig. 13.5; see also Fig. 13.1c, d). A number of these amino acids correspond to positions that interact with the incoming nucleotide triphosphate and the 3′ end of the growing RNA chain and are thus thought to be critical for catalysis. Among these are amino acids at the tip of the trigger loop and in the bridge helix that are not simply substituted but missing altogether in Pols IV and V (Landick 2009).

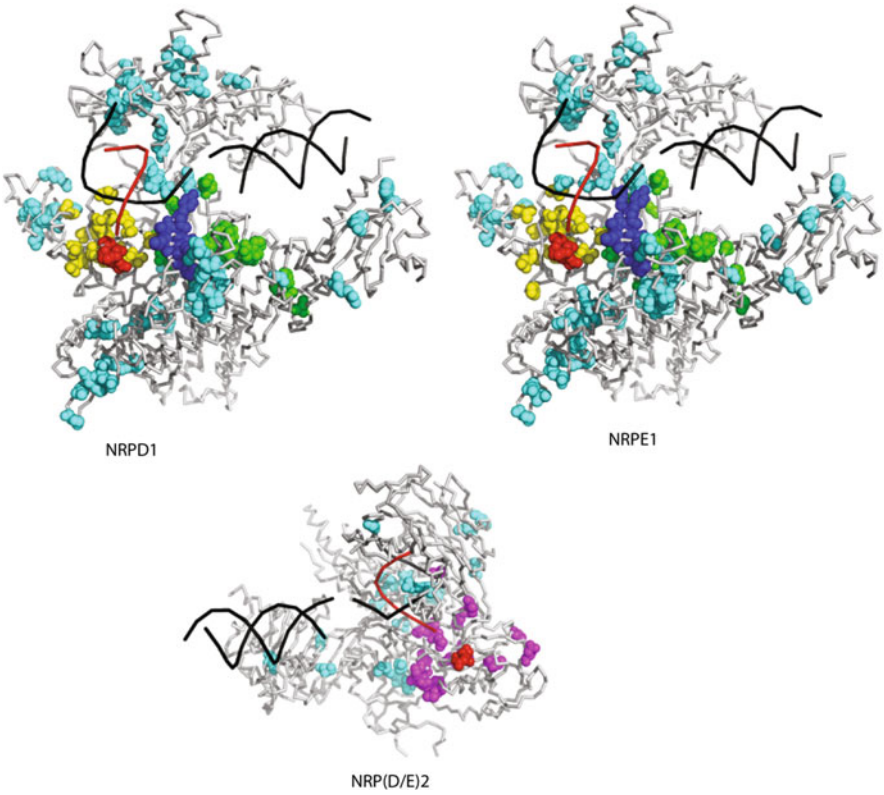

Fig. 13.5 Sequence divergence in Pols IV and V at amino acid positions that are invariant in Pols I, II, and III. The three images show these substituted positions as space-filling spheres mapped onto the Rpb1 and Rbp2 subunit structures of a yeast Pol II elongation complex determined in the Kornberg laboratory (Protein Data Bank structure 1R9T). The dsDNA substrate is shown in *black* and the RNA product in *red*. Amino acids colored *red mark* the positions of the invariant Metal A and Metal B sites in the largest and second-largest subunits, respectively. Substituted amino acids in the cleft, bridge helix, and active site domains of the largest subunits are colored *green*, *blue*, and *yellow*, respectively. Substituted amino acids in the hybrid-binding domain of the second-largest subunit are colored *magenta*. Substituted amino acids in the largest and second-largest subunits that are located outside of these domains are colored *cyan*. These images are reprinted from the open access article by Haag et al. (2009), as permitted under the Creative Commons Attribution License

Despite their lack of activity using conventional DNA templates, and their divergent amino acid sequences, *Arabidopsis* Pols IV and V are indeed functional RNA polymerases in vitro. Using templates in which an RNA primer is annealed to a DNA template oligonucleotide, thus forming an 8 bp RNA–DNA hybrid, Pols IV and V will elongate the RNA in a templated fashion (Haag et al. 2012), as shown previously for Pols I and II (Kuhn et al. 2007; Lehmann et al. 2007). Unlike Pol II, Pols IV and V are insensitive to the fungal toxin, alpha-amanitin, consistent with the divergence of multiple amino acids within the alpha-amanitin binding pocket of

Pol II (Haag et al. 2012). If double-stranded DNA is present downstream of the template DNA–RNA hybrid region, Pols II and IV are able to generate transcripts, but Pol V cannot, suggesting that Pol V cannot displace a non-template strand during transcription (Haag et al. 2012).

Our inability thus far to coax affinity-purified Pols IV and V to initiate transcription on DNA templates in the absence of an RNA primer may simply be due to the absence of unidentified helper proteins that do not copurify with the polymerases. However, cytological experiments have shown that Pols IV and V become mislocalized in RNase A-treated nuclei, unlike Pol II (Pontes et al. 2006), consistent with some form of RNA involvement, possibly as a template or primer.

13.9 Missing Pieces

Pols IV and V are remarkable enzymes given their evolution as specialized forms of Pol II but their substantial divergence at amino acid positions that are otherwise invariant among Pols I, II, and III. Approximately 120 of these normally invariant amino acids are substituted, or missing, in the largest subunits of *Arabidopsis* Pols IV and V, and another 40 are altered in the second-largest subunit (see Fig. 13.5). Moreover, comparison of the largest subunit sequences of Pols IV and V in a variety of plants reveals that they are evolving rapidly, with amino acid substitution rates occurring at 20 times the rate observed for the largest subunits of Pols I, II, or III (Luo and Hall 2007). Collectively, these observations indicate that there are fewer functional constraints on the evolution of Pols IV and V than for other polymerases.

DNA-dependent RNA polymerases perform a variety of functions in addition to RNA synthesis. These include pausing at specific sequences, backtracking along the template, RNA cleavage, and proofreading. These activities allow a polymerase to overcome barriers in the template or correct misincorporated nucleotides (Sydow and Cramer 2009). It is unknown if Pols IV and V possess any of these activities in vivo or in vitro. Given their involvement in noncoding RNA-mediated silencing processes, perhaps Pols IV and V do not need to be high-fidelity enzymes with proofreading or backtracking capabilities. In fact, error-prone transcription might actually be advantageous in the production of siRNAs that participate in the silencing of large transposon families, whose sequences are similar, but not identical. Perhaps by making use of RNA primers derived from Pol II (or other polymerase) transcripts, the catalytic centers of Pols IV and V have evolved to accommodate binding to DNA–RNA hybrid templates and only need to elongate RNAs rather than initiate their synthesis de novo from nucleoside triphosphates. This might also remove constraints on the evolution of the Pol IV and Pol V catalytic centers. Likewise, the possibility that Pols IV and V recognize methylated DNA, or chromatin templates bearing specific histone modifications, may contribute to their different amino acid sequences compared to Pol II. Such speculations should be possible to test upon further development of in vitro assays.

In vivo, identification of full-length Pol V or Pol IV transcripts through strand-specific RNA deep sequencing in appropriate wild-type and mutant backgrounds (coupled with comparative bioinformatics analyses) has not yet been accomplished. However, this basic information is critically important. Knowing where Pol IV and Pol V transcripts begin and end will define their transcription units and allow for more focused analyses of potential consensus motifs in the vicinity of these transcription units, possibly revealing promoters or other regulatory elements that can be tested. Moreover, knowing if Pol IV or Pol V transcripts are co- or post-transcriptionally modified or processed may reveal insights into their channeling, trafficking, and stability.

Understanding the functions of subunits that differ between Pols II, IV, and V is also likely to yield important new insights. Evidence that multiple structurally distinct subtypes of the different polymerases exist, based on alternative choices for many of their subunits, is intriguing. By exploiting the non-lethality of Pol IV- or Pol V-specific subunit mutations, the roles of different subunits in DNA methylation and chromatin modification in vivo, and transcription in vitro, can be explored, potentially revealing new insights, particularly into the functions of non-catalytic subunits. Last but not least, understanding the functions of the unique CTDs of the Pol IV and Pol V largest subunits should also be illuminating, potentially revealing mechanisms that couple transcription with noncoding RNA processing and chromatin modification.

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Chapter 14
Structure, Dynamics, and Fidelity of RNA-Dependent RNA Polymerases

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Abstract The RNA-dependent RNA polymerase (RdRp) is responsible for replicating the genomes of RNA viruses. The overall structure and function of RdRps is similar to other nucleic acid polymerases, although some RdRps employ unique initiation mechanisms. Recent biophysical studies indicate that the internal motions of RdRps, and other nucleic acid polymerases, are critical for their catalytic function and fidelity. In particular, these studies have suggested that the closing of the active site in preparation for catalysis involves the movement of the motif-D loop to help reposition a highly conserved lysine, enabling this residue to act as a general acid to protonate the pyrophosphate leaving group. Binding of incorrect nucleoside triphosphate does not induce the same structural changes in the motif-D loop, indicating a role for this loop in nucleotide discrimination. Indeed, substitution at the motif-D lysine increases polymerase fidelity and, intriguingly, decreases viral pathogenesis. The highly conserved nature of this lysine thus suggests a universal mechanism for rational vaccine design based on generating variants at this position. Moreover, substitutions elsewhere in the RdRp structure, including those remote from the active site, likewise lead to changes in polymerase fidelity and decrease viral pathogenesis. In these cases, the amino acid substitutions alter internal protein motions (including those in the motif-D loop) without substantially affecting the polymerase structure. A picture emerges in which RdRps and other nucleic acid polymerases can be viewed as "small world" networks of amino acids; communication pathways connect from the surface of the protein all the way to the catalytic center. These networks can be impacted by amino acid substitutions, inhibitor binding, and/or binding of accessory replication proteins to regulate RdRp catalysis and fidelity.

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Abbreviations

CRE	Cis-acting element
DCCM	Dynamic cross correlation map
ds	Double stranded
FMDV	Foot-and-mouth disease virus
HCV	Hepatitis C virus
MD	Molecular dynamics
NMR	Nuclear magnetic resonance
NTP	Nucleoside triphosphate
NV	Norovirus
PCA	Principal component analysis
PV	Poliovirus
RdRp	RNA-dependent RNA polymerase
RT	Reverse transcriptase
SDKIE	Solvent deuterium kinetic isotope effect
ss	Single stranded
VPg	Virion protein genome linked
WNV	West Nile virus
WT	Wild-type

14.1 Introduction

The RNA-dependent RNA polymerase (RdRp) is responsible for the replication of viruses with RNA genomes. These viruses include some of the best known and most costly to human health and productivity, including rhinovirus (Gavala et al. 2011) and the influenza virus (Barik 2012) that are responsible for the common cold and "flu," respectively; hepatitis C virus (HCV) (Miller et al. 2012) that is a major cause of liver disease; and poliovirus (PV) (Aylward and Tangermann 2011) in which a decade-spanning effort has been directed towards its global eradication. The fast rates of replication, large virus yields, and high genetic diversity of these viruses make therapeutic interventions against these "moving targets" difficult. The catalytic efficiency and fidelity of their RdRps contribute to some extent to all of these factors.

Although RdRps have similar levels of nucleotide incorporation fidelity as the catalytic cores of other nucleic acid polymerases, their lack of a proofreading exonuclease activity leads to a higher mutation rate, approaching two nucleotide changes per RNA genome (Castro et al. 2005). Antiviral therapies may be compromised by these high mutation rates through resistant variants that arise

from this vast genetic reservoir. The intrinsic genetic variation among these viruses is also critical for viral pathogenesis; it has been observed that viruses that express RdRp variants that are more faithful are less pathogenic (Pfeiffer and Kirkegaard 2003; Vignuzzi et al. 2006). Genetic diversity within the RNA virus population may allow these viruses to escape bottlenecks and defenses of the host that would otherwise force the virus into extinction.

Antiviral therapies can take advantage of the RdRp's lack of proofreading mechanism. For instance, the most successful drug combination against HCV involves the synthetic nucleotide analogue ribavirin, which becomes directly incorporated into viral RNA. Ribavirin can template equally well with cytidine and uracil, leading to increased mutation rates and the failure of the virus through "lethal mutagenesis" (Cameron and Castro 2001; Graci and Cameron 2002, 2008). Manipulating the fidelity of RdRps may also serve as critical starting points in the rational generation of new vaccine strains (Vignuzzi et al. 2006, 2008). Small animal studies have demonstrated that modification of the RdRp by changing its catalytic activity and/or nucleotide addition fidelity can lead to viral strains incapable of causing disease while providing immune protection to lethal dosages of "wild-type" (WT) virus (Van Slyke et al. 2012; Gnadig et al. 2012; Coffey et al. 2011; Coffey and Vignuzzi 2011; Vignuzzi et al. 2006, 2008; Pfeiffer and Kirkegaard 2003; Weeks et al. 2012). The importance of drug and vaccine developments based on knowledge of RdRps is further underscored by the finding that the virulence and pathogenicity of RNA viruses is directly linked to the function of their respective RdRps; increased virulence for influenza (Zhu et al. 2012; Leung et al. 2010) and West Nile virus (WNV) (Van Slyke et al. 2012) have been correlated to increased RdRp catalytic activity. Continued development of antiviral therapies will no doubt require a deeper understanding of RdRp catalytic function and fidelity.

An emerging theme in RdRp function, and nucleic acid polymerases in general, is the importance of internal protein motions for catalytic activity and regulation. As observed in other enzymes, internal motions may not be random, but rather they may have evolved to direct ligand binding, substrate selection, and chemical catalysis itself (Silva et al. 2011; Schramm 2011; Nashine et al. 2010; Villali and Kern 2010; Boehr et al. 2006a, b, 2009). With RdRps and other nucleic acid polymerases, it has long been recognized that conformational changes before and after nucleotide addition are critical for their function; nucleotide discrimination and phosphodiester bond formation may also depend on faster, more localized fluctuations. Computer simulations and biophysical techniques, such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and single-molecule fluorescence (e.g., Moustafa et al. 2011; Yang et al. 2010; Seckler et al. 2009; Zhou et al. 2012), are just beginning to reveal the rich dynamic behavior of RdRps and other nucleic acid polymerases. Indeed, these techniques indicate that RdRps may be especially dynamic proteins. Thermodynamic melting data (Thompson et al. 2007) and NMR spectra (Yang and Boehr, unpublished results) are both consistent with PV RdRp behaving similar to a "molten globule," at least in their apo states. Such behavior would be consistent with the view that viral proteins possess unique biophysical characteristics that help them

to compensate for high mutation rates and give them the ability to adapt to host defenses (Tokuriki et al. 2009).

14.2 Fundamentals of RdRp Structure and Function

14.2.1 Overall Structural Architecture of RdRps

RdRps are structurally similar to other template-directed nucleic acid polymerases, adopting the typical "cupped right-hand" structure with fingers, thumb, and palm subdomains (Hansen et al. 1997; Ago et al. 1999; Bressanelli et al. 1999; Lesburg et al. 1999; Butcher et al. 2001; Ng et al. 2002, 2008; Tao et al. 2002; Ferrer-Orta et al. 2006a, b, 2007, 2009; Choi et al. 2004, 2006; Yap et al. 2007; Malet et al. 2007; Campagnola et al. 2008; Zamyatkin et al. 2009; Gong and Peersen 2010; Lee et al. 2011; Mosley et al. 2012; Lescar and Canard 2009; McDonald et al. 2009). One major difference between RdRps and other nucleic polymerases is the extension of the fingers through the "fingertips" that helps to more fully enclose the active site (Fig. 14.1a). The full closure of the active site likely restricts large-scale conformational changes between the fingers and thumb subdomains and may have consequences in terms of protein stability (Thompson et al. 2007), their high processivity (Arnold and Cameron 2000; Rodriguez-Wells et al. 2001), and other features of RdRp function.

The fingers and thumb subdomains are primarily responsible for RNA primer and template binding, whereas the palm domain contains residues important for positioning the primer 3′-end, divalent cations, templating, and incoming nucleotide. Recent crystal structures of RdRps have identified channels necessary for template and NTP access and for the egress of newly synthesized RNA (Gong and Peersen 2010; Ferrer-Orta et al. 2007).

Conserved structural motifs A–G are also crucial for polymerase function (Table 14.1). Four of these motifs (A–D) are located in the palm subdomain and are conserved with other nucleic acid polymerases (Poch et al. 1989; O'Reilly and Kao 1998). Metal ion binding is mediated through the absolutely conserved Asp residues in motifs A and C. Other residues within motifs A and B are important for nucleobase and sugar selection (Gohara et al. 2000, 2004; Korneeva and Cameron 2007). Motifs D and F contain residues important for positioning of the nucleoside triphosphate moiety and may be important for stabilization of the pentaphosphate transition state (Castro et al. 2009; Gong and Peersen 2010; Iglesias et al. 2011; Yang et al. 2012).

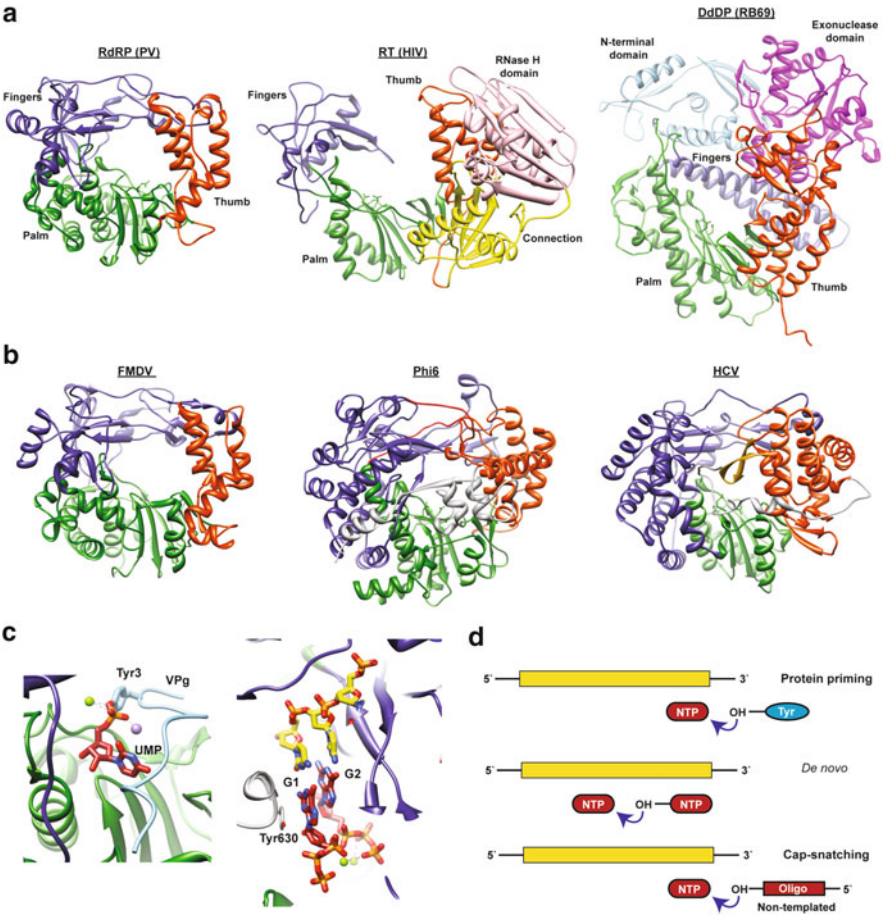

Fig. 14.1 Polymerase structures and initiation mechanisms. (**a**) Similarities and differences between the structures of nucleic acid polymerases. Structures of RdRp from PV (PDB code 1RA6) (Thompson and Peersen 2004), RT from HIV (PDB 1RTD) (Huang et al. 1998), and DdDp from RB69 (PDB 1IG9) (Franklin et al. 2001) are shown and labeled. The three polymerases revealed the canonical right-hand polymerase structure with the fingers (*blue*), palm (*green*), and thumb (*orange red*) subdomains. RdRps possess a unique feature compared to DdDps and RTs in that RdRp adopts an enclosed conformation caused by extensive interactions between the fingers and thumb subdomains, leading to a completely encircled active site. The structure of RB69 DdDp has two additional domains: the N-terminal domain (*cyan*) and the exonuclease domain (*purple*). The structure of HIV RT also has two additional domains: the RNase H domain (*pink*) and the connection domain (*yellow*), which connects the RNase H domain to the thumb. The metal-binding Asp residues are depicted as *sticks* to indicate the active site of the different polymerases. (**b–d**) RdRps from different RNA viruses employ different initiation mechanisms. (**b**) Crystal structures of RdRps from FMDV Picornaviridae (PDB 2F8E) (Ferrer-Orta et al. 2006a), bacteriophage Φ6 Cystoviridae (PDB 1HHS) (Butcher et al. 2001), and HCV Flaviviridae (PDB 1C2P) (Lesburg et al. 1999) are shown. The subdomains conserved among all polymerases are highlighted in different colors: fingers (*blue*), palm (*green*), and thumb (*orange red*). Φ6 RdRp and HCV RdRp have additional characteristic structural elements that are absent from FMDV RdRp. Φ6 RdRp has a C-terminal domain (*gray*) and two loops (*red*) protruding from the thumb interconnecting different parts of the fingers; HCV RdRp has C-terminal residues (*gray*) that

Table 14.1 Conserved sequence/structure motifs and their functional importance

Subdomain	Conserved structural/ functional regions	Sequence[a]	Function
Fingers	G	113 STSAGYPY 120	Template binding[b]
	F	153 PLVT**YVK**D**E**LRSKT**K**VEQ-GKS**R**LIE**A** 178	NTP and template binding
	I	107 LEALDL 112	Template binding
	II	184 SVAMRMAFGNLYAAFHK 200	Template binding
Palm	A	229 LFAF**D**YTGY**D**AS 240	2′-OH of NTP and metal-B binding
	B	293 TSIF**N**SMINNLIIRTLLLKT 312	Base of NTP and template binding
	C	323 MIAY**GDD**VIAS 333	Primer and metal-A binding and catalysis
	D	338 VDASLLAQSGK-DY**G**LTMTPAD**K**SAT 362	Triphosphate of NTP binding and catalysis
	E	363 FETVTWENVT**F**L**K**RF**F**RA 380	Nascent RNA 3′-end binding
Thumb	III	405 KDPRNTQDHVRSLCLL 420	Nascent RNA duplex binding

[a]The residues shown in double underlined and bold-faced type are completely conserved among RdRps; those residues representing signatures of the conserved sequence motifs are shown in black bold-faced type and single underlined. Residue number corresponds to PV RdRp

[b]The identification of residues' functions was based on the crystal structure of RdRp–RNA–NTP complex of FMDV RdRp (PDB 1WNE)

14.2.2 Structural Differences Among RdRps

RNA viruses can be divided into four basic classes: plus-sense single stranded (ss), minus-sense ss, double stranded (ds), and retroviruses; RdRps from these classes may differ structurally to accommodate the different replication challenges these

Fig. 14.1 (continued) constitute part of the polypeptide linking the polymerase to the membrane anchor and β-loop insertion (*gold*) within the thumb that protrudes into the polymerase active site. (**c**) The initiation complex of FMDV RdRP–VPg–UTP (PDB 2F8E), which employs the protein-priming initiation mechanism (*left panel*), and the initiation complex of Φ6 RdRp–DNA template–GTP (PDB 1HI0) (Butcher et al. 2001), which employs the de novo initiation mechanism (*right panel*), are shown. In the FMDV complex, the VPg primer protein (*cyan*) fits into the polymerase active site projecting Tyr3 (*cyan sticks*) that is covalently linked to the UMP molecule (*sticks* with *dark red* carbon atoms). In the Φ6 complex, two molecules of GTP substrate are bound, G1 and G2, and base paired to the thymine bases of the bound DNA oligomer (model of the preferred RNA template); Tyr630 stacks with the base of G1. The divalent catalytic ions are shown as *spheres*: magnesium (*green*) and manganese (*magenta*). (**d**) Schematic representations of the protein-priming and de novo initiation mechanisms shown in (**c**) and the cap-snatching initiation mechanism proposed for influenza polymerase

viruses face (Fig. 14.1b). Retroviral reverse transcriptases (RTs) are the subject of another chapter and will not be discussed in depth here, although it is important to point out the overall structural similarity between RdRps and RTs (Fig. 14.1a) and to note that mechanistic studies in one class are likely revealing for the other class of RNA-templated polymerases.

In plus-sense ssRNA viruses, the genomic RNA can be used directly as mRNA in infected cells to produce viral proteins. Only small amounts of minus-sense ssRNA are produced to guide the synthesis of multiple copies of plus-sense ssRNA (Uchil and Satchidanandam 2003; Choi 2012). The size of the thumb subdomain varies widely among RdRps from plus-sense ssRNA viruses and generally correlates with the polymerase initiation mechanism. RdRps from Flavivirus (also known as NS5B or NS5), including HCV and WNV, initiate replication de novo with the first two NTPs in sequence and generally have a larger thumb subdomain and/or additional structural elements that help to restrict access to the template-binding channel (Fig. 14.1b), only allowing ssRNA into the active site during de novo initiation (Ago et al. 1999; Bressanelli et al. 1999; Lesburg et al. 1999; Choi et al. 2004; Malet et al. 2007; Yap et al. 2007). These RdRps also have a GTP-binding site adjacent to the active site that helps to position the 3′-OH of the initiation NTP for nucleophilic attack by the second NTP (Choi et al. 2004; Ranjith-Kumar et al. 2002; Kao et al. 1999; Luo et al. 2000). Interestingly, widening the template-binding channel through removal of the C-terminal residues in HCV RdRp allows the use of a dsRNA template–primer complex for primer-dependent initiation (Hong et al. 2001).

In contrast, RdRps from Picornaviruses (also known as $3D^{pol}$), including those from PV (Hansen et al. 1997; Thompson and Peersen 2004; Thompson et al. 2007; Gong and Peersen 2010), foot-and-mouth disease virus (FMDV) (Ferrer-Orta et al. 2006a, 2007), and coxsackievirus (Campagnola et al. 2008), have smaller thumb subdomains, creating a larger template-binding channel that would accommodate the peptide primer necessary for initiation. These RdRps use a virally encoded 22 amino acid peptide called VPg (virion protein genome linked) to initiate replication. The cis-acting replication element (CRE) or ori-I is first used as a template for uridylation of VPg, and then the uridylated peptide primer is transferred to the 3′-end of the viral RNA template (Paul et al. 2000, 2003; Steil and Barton 2009; Ferrer-Orta et al. 2006a, 2009; Choi 2012) (Fig. 14.1c). RdRps from Caliciviruses use a similar protein-based initiation mechanism, but the details of the process are not well understood.

For minus-strand ssRNA viruses, no full crystal structure of any RdRp is currently available. This situation is unfortunate because minus-strand RNA viruses include some of the most prevalent and evasive viruses known, including influenza, measles and mumps viruses, rabies virus, and Ebola virus, and these structures could provide insight into some of the unique characteristics of RNA replication in these viruses. For instance, the functional influenza virus polymerase is a heterotrimer with one subunit containing endonuclease activity that is able to bind cellular mRNA cap and cleave it 10–15 nucleotides downstream from the 5′ end (Boivin et al. 2010). This action provides the RNA primer necessary for

initiation through a mechanism known as "cap snatching" (Choi 2012) (Fig. 14.1d). The interactions between the RdRp and endonuclease may be important for coordinating initiation of RNA transcription. A structural description of these interactions may provide new avenues for antiviral therapies.

RdRps from dsRNA viruses, including reovirus and rotavirus (Tao et al. 2002; McDonald et al. 2009) and bacteriophage Φ6 (Butcher et al. 2001; Salgado et al. 2004; Poranen et al. 2008), are structurally similar to those characterized from plus-strand ssRNA viruses. One interesting RdRp variant comes from birnavirus. Here, the palm domain motifs are permuted such that the canonical A-B-C amino- to carboxyl-terminal order is changed to C-A-B (Garriga et al. 2007). Structural rearrangements to accommodate this new order lead to the motif-B loop occluding the active site in the ligand-free enzyme. Binding of the accessory protein VP3 leads to conformational changes in the motif-B loop to open the active site, which then allows RdRp catalysis (Garriga et al. 2007).

Structural differences among RdRps are generally related to differences in RNA synthesis initiation. In contrast, RNA elongation is predicted to occur through the same general mechanisms. As such, the rest of the chapter will focus mainly on PV RdRp, which has established kinetic and catalytic mechanisms, and crystal structures provide snapshots of most of the structural intermediates along its nucleotide addition cycle.

14.2.3 The Kinetic Mechanism of PV RdRp Highlights Fidelity Checkpoints

The first full description of the kinetic mechanism for a RdRp (Arnold and Cameron 2004) was enabled by the use of a symmetrical primer–template RNA substrate ("sym/sub") with a six base pair duplex and four nucleotide 5′ overhangs (Arnold and Cameron 2000). The use of this RNA substrate overcame previous difficulties in assembling stable RdRp–primer/template complexes and enabled single nucleotide incorporation events to be monitored and kinetic parameters, k_{pol} (i.e., the maximal rate constant for nucleotide incorporation) and $K_{d,app}$ (i.e., the apparent dissociation constant for incoming NTP), to be derived. These original studies set the stage for a more mechanistic understanding of RdRp catalysis and fidelity on par with what was known for DNA polymerases. Similar RNA substrates have since been used to characterize other viral RdRps (e.g., Arias et al. 2008; Jin et al. 2011, 2012). Newer RdRp assays have also been devised that are more amenable to high-throughput screening efforts and have the potential to provide new lead compounds for antiviral therapies (Mestas et al. 2007; Gong et al. 2009; Campagnola et al. 2011).

Pre-steady state kinetic experiments have established five key intermediates in the RdRp nucleotide addition cycle (Fig. 14.2a) (Arnold and Cameron 2004). The kinetic mechanism begins with enzyme bound with primer/template RNA

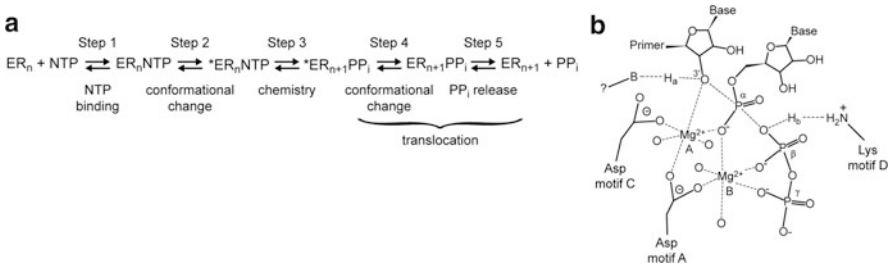

Fig. 14.2 Polymerase-catalyzed nucleotide incorporation. (**a**) Five-step kinetic mechanism for RdRp-catalyzed nucleotide incorporation. (**b**) Chemistry of nucleotidyl transfer. Initially, a nucleoside triphosphate bound by a divalent Mg^{2+} cation (metal B) enters the active site. Metal B is coordinated by the β- and γ-phosphates of the nucleotide, the conserved Asp residue located in structural motif A, and, likely, water molecules (indicated as oxygen ligands to metal B without specific designation). Metal B orients the triphosphate in the active site and contributes to charge neutralization during catalysis. Once the nucleotide is in place, a second divalent Mg^{2+} cation binds (metal A). Metal A is coordinated by the RNA primer 3′-OH, the α-phosphate of the nucleotide, and the Asp residues of structural motifs A and C. Metal A lowers the pK_a of the RNA primer terminus 3′-OH (denoted as H_a), facilitating nucleophilic attack on the nucleotide α-phosphorus atom. As the transition state of nucleotidyl transfer is approached (indicated by *dashed lines*), the primer 3′-hydroxyl proton, H_a, is transferred to a base, the identity of which is unknown, but that is hypothesized to be the conserved Asp residue in motif C. A conserved amino acid residue, a Lys, located in motif D of RdRps and RdDps, or helix O or helix P of DdDps or DdRps, respectively, serves as a general acid catalyst by donating a proton (denoted as H_b) to the pyrophosphate leaving group, which enhances the efficiency of nucleotidyl transfer (Castro et al. 2009)

(E:RNA); incoming NTP then binds to form a ternary complex (E:RNA:NTP) that induces a conformational change into a more active "closed" enzyme conformation (E*:RNA:NTP). Following phosphodiester bond formation (E*:RNA_{n+1}:PP_i), another conformational change occurs (E:RNA_{n+1}:PP_i) to prepare the enzyme for pyrophosphate release (E:RNA_{n+1}). Translocation along the RNA may be coupled to the post-chemistry conformational change and/or pyrophosphate release, before the cycle begins again. These kinetic steps are identical to those found for other nucleic acid polymerases (Joyce and Benkovic 2004).

In PV RdRp, both the pre-chemistry conformational change and the chemistry step itself are partially rate limiting when Mg^{2+} is used as the metal cofactor (Arnold and Cameron 2004). In the presence of Mn^{2+}, the chemistry step is fully rate determining, but the polymerase fidelity is substantially decreased (Arnold et al. 2004), presumably owing to the loss of the pre-chemistry fidelity checkpoint. The contributions of the pre-chemistry and chemistry steps to the overall rate can also be assessed through solvent deuterium kinetic isotope effects (SDKIE), given that the SDKIE can report on proton transfer steps during chemistry (see Fig. 14.2b). For PV RdRp, correct NMP incorporation (in the presence of Mg^{2+}) is associated with a significant SDKIE (~3) (Castro et al. 2007, 2009), consistent with the chemistry step making a substantial contribution to the overall rate. However, incorporation of NMP with incorrect nucleobase is associated with a much reduced SDKIE (~1.3) (Yang et al. 2012), indicating that the chemistry step is

making a smaller contribution to the overall rate and suggesting that the pre-chemistry conformational change is becoming more rate limiting. Misincorporation further slows down the pre-chemistry conformational change for subsequent nucleotide addition as evidenced by the ~10-fold decrease in k_{pol} and the complete loss of the SDKIE (~1.0) (Yang et al. 2012). Misincorporation is also known to affect subsequent nucleotide addition in DNA polymerases (Kunkel and Bebenek 2000; Johnson and Beese 2004). These studies serve to highlight the importance of the pre-chemistry conformational change as a fidelity checkpoint.

Triggering the conformational changes necessary for catalysis likely depends on recognition of both the correct nucleobase and correct sugar (Castro et al. 2005). Disruption of a key hydrogen bond in PV RdRp between the 2′-OH of the incoming ribose and the carboxamide functional group of Asn297 through mutagenesis not only decreases sugar discrimination but also leads to ~30-fold decrease in k_{pol} for correct NMP incorporation (Gohara et al. 2004). Recognition of the 3′-OH is also critical; disruption of the interactions between Asp238 and the 3′-OH results in >2,000-fold decrease in k_{pol} (Gohara et al. 2004).

14.2.4 Crystal Structures Provide Molecular Snapshots of the Conformational Changes Along the Nucleotide Addition Cycle

RdRps catalyze phosphodiester bond formation through the two-metal ion mechanism, as previously suggested for other nucleic acid polymerases (Fig. 14.2b). Crystal structures of FMDV, NV, and PV RdRps have begun to reveal the structural changes that accompany NMP incorporation (Ferrer-Orta et al. 2007; Zamyatkin et al. 2009; Gong and Peersen 2010). The Peersen lab has been able to monitor structural changes to PV RdRp before and after NMP incorporation (Gong and Peersen 2010). Nucleotide addition resulted in local conformational changes in the palm region involving motifs A–C (Fig. 14.3a). Changes included a realignment of the motif-A backbone to allow for the formation of a complete three-stranded β-sheet with motif C and a repositioning of Asp233 in motif A to allow it to coordinate both Mg^{2+} metals in the active site. Upon NTP binding, there is also a repositioning of the side chain of Asp238 that induces a conformational change in Ser288, such that both Asp238 and Ser288 can form H-bonds to the ribose hydroxyls. Importantly, no significant changes in these active-site residues were observed in the presence of the corresponding 2′-dNTPs, suggesting a mechanism for correct sugar selection. Similar structural rearrangements have been observed for FMDV and NV RdRps (Ferrer-Orta et al. 2007; Zamyatkin et al. 2009).

One key question to address is what structural rearrangement(s) are important for the fidelity checkpoints highlighted by the kinetic experiments. It is unlikely that the pre-chemistry conformational change is reporting on a global repositioning of the fingers and thumb subdomains, considering that the fingertip interactions with the

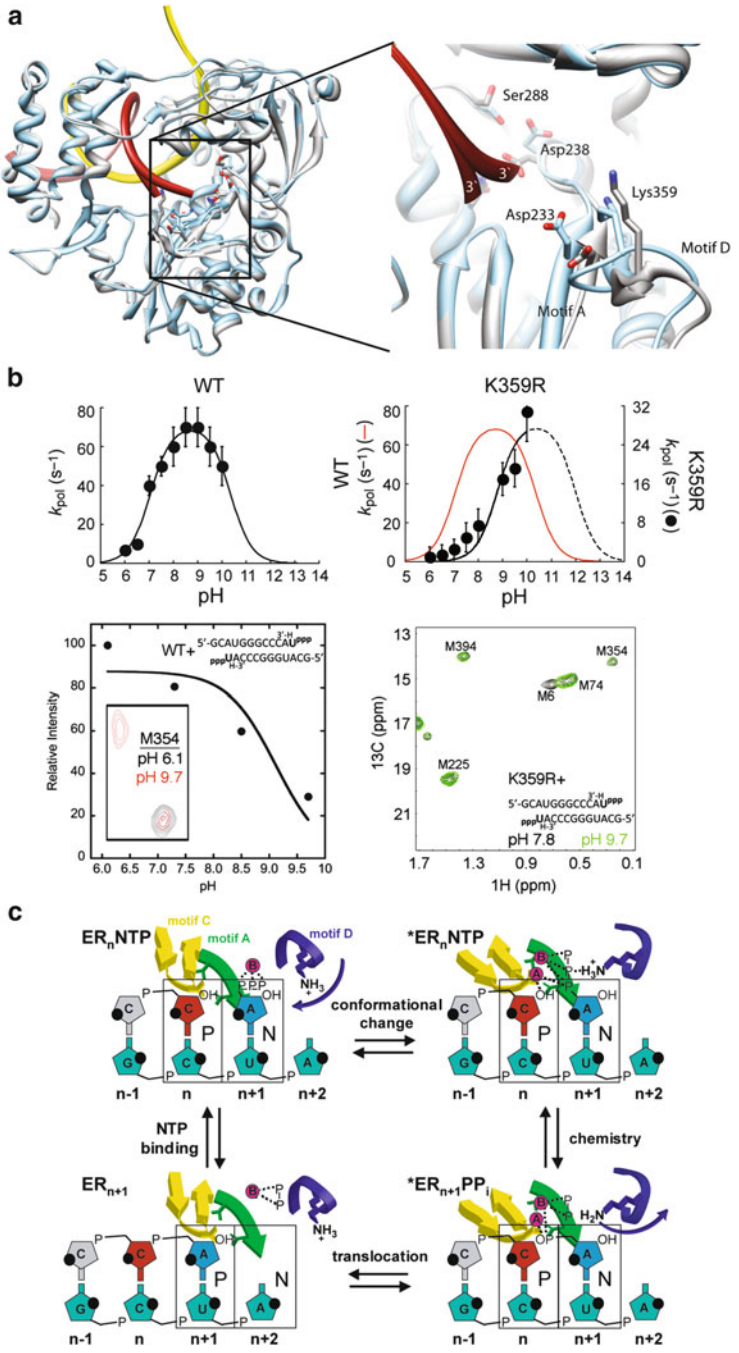

Fig. 14.3 Conformational changes of motif D and its functional role. (**a**) Conformational changes of RdRp revealed by X-ray crystallography. (*Left panel*) The PV RdRp binary (PDB 3OL6, *gray*) and ternary (PDB 3OL7, *cyan*) complexes are shown (Gong and Peersen 2010); the template–primer RNA are depicted as *yellow* and *dark red ribbons*, respectively. Subtle differences were observed between

thumb in RdRps would restrict such motions. Maintenance of the fingertip–thumb interactions in PV RdRp are also important for the protein's thermodynamic stability (Thompson et al. 2007), and even in molecular dynamics (MD) simulations, the interactions are kept intact while other regions undergo dynamic fluctuations (Moustafa et al. 2011).

The pre-chemistry conformational change is likely related to more subtle, local rearrangements in the palm subdomain, including the structural rearrangements in palm motifs A and C to bind magnesium and/or a reorientation of the triphosphate moiety of the incoming NTP into its catalytically active conformation; structural rearrangements within motifs D and F to reposition critical Lys and Arg residues may be important for proper alignment of the triphosphate for nucleophilic attack by the primer 3′-OH.

14.3 Newer Aspects in RdRp Structure and Function

14.3.1 The Catalytic Mechanism of RdRps Include General Acid Catalysis

One often overlooked aspect of the two-metal ion mechanism for DNA/RNA polymerases is the importance of proton transfer events that de-protonate the primer's 3′-OH to create a better nucleophile and protonation of the β-phosphate

Fig. 14.3 (continued) the binary and ternary complex structures; the protein chains in the two complex structures superimpose with an RMSD value of 0.53 Å. The key residues that revealed conformational changes in the ternary relative to the binary complex, resulting in closure of the polymerase active site, are shown as *sticks*. (*Right panel*) Zoom-in view of the region that showed conformational changes, enclosed by a *rectangle* in the *left panel*. The observed conformational changes involved motifs A and D, and repositioning of the side chains of Asp233, Asp238, Ser288, and Lys359, which are known to be important for the polymerase function. (**b**) Comparison of pH curves for WT and Lys359Arg PV RdRp from kinetics and NMR. Kinetics (*top panel*): pH rate profiles for WT and Lys359Arg PV RdRp obtained from evaluating the kinetics of nucleotide incorporation. WT PV RdRp yields two pK_a values of 7.0 ± 0.1 and 10.5 ± 0.1 (Castro et al. 2009). Lys359Arg RdRp yields a pK_a value of 8.8 ± 0.3 (*solid line*) with a second predicted pK_a value of 12.0 (*dashed line*). The *red line* shows the curve from WT PV RdRP for comparison. NMR (*bottom panel*): the open to closed transition of the WT PV RdRp–RNA-ternary complex is associated with a pK_a of 9.2, as obtained from the relative peak intensity of the Met354 resonance in the closed complex position. Met354 resonances for the RdRp–RNA-ternary complex at pH 6.1 (*black*) and 9.7 (*red*). Formation of the closed complex at high pH is restored by substitution of Lys359 with Arg, consistent with the higher pK_a of Arg (Yang et al. 2012). Met resonances for Lys359Arg RdRp–RNA–UTP ternary complexes at pH 7.8 are in *black* and at 9.7 are in *green*. The Met354 resonance remains unchanged between different pH regimes and is consistent with formation of the closed complex. (**c**) The stages of RNA synthesis and model for the role of motif D. During the conformational-change step, thought to be reorientation of the triphosphate for catalysis, Lys359 on motif D moves into a position both to serve as a proton donor and contribute to charge neutralization that develops during the transition state. After chemistry, loss of the proton on Lys359 triggers translocation to occur that facilitates PP_i release and reprotonation of Lys359

that would create a better leaving group. The pH rate profile for PV RdRp is consistent with acid/base catalysis (Fig. 14.3b), and proton inventory studies indicate that there are two proton transfer events in the rate-limiting step(s) (Castro et al. 2007, 2009). The identity of the general base is currently unknown, but recent studies indicate that the conserved motif-D Lys (Lys359 in PV RdRp) serves as the general acid. This residue is nearby the incoming NTP triphosphate, and substitutions at this position lead to changes in the SDKIE, proton inventory, and pH rate profile, all consistent with this Lys acting as a general acid (Fig 14.3b) (Castro et al. 2009).

These functional studies have been somewhat surprising given that in crystal structures of PV RdRp, the ε-amino group of Lys359 is out of reach (>5 Å away) of the β-phosphate (Gong and Peersen 2010). Nonetheless, additional structural rearrangements in motif D, such as those observed for NV RdRp, could reposition Lys359 within proper contact distance away, similar to the arrangement observed in telomerase (Gillis et al. 2008). Molecular dynamics (MD) simulations of HIV RT also demonstrate conformational fluctuations that would bring its motif-D Lys (i.e., Lys220) into proper catalytic register (Michielssens et al. 2011). Solution-state NMR experiments suggest that there are additional structural rearrangements in motif D, not observed in the currently available crystal structures (Yang et al. 2012). In bacteriophage Φ6 RdRp, NMR studies have demonstrated that Ile488 in motif D is undergoing conformational exchange on the same timescale as catalysis (Ren et al. 2010).

In PV RdRp, conformational rearrangements in motif D can be tracked by monitoring the chemical shift and relaxation behavior of the NMR peak for the nearby Met354, also located in motif D (Yang et al. 2012). The Met354 resonance shows a large, pH-dependent chemical shift change upon binding correct NTP to the RdRp/RNA complex (Yang et al. 2012). The pK_a associated with this conformational change closely matches the pK_a derived from the pH rate profile and assigned to Lys359 (Fig. 14.3b). An Arg substitution at this position leads to increases to both the kinetically and NMR-derived pK_as (Fig. 14.3b), indicating that the general acid Lys359 is intimately involved in the structural rearrangements in motif D that, in turn, help to position this residue for catalysis. The NMR studies also indicate that a similar conformational change does not occur when incorrect NTP binds, consistent with this step being kinetically slower for NMP misincorporation as demonstrated by slower overall rates and a decrease in the SDKIE mentioned above (Yang et al. 2012). De-protonation of the motif-D Lys and the loss of the electrostatic interaction with the pyrophosphate leaving group may trigger the post-chemistry conformational change, pyrophosphate release, and RNA translocation (see model in Fig. 14.3c).

General acid catalysis through a conserved Lys residue appears to be a common mechanism among DNA/RNA polymerases (Castro et al. 2009). In the case of A and B family DNA polymerases, the Lys is present on the O- and P-helices, respectively. These α-helices are repositioned only upon binding the correct NTP, and this serves as an important fidelity mechanism (Kiefer et al. 1998; Li et al. 1998; Tahirov et al. 2002; Temiakov et al. 2004; Yin and Steitz 2002); a

similar helix is absent in RdRps and RTs. In an analogous manner to what has been observed with Lys359 in PV RdRp, Lys758 in the O-helix in *Escherichia coli* DNA polymerase I has been proposed to be involved in conformational changes in the ternary complexes before and after nucleotide addition (Kaushik et al. 1996). These studies indicate that the motif-D loop in RdRps and RTs plays an analogous role to the O/P "fidelity" helix in the DNA polymerases (Yang et al. 2012).

14.3.2 Polymerase Fidelity Is a Critical Determinant of Viral Pathogenesis

Alteration of the motif-D Lys may be a universal vaccine strategy for RNA viruses; a PV strain containing an Arg at this position was attenuated but provided immune protection to mice, along the same order of magnitude as the clinically used Sabin I vaccine strain (Weeks et al. 2012). The PV variant was delayed in growth and replicated with high fidelity, consistent with the in vitro kinetics of the Lys359Arg RdRp. As a side note, the Sabin I vaccine strain itself contains a mutation in motif D (Thr362Ile) that could also impact the function of RdRp and contribute to viral attenuation (Nomoto et al. 1982; Georgescu et al. 1995).

Previous studies had established RdRp fidelity as a determinant of viral pathogenesis and virulence. Serial passages of PV against the antiviral compound ribavirin led to the selection of a resistant variant of PV with a single amino acid change to the RdRp (i.e., Gly64 to Ser) (Pfeiffer and Kirkegaard 2003; Vignuzzi et al. 2006; Arnold et al. 2005). This variant also led to higher RdRp fidelity and decreased pathogenesis in mice (Arnold et al. 2005; Vignuzzi et al. 2006; Pfeiffer and Kirkegaard 2003). Other substitutions have since been discovered that also impact RdRp function and contribute to viral attenuation (Gnadig et al. 2012). Some of these changes have an opposite effect on polymerase fidelity compared to the Gly64Ser and Lys359 variants, i.e., they decrease polymerase fidelity, yet still decrease pathogenicity and virulence (Gnadig et al. 2012). Together, these studies suggest that there is a small window of genetic variation that is optimum for virus biology and which is finely tuned by RdRp fidelity (Fig. 14.4).

14.3.3 RdRp Dynamics: The Next Frontier in Understanding Polymerase Catalytic Function and Fidelity

Perhaps surprisingly, the Gly64 to Ser substitution that leads to ribavirin resistance in PV is not located in the active site and would not be predicted to make direct interactions with ribavirin. Instead, Gly64 is involved in a hydrogen-bonding network that includes Gly1 in the N-terminal β-strand, and Ala239 and Leu241 in motif A (Fig. 14.5a). It was originally predicted that the Gly64Ser amino acid

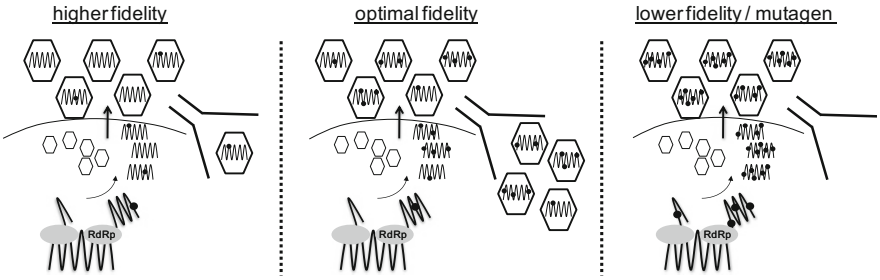

Fig. 14.4 Quasispecies nature of RNA viruses and effect of RdRp fidelity on virus biology. During viral replication, the number of mutations per genome is optimized and determined by the intrinsic error frequency, or fidelity, of the virus-encoded RdRp. A higher-fidelity RdRp leads to a decreased number of mutations per genome, limited diversity, bottleneck restriction, and lower viral fitness. A lower-fidelity RdRp or alternatively treatment with a viral mutagen, such as ribavirin, leads to an increased number of mutations per genome; this can exceed the tolerable threshold of mutations and reduce viral fitness

substitution would result in structural changes to the N-terminal β-strand and/or motif A that would change RdRp function and fidelity (Arnold et al. 2005). However, comparison of the X-ray crystal structures of WT and Gly64Ser RdRp did not reveal any substantial structural changes in these regions (Fig. 14.5b), although the modification to Ser enables the formation of additional hydrogen bond interactions with Glu2 (Marcotte et al. 2007). These results prompted the suggestion that static structures derived by X-ray crystallography were, by themselves, insufficient to explain the altered RdRp fidelity induced by the Gly64Ser substitution. Instead, changes to the dynamic excursions from the ground-state structure generated by the mutation may be responsible (Cameron et al. 2009). Consistent with this view, MD simulations have suggested that the Gly64Ser substitution leads to widespread ps–ns timescale dynamics in RdRp (Fig. 14.5c) (Moustafa et al. 2011). The observed dynamic changes with Gly64Ser RdRp appeared to be more substantial than dynamic differences between evolutionary distinct WT RdRps (Moustafa et al. 2011). Regions with altered flexibility were all associated with known functional regions of the enzyme, including motifs A, D, and F, and regions associated with the NTP- and RNA-binding channels (Fig. 14.5d). These results were consistent with dynamic NMR experiments that also showed that the Gly64Ser substitution induces global changes to the structure/dynamics of the enzyme (Yang et al. 2010).

The MD simulations may also be able to explain why other active-site remote mutations lead to changes to RdRp function; regions of flexibility were identified that had no previously known functional relevance to phosphodiester bond formation (Moustafa et al. 2011) but contained residues known to contribute to viral fitness (Diamond and Kirkegaard 1994) (Fig. 14.5e, f). One of these residues is Asp53, and it is worth noting that the Asp53Asn substitution is encoded by the Sabin I vaccine strain (Nomoto et al. 1982); the other RdRp substitutions are Tyr73His, Lys250Glu, and Thr362Ile. Tyr73 is next to Met74 that changes its chemical shift in response to RNA and NTP binding (Yang et al. 2010, 2012).

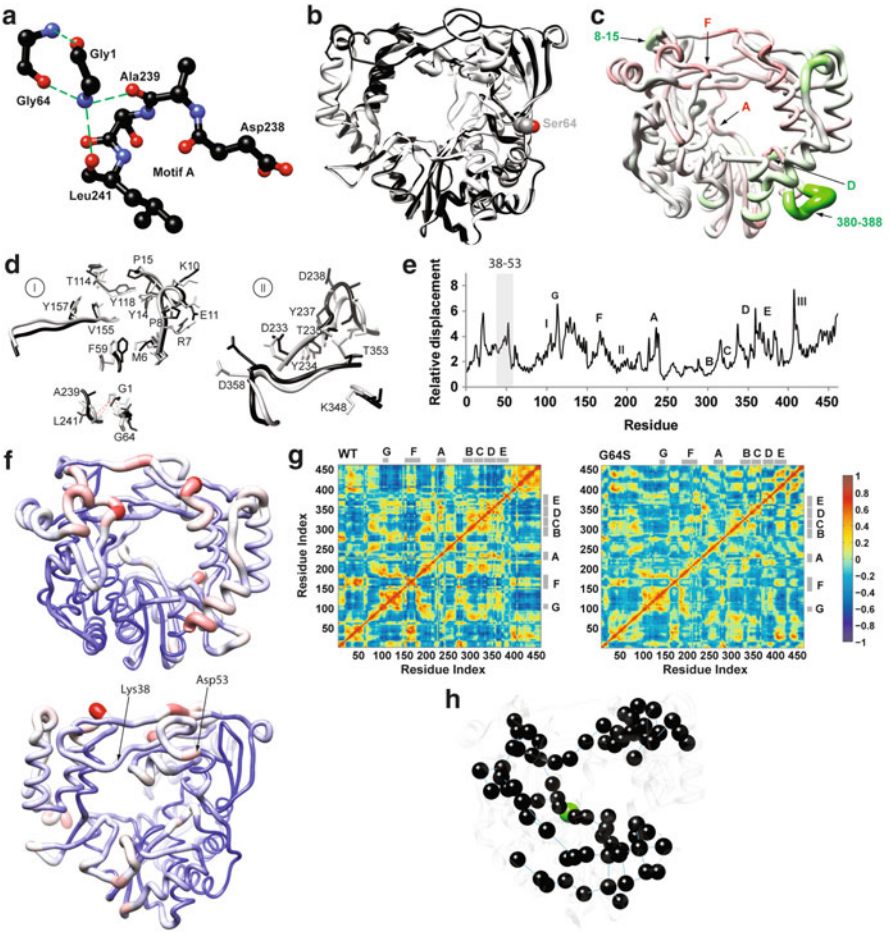

Fig. 14.5 Role of dynamics in RdRp function. (**a–d**) The high-fidelity derivative Gly64Ser revealed WT structure, but MD simulations showed global changes in dynamics of Gly64Ser compared to the WT enzyme. (**a**) Gly64 in PV RdRp (PDB 1RA6) plays a role in positioning the N-terminal Gly1 via H-bonding between the backbone atoms; Gly1 in turn stabilizes the motif-A conformation by forming H-bonding interactions with the backbone atoms of Ala239 and Leu241. Substitution of Gly64 by Ser was expected to alter motif-A conformation. (**b**) The crystal structure of Gly64Ser RdRp (PDB 2IJF, *gray*) (Marcotte et al. 2007) has been shown to be almost identical to that of the WT enzyme (*black*), RMSD value of 0.3 Å. (**c**) The differences between relative flexibility of Gly64Ser and WT RdRp obtained from MD simulations are mapped onto the WT structure. The differences are shown as color gradients of *green* (regions that are more flexible in Gly64Ser relative to WT RdRp) and *red* (regions that are less flexible in Gly64Ser relative to WT RdRp); the structure is rendered as a tube whose radius corresponds to the magnitude of flexibility differences between the two enzymes. The labeled *green* regions (motif D, residues 8–15, and residues 380–388) are much more flexible in Gly64Ser compared to WT RdRp, whereas the labeled *red* regions (motif A and motif F) are much less flexible in Gly64Ser compared to WT RdRp. (**d**) The average structures calculated from MD simulations for WT (*black*) and Gly64Ser (*gray*) RdRp are superimposed and regions that revealed structural perturbations are shown. Changes from position 64 to the N-terminal residues (8–15) and motif F are shown in **d-I**; changes in motif A and motif D are shown in **d-II**. Note that the hydrogen-bonding network involving residue at position 64 and the residues Gly1, Ala239, and Leu241 remain in both WT and

Lys250 is next to Met251, and NMR studies indicate that the Met251Ile substitution can have long-range structural and/or dynamic effects (Yang et al. 2010). Thus, it is tempting to speculate that the Sabin I substitutions may alter the structural dynamics of RdRp to impact catalytic function and fidelity.

Analysis of the MD simulations gives further insight into the functioning of the RdRp and, by extension, other nucleic acid polymerases. One important dynamic mode in RdRps involves the motions of the fingers and thumb subdomains relative to the palm subdomain such that the nascent RNA duplex channel expands and contracts (Moustafa et al. 2011). These motions appear to be "anticorrelated" to the opening/closing of the NTP-binding channel; when the RNA channel is open, the NTP channel is closed and vice versa. These coordinated motions may be important for binding of NTP, release of pyrophosphate, and/or translocation of the RNA.

14.3.4 RdRps Are "Small World" Networks of Amino Acid Residues

The MD simulations further suggest that there are long-range amino acid interaction networks important for RdRp function as demonstrated by the correlation of motions throughout the RdRp structure (Fig. 14.5g) (Moustafa et al. 2011).

Fig. 14.5 (continued) Gly64Ser RdRp. (**e**, **f**) Dynamics can explain biological effects caused by other remote-site mutations. (**e**) MD simulations revealed that functional motifs are largely contributing to the major motions observed during the course of simulations. The relative displacements of Cα atoms derived from principal component analysis (PCA) of the MD trajectory are plotted for each residue. Peaks correspond to regions that have large contributions to the major motions observed in the simulations. The region comprising residues 38–53 are highlighted by *gray shade*; mutations in this region have been shown to affect replication of PV. (**f**) Two views of PV RdRp structure (PDB 1RA6) are shown: (*top panel*) looking through the template–nascent RNA duplex channel and (*bottom panel*) looking through the NTP channel. The structure is rendered as a tube to indicate flexibility; the tube is colored in *blue-to-red* gradients with varying radii corresponding to the magnitude of the relative displacements shown in (**e**). The largest radius corresponds to the most flexible region, the smallest radius corresponds to the least flexible region. Positions of Lys38 and Asp53 are indicated in the *bottom view*. (**g**, **h**) Long-range network in RdRp revealed by MD simulations. (**g**) The color-coded dynamic cross correlation map (or DCCM), which measures the correlation between the displacements of Cα atoms, calculated from MD simulations of WT (*left panel*) and Gly64Ser (*right panel*) PV RdRp are shown. For each enzyme, the calculated matrix whose elements are the pairwise correlation scores between its residues is visualized as a colored map. The correlation scores are encoded with a color gradient from −1 (*blue*, completely anticorrelated) to +1 (*red*, completely correlated). Correlations between conserved structural motifs (*marked* with *gray bars*) can be observed in the maps. Interestingly, the single amino acid substitution in PV RdRp resulted in noticeable differences between the calculated DCCM maps of WT and Gly64Ser. (**h**) Residues shown to be correlated in the calculated DCCM are depicted as *black spheres* in the PV RdRp structure, shown as *transparent ribbon*. Of note, Gly64 (*green sphere*) falls along the inferred long-range network of polymerase residues

Additional evidence for these long-range interactions can be derived from NMR and mutational studies. In bacteriophage Φ6 RdRp, residues in motifs C, D, and E, along with residues in the outer edge of the template-binding channel, experience correlated dynamics on the μs–ms timescale as evidenced by their similar kinetic exchange parameters from NMR relaxation experiments (Ren and Ghose 2011; Ren et al. 2010). For PV RdRp, Met6 in the N-terminal β-strand and Met187 on a fingers subdomain helix are also conformationally coupled. These Met side chains occupy two conformations on the slow NMR chemical shift timescale, as evidenced by the presence of two resonances under some circumstances, and amino acid substitutions remote from either site have a similar effect on the dynamic equilibrium between these two conformations (Yang et al. 2010). This finding is intriguing considering that conformational changes in the N-terminal β-strand, mediated by Trp5, have been proposed to be involved in maintaining an elongation-competent complex (Campagnola et al. 2008; Hobdey et al. 2010).

RdRps, and other nucleic acid polymerases, can thus be viewed as having highly connected amino acid networks that provide communication pathways from the surface of the enzyme all the way to the catalytic center (Fig. 14.5h). Disruption of these networks through amino acid substitutions, as observed for Gly64Ser RdRp, or small molecule binding can have substantial impact on RdRp function. For the latter aspect, MD simulations for HCV RdRp have shown that inhibitor binding to an allosteric binding pocket influences internal fluctuations throughout the protein structure (Davis and Thorpe 2012). These networks also provide avenues for biological regulation; binding of accessory proteins in larger replication complexes and/or association of these replication complexes with the lipid membrane (Hsu et al. 2010) may alter RdRp structure, dynamics, and function. The evolution of the structure–function paradigm into a structure–dynamics–function paradigm may be the final frontier to a comprehensive understanding of nucleic acid polymerase function. Such studies have the potential to reveal new binding modes for RdRp inhibitors and give insight into how RdRp fidelity can be tuned through active-site remote mutations that may serve as the starting point for rational vaccine design.

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