DNA Polymerases as Engines for Biotechnology

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ABSTRACT

This review examines the DNA polymerases, a class of enzymes that has been an essential tool for molecular biology research. Several families of DNA polymerases have been defined based on amino acid sequence comparisons, and new enzymes are continually being discovered, driving the expansion of the current classifications. Structural similarities among the enzymes are examined, as well as the functions of the various subunits and enzyme domains. The natural variety of polymerase activities has been harnessed for applications such as amplification, labeling, and detection of DNA sequences. In addition, enhancements to DNA polymerases by genetic engineering will be described, such as enzymes specifically designed for DNA sequencing by improving the incorporation of dideoxynucleotide terminators. Reverse transcription, the ability to use RNA as a template for DNA synthesis, is described for the application of making cDNA. We believe that new and unanticipated applications will emerge as new polymerases and mutated polymerases are created and characterized.

INTRODUCTION

Advances in biotechnology have enabled the research community to uncover and accumulate vast amounts of data at an ever-increasing pace. Obtaining the sequence of the human genome was once a "Holy Grail" project. Now that the draft sequence is available well ahead of schedule, it is evident that the work has only just begun—the greater the continent of knowledge, the greater is the shore of the unknown. Having the sequence of a representative human genome fuels the search for the differences between individuals; it is the hope of pharmacogenomics that these differences between individuals can be harnessed for prognostic and therapeutic purposes. Sequence comparisons of model organisms in which genetic manipulations can be done with ease, such as the mouse, the worm, and the fruit fly, will accelerate attempts at discovering gene function. Obtaining the sequences of microbes will display the diversity of life and perhaps uncover clues to its origin.

This review will examine the class of enzymes that has been an essential tool for molecular biology research, the DNA polymerases. A brief examination of the flow of a genome project will reveal the need for polymerases at every step. To prepare templates for sequencing, amplification is frequently used, and sequencing itself is an adaptation of the replication function of polymerase. To accelerate the process of gene discovery, the reverse transcriptase activity of DNA polymerases has

been exploited to look at transcription by making cDNA. Shortcuts to cloning can be made with PCR even if a limited amount of sequence information is available. Mutagenesis is a powerful technique to understand gene function, and polymerases play a key role in constructing gene variations in vitro, by site directed and random mutagenesis.

Advances in genetic engineering of DNA polymerases will be explored, which have led to the acceleration of the pace of genome projects. These include improved polymerases for DNA sequencing, for amplification, and for reverse transcription. These applications have increasingly specialized demands for polymerases. Characteristics such as nuclease activity, thermostability, processivity, strand displacement activity, and fidelity are important in some roles but unneeded or undesired in others. Polymerases can be tailored to incorporate modified nucleotides as well. The biotech industry strives to fill these niches by engineering changes in the current repertoire of polymerases and by prospecting a diverse microbial world for new and promising enzymes. Before looking at improvements related to specific applications, a background will be given on the multiple families of DNA polymerases and their common structural elements and activities.

BACKGROUND

Braithwaite and Ito (16) have segregated DNA polymerases into four families: A, B, C, and X. Families A, B, and

C are arranged based on their amino acid sequence homologies to *E. coli* polymerases I, II, and III, respectively. Family X has no homologous *E. coli* polymerase.

Family A includes *E. coli* pol I, which has roles in recombination, repair, and replication; phage polymerases such as T5 and T7; and mitochondrial DNA polymerase pol gamma. These polymerases have simple subunit structures, usually a single subunit.

Family B includes *E. coli* pol II; Archaeal polymerases such as *Pyrococcus furiosus*; ϕ 29 and T4 phage polymerases; eukaryotic polymerases, α , Δ , ϵ , and many viral polymerases. Pol IIs have DNA repair roles and are usually single-subunit enzymes. Exceptions are the eukaryotic pol α , Δ , ϵ , which are all multiple-subunit enzymes and have roles in DNA replication.

Family C includes polymerases that are homologous to either α or ϵ subunits of the *E. coli* pol III. Together with the smaller subunit θ , these three subunits make up the pol III core. Pol III has at least seven accessory proteins and is the main replicative polymerase of *E. coli* (55). This family can be subdivided into three classes (43): Class 1, in which α and ϵ are separate subunits; Class 2, in which they are combined into one unit; and Class 3, in which they are expressed as separate proteins and joined by intein splicing (106). The placement of each functional domain within the primary sequence varies from class to class.

Family X was established to contain pol β , a eukaryotic repair enzyme that has no corresponding *E. coli* DNA polymerase (16). While its primary amino acid sequence places it in a unique family, pol β can carry out some pol I-type functions. When the gene encoding pol β is included in a plasmid containing an *E. coli* origin of replication, expression of the polymerase can confer plasmid maintenance in the absence of functional pol I (87). In addition, pol β is capable of gap filling between Okazaki fragments, as well as excision repair (85,86).

New polymerases continue to be discovered. Occasionally, their sequence-based placement into one of the above families proves difficult. Eukaryotic pols η and τ do not fit well into the

first four categories; however, they are homologous to two newly discovered *E. coli* polymerases that are designated pol IV and V (44,94,101). *E. coli* DNA polymerases IV and V, the yeast proteins Rev1 and Rad30, and eukaryotic polymerases ξ , η , θ , and τ form a group of loosely homologous enzymes involved in damage bypass replication (37,44). These enzymes have specialized activities such as the replication of abasic or thymine dimer sites. In 1997, Uemori and colleagues (99) discovered a novel DNA polymerase with two subunits in *Pyrococcus furiosus*. This enzyme was discovered by polymerase activity and not by sequence identity with previously known polymerases.

Table 1 describes the classification and characteristics of widely used DNA polymerases. The polymerases from families A and B have seen the most utility in the biotech industry, particularly those with a single subunit. There are several advantages to working with the single subunit polymerases from these groups. Cloning these proteins from new species is simplified, as are construction of expression vectors and protein purification. There are regions in the pol I sequence that are sufficiently conserved to allow amplification from a variety of bacterial genera (98).

A survey of the tertiary structures of DNA polymerases suggests a more ordered picture than does a comparison of their primary sequences. While primary sequences and function have diverged, the structural similarities remain. In fact, family X may have developed a structure similar to the other families through convergent evolution (47). The collection of higher-resolution structural data continues to expand, and researchers have been able to co-crystallize polymerases with their duplex DNA substrates (27,50). In one case, the crystals were shown to be biologically active and capable of nucleotide incorporation and translocation (50). Co-crystallization of T7 DNA polymerase with template and ddNTP gives structural insights as to how it can incorporate the chain-terminating nucleotide with an activity that is thousands-fold higher than many other family A polymerases (27). Recently, Kornberg and colleagues (22) have succeeded in obtaining a structure

for the first multiple-subunit polymerase, a 10-subunit RNA polymerase from *Saccharomyces cerevisiae*.

It is the monomeric DNA polymerases from families A and B that have been the most useful engines for biotechnology. Polymerase function can be considered to be modular. Activities such as the 5'→3' exonuclease activity of pol I or the RNase H activity of reverse transcriptases are found either in separate domains or separate subunits and are not part of the structure of the polymerase domain. A mini-review by Joyce and Steitz (47) elaborates on the common structures of polymerase domains and on the common functions of the subdomains. The classic polymerase domain is often compared in shape to a partially closed right hand (70), with the thumb, palm, and fingers representing subdomains.

The palm subdomain is the catalytic portion of the polymerase. Three carboxylate side chains are key to this active site. These residues are postulated to bind two metal ions (Mg^{++}) that play critical roles in the catalytic step (47, 82). The polymerase catalyzes a phosphoryl transfer reaction in which the alpha phosphate of the incoming dNTP undergoes nucleophilic attack from the OH primer terminus. One metal ion may facilitate deprotonation of the OH terminus, while the other contributes to the formation of a transition state at the alpha phosphate of the dNTP. While the primary amino acid sequence varies among polymerases, the shape of active sites, especially the positions of the carboxylates, is superimposable (i.e., the shape is conserved among family A and B polymerases, as well as reverse transcriptases) (47,52,80,83,104). The similarity of the palm active sites between RNA polymerases, DNA polymerases, and reverse transcriptases suggests that the mechanism by which the phosphoryl transfer reaction is catalyzed may be common to all polymerases. Even pol β , which shows little sequence identity to the other classes of polymerases, has equivalently positioned carboxylates in the active site.

Relatively minor changes in sequence within this structural region can affect substrate specificity. A single residue change in pol I polymerases results in more than a thousand-fold in-

crease in ddNTP incorporation (88), thus facilitating chain termination sequencing. Single amino acid substitutions in pol I Klenow, ϕ 29, and Vent (*Thermococcus litoralis*) DNA polymerase allow significant increase in ribonucleotide incorporation (5,15,36), although these same changes do not improve the polymerase's ability to extend from a ribonucleotide at the 3' end. A recent work (72) describes point mutations in *Taq* DNA polymerase that allow for the incorporation of successive ribonucleotides.

The thumb domains are less conserved but have the common feature of having largely helical structures (47). The thumb is believed to interact with the minor groove and also with the incoming nucleotide.

The finger subdomain shows the greatest structural diversity of the three polymerase subdomains. The finger domain of Klenow is mainly α helices, while that of HIV-RT has a higher content of β strands. The finger domain may play a role in template fixation and template specificity; like the thumb, it is likely to interact with the incoming nucleotide.

An alignment of all types of polymerases reveals two motifs, A and C (not to be confused with the families described above), which are conserved among DNA-dependent RNA and DNA polymerases, as well as RNA-dependent DNA and RNA polymerases (23). A third motif, found in the finger sub-domain, is associated only with the DNA-dependent polymerases (23,47). This is consistent with the idea that the palm contains a catalytic activity that is universal to all polymerases and that, while the fingers may also have a universal role in template binding, this subdomain is structurally adapted to the specific template involved (47).

ACTIVITIES OF DNA POLYMERASES AND THEIR APPLICATIONS

3'→5' Exonuclease Proofreading Activity-Fidelity for PCR

Dozens of DNA polymerases have been cloned from organisms that grow at high temperatures. These include

family A polymerases from thermophilic and hyperthermophilic bacteria, such as those from *Thermus aquaticus*, *Thermus thermophilus*, *Thermus flavus*, *Thermotoga maritima*, *Bacillus stearothermophilus*, and *Bacillus caldolenax*, as well as family B polymerases from *Archaea* such as *Thermococcus litoralis*, *Thermococcus* sp. 9°N, *Pyrococcus furiosus*, and *Pyrococcus woesei*. Like their well-studied mesophilic counterparts, the thermostable polymerases characterized for PCR applications also contain either 5'→3' exonuclease activity, 3'→5' exonuclease activity, or both in addition to polymerase activity. Protein sequence alignments suggest that the 3'→5' exonuclease active site is highly conserved in all polymerases with this editing function (45,46); however, the spatial relationship between this activity and the polymerase active site is less well understood. The spatial separation of this activity from the polymerase function is also widely variable, with *E. coli* DNA polymerase I activities separated by 25–30 Å, while in the family B polymerases such as ϕ 29, the activities located in the binding cleft are separated by a single nucleotide (26). The two active sites may be functionally interdependent, as in the case of *Pfu* DNA polymerase (53), or actually share common residues such as ϕ 29 (14). It is interesting to note that while the important active site residues are highly conserved, the rest of the protein scaffold within this region is divergent. There is currently little understanding of the mechanism involved in the switching of the primer strand between the polymerase and the 3'→5' exonuclease sites. Efforts have been made to identify possible residues involved in this transfer for ϕ 29 (97) and in T4 (6,84), but these residues or a suggested new motif (97) have not been identified in other related DNA polymerases (26).

The 3'→5' exonuclease activity associated with many polymerases conveys proofreading capability, which enhances polymerase fidelity, expressed as proportion of misincorporation. Reported fidelity ranges between 10^{-4} and 10^{-6} for the thermostable polymerases. There have been substantial differences between reported values, due in part to the influence of factors including

primer-template combination, temperature, and ion effects (54,93).

The fidelity is a function of the rates of the polymerase and exonuclease activities, which may be influenced by the rate of dissociation, the rate of conformational change before catalysis in the presence of mismatched primer terminus, and the rate of polymerization after misincorporation. The relationship between the 3'→5' exonuclease activity and polymerization rate can be described as a partitioning of the primer-template between the two active sites. Switching between these sites is controlled in part by the dNTP concentration, although there is only partial suppression of proofreading in the presence of high dNTP concentrations; a phenomenon referred to as the "next-nucleotide effect" (21,28,31,39). The competition between polymerization and proofreading is related to many factors, including increased rate of dissociation of mismatched dNTPs, decreased polymerization rate, ability of the enzyme to assume the correct conformation in presence of mismatch at 3' terminus, and melting of the primer terminus in preparation for excision (38,73).

The roles that sequence and structure of the template play have also been investigated (96). The DNA sequence of the primer terminus results in observed mutational hotspots. These hotspots are a reflection of local DNA stability in which stable DNA is less easily melted out to the single-stranded form required for 3'→5' exonuclease activity.

Selected applications require gene copies with a minimum of errors. The error rates in polymerases lacking 3'→5' exonuclease activity, such as *Taq* DNA polymerase of $1-2 \times 10^{-4}$ (73), can be sufficiently high that 20%–40% of the amplification products of a 200-bp PCR product will contain mutations after 20 cycles (18,58). *Pfu* and *Tli* DNA polymerases, with their associated 3'→5' activity, have found heavy use in applications requiring high fidelity. Although these enzymes may extend a misincorporated nucleotide, they may correct the error up to five nucleotides after the misincorporation (38). This can also result in higher PCR yields because non-proofreading enzymes such as *Taq* DNA polymerase may fail to extend 3' termini with mismatches, result-

ing in non-participation of these fragments in further cycles of extension.

Circumstances that favor proofreading can, depending on the enzyme, correct 95%–99.5% of single-base substitution errors (32) but comes at the cost of increased removal of correct nucleotides (32) and hence a reduced net extension rate (93). Another drawback to the use of enzymes harboring this activity is the degradation of single-stranded primers by the 3'→5' exonuclease activity. Unannealed primers can be degraded, thereby reducing the number and rate of successful initiations, and could also remove intended mismatches near the 3' end of the primer that were intended to generate mutations. By paying careful attention to the biochemical characteristics of the polymerases, proofreading enzymes have been used to generate PCR products of approximately 10 kb (54). However, products of 20–40 kb, which are defined as long PCR products, require a more refined approach. It was suggested that the errors that could occur approximately every few kilobases (depending on the error rate of the polymerase) were constantly removing template from the extension pool before the synthesis of the full-length product (9). To achieve long PCR and maximize fidelity, a mixture of polymerases with different activities has proven effective. By using a small amount of proofreading polymerase in conjunction with a non-proofreading enzyme such as *Taq* DNA polymerase, long PCR yields were optimized (9,18,20). In this way, primer degradation was minimized, more stringent annealing conditions were realized, and the proofreading function of the minority polymerase removed mismatched nucleotides.

5'→3' Exonuclease for Labeling and Detection of Sequences

The 5'→3' exonuclease activity of *E. coli* DNA polymerase I has long been used to label DNA by nick translation. In this application, DNA polymerase binds to a nick in the double-stranded substrate. These occur in vivo during lagging strand synthesis of replication or upon removal of RNA primers from Okazaki fragments. If

nicks are present, the polymerase function will add nucleotides to the template, while the 5'→3' exonuclease will concurrently remove nucleotides ahead of the polymerase. In vitro, this reaction has been harnessed by adding labeled nucleotides into the reaction mixture, such that radioactive, biotinylated, or fluorescent nucleotides become incorporated into the DNA.

The same domain that is responsible for 5'→3' exonuclease has been shown also to have structure-specific endonucleolytic activity. In this reaction, strands that have been displaced can be cleaved (61). Applications were noted in Lyamichev et al. (62), such as the ability to create cleavage reactions at a desired sequence. This is the basis for the Invader method of sequence detection (63) and mutation detection by fragment length polymorphism (69).

Structure-specific cleavage is the basis for the TaqMan™ assay (Roche Molecular Systems, Alameda, CA, USA). This mode of sequence detection involves binding of an oligonucleotide probe with both a reporter fluor and a quencher present (57). When a DNA polymerase synthesizing in the 5'→3' direction encounters such a bound oligonucleotide, strand displacement of the oligonucleotide begins, followed by cleavage of the displaced end, resulting in the liberation of the detection fluor from its quencher. This activity involves a combination of strand displacement, followed by cleavage (41) via the 5'→3' structure-specific endonuclease. Mutations that inactivate this activity are described below.

Polymerases for DNA Sequencing

Chain termination sequencing (76) relies on the polymerase function, initiating at specific primers. Dideoxynucleotides are used to terminate synthesis prematurely so that a collection of sequence-specific fragments differing in length is generated. Labels for the detection of the DNA fragments can be present on the primer or on the terminators, posing a challenge to the polymerase to incorporate these unusual substrates. Incorporation efficiency of the dideoxynucleotides is a vital consideration in selecting a polymerase as a sequencing enzyme. In addition, the

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Table 1. Classification and Characteristics of Widely Used DNA Polymerases

Enzyme	Family	3'→5' Exo	5'→3' Exo	Reverse Transcription	Strand Displacement	References
<i>Bacillus stearothermophilus</i> pol I	A	-	+		+	4,16,65,74
<i>E. coli</i> pol I	A	+	+	+	+	11,16,48,55
Exonuclease free Klenow	A	+	+			51,102
Klenow	A	+	-		+	51,102
Mitochondrial pol gamma	A	+				16,34
<i>Thermoanaerobacter thermohydrosulfuricus</i> pol I	A	-	+	+	+	64
T5	A		-			16,77
T7	A	+	-			16,49,90,92
<i>Thermus aquaticus</i> pol I	A	-	+			8,16,100
<i>Thermus flavus</i> pol I	A		+			3,16,40
<i>Thermus thermophilus</i> DNA polymerase	A			+		66
Bacteriophage phi 29	B	+	+		+	13,14,16,26,55,79
Bacteriophage T4	B	+	-		-	6,16,17,42,54,84
<i>E. coli</i> pol II	B	+	-		-	16,17,45,55
Eukaryotic pol δ	B	+				16,78
Eukaryotic pol ϵ	B	+				16,44
<i>Pyrococcus furiosus</i> DNA pol	B	+				16,53
<i>Pyrococcus</i> sp. Strain KOD1	B	+	-			93
<i>Pyrococcus woesei</i> DNA pol	B	+				16,53,73,93
Eukaryotic pol α	B					12,16
<i>Thermococcus litoralis</i>	B	+	-		+	16,54
<i>Thermococcus</i> sp. 9°N	B	+				81
<i>Bacillus subtilis</i> pol III	C	+				10,16
<i>E. coli</i> pol III	C	+	-		+(HE+SSB)	16,17,55
Eukaryotic DNA pol β	X	-	-			1,71,85–87
Moloney murine leukemia virus RT				+	+	56,105

E. coli pol III holoenzyme (HE) has strand displacement activity in the presence of single strand binding (SSB) protein.

ideal sequencing enzyme should be free of 5'→3' as well as 3'→5' exonuclease activities.

The 5'→3' nuclease activity is detrimental to sequencing, whether the label for detection is on the 5' end of the sequencing fragment, incorporated into the fragment as an internal label, or is on the terminator. Primer labels could be removed by the 5'→3' exonuclease activity whenever a primer or nascent sequencing fragment is annealed to the

template, impairing detection of the fragments. Endonucleolytic cleavage will shorten the sequencing fragment; these fragments will go undetected if only the 5' end of the primer is labeled. However, if the label is internal or on the terminators, this reaction will lead to labeled fragments of varying size, contributing to background noise. To interpret a sequencing ladder correctly, all fragments must have the same 5' end, beginning with the first nucleotide

of the primer, and terminating with a dideoxynucleotide.

In some cases, the domain of the polymerase that has 5'→3' exonuclease activity is absent, as in the case of T7 Sequenase™ DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) (92). The domain on which this activity resides can be removed by protease cleavage, as first demonstrated by Klenow with *E. coli* DNA polymerase I (51), or by deletion

as in Thermo Sequenase™ polymerase (Amersham Pharmacia Biotech) (100). Alternatively, point mutations can be introduced into the enzyme, which inactivate this activity. There are conserved regions in the 5'→3' exonuclease domain (40), and mutations in these residues can lead to the inactivation of the 5'→3' exonuclease activity, as in Thermo Sequenase II™ DNA polymerase (Amersham Pharmacia Biotech). Other mutations such as *Taq* G46D lie outside of these conserved regions (2), and this mutation is found in Amplitaq FS™ DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). Removal of the 5'→3' exonuclease activity can have other consequences for DNA sequencing. In some cases, enzymes with deletions have also suffered losses in processivity. On the other hand, in the case of *Taq* DNA polymerases with amino-terminal deletions, some of these enzymes enjoy greater thermostability than the full-length enzymes (100), as well as greater fidelity (8). Thermostability is critical for cycle sequencing, in which the reaction temperature is cycled to obtain a linear amplification of the sequencing products. Greater thermostability permits longer enzyme survival under these conditions, which leads to greater sensitivity and improved signal strength.

While 3'→5' exonuclease activity has utility for PCR, this activity is not desirable in sequencing enzymes. As described above, errors in PCR can be amplified, whereas those in a sequencing reaction are not, because the fragments generated do not serve as templates in subsequent rounds. Misincorporations in sequencing reactions are silent, unless the fragment is erroneously terminated with the incorrect ddNTP. In the latter case, the vast majority of fragments will be correctly terminated, and the misincorporation will still go undetected. The 3'→5' exonuclease prefers the unpaired 3' terminus of ssDNA as a substrate or the unpaired (mispaired) 3' terminus of dsDNA. While removal of the occasional mispaired nucleotide may seem a benefit, this is outweighed for sequencing by the reduced rate of polymerization in the presence of the proofreading activity. The 3'→5' exonuclease activity is detri-

mental to sequencing by hydrolyzing single-stranded sequencing primers.

Conserved regions that are immediately upstream from the polymerase domain encode the 3'→5' exonuclease activity. This activity cannot be removed by deletion of this domain because large deletions from this region of the enzyme cause inactivation of the polymerase activity as well. Point mutations and small deletions have been identified that inactivate the 3'→5' exonuclease activity, as has been done with T7 (90). Some type I DNA polymerases, such as those derived from *Thermus aquaticus* and *Thermus thermophilus* lack this activity, as the conserved motifs are not present in these enzymes. Derbyshire et al. (24) have reviewed conserved motifs and the effects of mutations therein.

The exonuclease activities are detrimental to chain termination DNA sequencing; however, there are several other qualities that enhance the performance of a sequencing enzyme. The ability to incorporate chain terminators, such as ddNTPs, is critical for the Sanger sequencing method (75). Most polymerases naturally discriminate against ddNTPs—given a mixture of the natural substrate dNTPs and ddNTPs at equal concentration, these enzymes will incorporate very little ddNTP. T7 DNA polymerase, which is a family A enzyme that readily incorporates ddNTPs (92), is a naturally occurring exception to this rule. Other enzymes used for sequencing, such as Klenow fragment, require a much higher ratio of ddNTP to dNTP concentration for sequencing. There is a correlation between discrimination against ddNTPs and uneven signal intensity in sequencing bands, which is characteristic of enzymes like Klenow but not of T7 Sequenase (35,91). Tabor and Richardson (88) explored the basis for the discrimination against ddNTPs as substrates. They discovered, by swapping motifs in the T7 enzyme with those in *E. coli* pol I and *Taq* DNA polymerase, that a single amino acid residue is responsible for discrimination against nucleotides lacking a 3' hydroxyl in the sugar moiety. When a tyrosine (Y) residue on the O-helix of T7 polymerase was introduced into the corresponding position to replace the phenylalanine (F) residue of *E. coli* pol

I and *Taq* DNA polymerase, it was found that these engineered enzymes no longer discriminated against ddNTP incorporation. This mutation has subsequently been introduced into an exonuclease-free version of *Taq* DNA polymerase, is marketed as Thermo Sequenase polymerase, and is also the basis of the Amplitaq FS sequencing enzyme. Uniformity of signal is also crucial to detection of heterozygotes or other mixed template sequences. Polymerases with the FY mutation have allowed for heterozygote and mutation detection (19,100).

Polymerization is a reversible reaction; pyrophosphate, a product of polymerization, is a substrate for the reverse reaction of pyrophosphorolysis. Pyrophosphorolysis can occur when substrate becomes limiting. Pyrophosphatase is therefore included in formulations for enzymes, which easily incorporate the terminators, thereby removing pyrophosphate from the reaction. Without pyrophosphatase, these enzymes can just as easily remove the terminators (89).

It is interesting to note that the Y residue in the O-helix is found in a motif that is conserved throughout all of the DNA-dependent polymerase families. However, at the exact position of the Y residue in T7, there is no corresponding residue in family B polymerases. Various groups have tried to improve the ddNTP incorporation by family B polymerases using mutagenesis, both site specific and random (29, 36). Modest success was attained, in that the incorporation of ddNTPs was improved 15-fold in the case of Vent™ DNA polymerase (New England Biolabs, Beverly, MA, USA) (36) and 150-fold in the case of *Pyrococcus furiosus* DNA polymerase (29). However, neither of these enzymes approaches the facility of ddNTP incorporation or the uniform signal intensity of members of the pol I family that have a tyrosine residue in the O-helix. Tabor and Richardson (88) achieved greater than 1000-fold improvement in the incorporation of ddNTPs by three members of the DNA pol I family.

Thermostability is desirable in a sequencing enzyme if a cycle sequencing protocol is utilized, in which reaction temperature is cycled as in PCR. In this way, the template is reused in every cy-

cle, providing a linear amplification of sequencing products. Among the type I polymerases, members of the *Thermus* family have been used as cycle sequencing enzymes because of their thermostability. Greater thermostability allows more cycles to be performed and, hence, greater sensitivity to low amounts of template. Indeed, using Thermo Sequenase polymerase and ^{32}P terminators, sufficient sensitivity is achievable to sequence directly from total chromosomal DNA from bacterial and yeast genomes, without cloning (30). Certain members of family B have even greater thermostability (i.e., those from the hyperthermophilic Archaea, such as *Pyrococcus furiosus* and *Thermococcus litoralis*). While the greater thermostability of family B enzymes holds the promise of direct sequencing from even larger genomes, their discrimination against ddNTPs is still too high for this to be practical for applications requiring the highest sensitivity. Some family B enzymes are currently used for cycle sequencing of cloned DNAs.

Further improvements in cycle sequencing have been achieved through the introduction of Thermo Sequenase II polymerase. This DNA polymerase is an exonuclease-free, full-length, thermostable enzyme that features improvements in salt tolerance and signal uniformity with dye-labeled terminators. Variations in salt tolerance and incorporation of dye terminators are found among different polymerases, and the molecular mechanisms for these differences are currently under investigation. Salt tolerance is favorable when the quality of template is variable because of salt carryover from sample preparation. Improved signal uniformity allows for greater accuracy of base calling, permitting longer read lengths, and better detection of mixed template sequences.

Strand Displacement

The recent advances in the ability to detect minute levels of infectious agents or to interrogate genomic DNA for rare mutations have been driven by nucleic acid amplification technologies. The majority of these diagnostic and detection methods use PCR-based thermal cycling techniques. However, methods such as strand displacement

amplification (103) and rolling circle amplification (33,59) have further stimulated new isothermal amplification methods. The advent of new methods puts new demands for another group of enzymes with specific properties different from those required for sequencing or PCR applications.

Strand displacement activity is a striking feature of some family A and B polymerases. This activity involves the unwinding of duplex DNA in the path of the advancing polymerase with no need for added protein co-factors such as helicases or single-strand binding proteins. Strand displacement activity has been studied by using two adjacent primers annealed to the template strand. When a polymerase reaches the 5' end of the second or blocking primer, there are three possibilities: (i) polymerization stops, (ii) the primer ahead of polymerization is degraded by the 5'→3' exonuclease activity, or (iii) the primer-template duplex in the path of polymerization is unwound and displaced as ssDNA, allowing polymerization to continue.

A unique aspect of strand displacement activity was revealed in temperature studies with *Tli* (Vent) polymerase (54). Here, it was revealed that there was polymerase activity, but no strand displacement activity at 55°C; the strand displacement activity increased with temperature through 72°C. An exonuclease-free mutant of the same enzyme also showed this apparent duality of temperature dependence for polymerase and strand displacement activity, although in this case the enzyme did not completely lose strand displacement activity at lower temperatures (54). Similar observations of temperature-dependent strand displacement activity were made for Deep Vent™ (New England Biolabs) polymerase and exonuclease-free *Pfu* DNA polymerase, in which strand displacement activity was not observed below 70°C, even though polymerization was functional at these temperatures (54). On the other hand, *Bst* and *Bca* DNA polymerases performed strand displacement at all temperatures tested (73). These differences in temperature optima between polymerase activity and strand displacement activity may reflect temperature-dependent conformational issues

or suggest that the activities may not be directly coupled within a single active site for all polymerases.

Localization of the intrinsic strand displacement activity has been attempted for a few enzymes. Specific mutagenesis studies with the *Bacillus subtilis* phage polymerase $\phi 29$ revealed that the strand displacement activity of that polymerase was reduced when mutations were introduced into the 3'→5' exonuclease active site (79). In that specific case, replacement of a highly conserved aspartic acid residue in the 3'→5' exonuclease domain inactivated the exonuclease activity and drastically decreased strand displacement activity. These authors suggested that the strand displacement activity resides in the N-terminal domain and overlaps the 3'→5' exonuclease domain (79). However, these results may not represent a general trend because work done with exonuclease-free versions of Vent™ DNA polymerase, *Pfu* DNA polymerase, and Klenow fragment suggest that even complete removal of the 3'→5' activity does not remove the intrinsic strand displacement activity in any of these enzymes (25,73). Clearly, there is at present no consensus as to the exact motifs or even domains in which this activity is localized.

The rolling circle replication mechanism used by viruses with circular genomes or plasmids illuminated many aspects of strand displacement activity in vivo. Rolling circle replication generates a single-stranded product that has many tandem copies of the complement of the circular template. This replication method inspired a number of new amplification techniques that distinguish themselves from PCR in that they are isothermal processes and rely on polymerases that have strong strand displacement activity.

One of the first embodiments of this is strand displacement amplification (102). The method uses intrinsic activities of restriction enzymes and exonuclease-free Klenow, which is devoid of 5'→3' and 3'→5' exonuclease activity. This enzyme could neither degrade single-stranded primers in solution nor degrade annealed primers in the path of polymerization. The amplification also relies on the fact that restriction enzymes, such as *HincII*, cannot cut phos-

phorothioate linkages. Hence, synthesis of a recognition site with dATP α S in one strand means that only the opposite strand (template) will be cleaved to generate a nick. The nick serves as the start for strand displacement. The amplification begins when displaced primer strand anneals with added template strand oligonucleotides and displaced template strand anneals with added primer strand oligonucleotides. New nicks are generated, and the extension of the oligonucleotide primers at the nicks results in more displaced strands that again act as targets for additional nicking and strand displacement activity, ultimately resulting in up to 10⁷-fold amplification (103).

Rolling circle amplification (RCA) is another novel isothermal amplification method. In this case, a primer is annealed to a single-stranded circular template and extended with a polymerase that has strong strand displacement activity to generate long tandem repeat copies of the original template (33,59). This so-called linear RCA with M13 template has yielded impressive results when ϕ 29 polymerase is used. ϕ 29 has extremely high processivity greater than 70 kb (13) and is capable of catalyzing production of linear product of $\geq$ 23 kb in 10 min (60). In similar assays, others have shown that the amplification continues in a linear fashion with a half-life of 11 h (7). The combination of strong sequence-independent and temperature-independent strand displacement activity and the extreme processivity of ϕ 29 makes the production of amplification products in the 400–500 kb size range possible (7). If a unique restriction site is present in the original circular template, then the linear product can be efficiently collapsed to one band with restriction digestion, using a complementary oligonucleotide to form the double-stranded restriction site (95).

Single-stranded circular templates for RCA can also be generated in situ. A single linear oligonucleotide is designed such that the 5' and 3' regions will base pair next to each other on the linear target strand. Ligation generates a closed circular oligonucleotide or so-called padlock probes that is catenated to the target sequence (60,67). The ligated probe can then be utilized for RCA amplification. Since ligases discrimi-

nate strongly between matched and mismatched annealed ends of the probe nucleotide, the method can be used to score genetic mutations (60,68,95).

Exponential amplification is also possible with the inclusion of a second primer that binds to the complement sequence of the original circular template. The reaction begins with the generation of linear tandem copies, then the second primer anneals and is extended by polymerase, making the linear product double-stranded. The ensuing strand displacement regenerates multiple binding sites where both primers can anneal. The result is a continuously expanding collection of branched DNA or hyperbranched DNA product connected to the original circle (60). The strand displacement also generates a discrete set of free dsDNA fragments corresponding to the unit length of the circle. The suitable subset of enzymes for this application with the appropriate strand displacement activity expands when circles of 150 bp or less are used (33,59). In this case, the torsional strain on the duplex product generated as the enzyme proceeds around the circle is such that the second strand is prone to peel away on its own. The result of this hyperbranched or cascade rolling circle method is a 10⁹-fold amplification in less than 90 min (60). Coupled to fluorescence detection methods (95), this exciting amplification technology will certainly find use in detection of point mutations in many genomic DNA applications, including the human genome.

Reverse Transcription

Several family A DNA-dependent DNA polymerases, including *E. coli* DNA polymerase I (48), *Thermus thermophilus* polymerase (66), and the pol I DNA polymerase of *Thermoanaerobacter thermohydrosulfuricus* (Tts) (64), have been shown to have reverse transcriptase activity (i.e., RNA-dependent DNA polymerase activity) under certain conditions. One application of such an enzyme is RT-PCR, in which an RNA template is used to generate a cDNA, which is amplified by PCR. Thermostable DNA-dependent DNA polymerases that can also use RNA templates, such as *Tth* DNA polymerase, allow RT-PCR to be carried out by a single en-

zyme. The reactions can be done in a single tube with fewer manipulations than are required for the traditional process.

A second application of these family A polymerases may be as tools for cDNA synthesis or cDNA labeling applications. Family A polymerases may offer several advantages over viral reverse transcriptases for these applications. First, viral reverse transcriptases have an associated RNase H activity. This endonuclease activity may degrade the RNA template during cDNA synthesis, resulting in truncated products (56). While the family A polymerases have 5'→3' exonuclease activity that will degrade RNA primers associated with lagging strand synthesis, this RNase H activity does not, by its nature, interfere with cDNA synthesis.

The thermostable family A DNA polymerases may provide additional advantages over the use of mesophilic RT enzymes such as those from Moloney murine leukemia virus (MMLV) and avian myeloblastosis virus (AMV). Conceivably, the higher reaction temperature may allow for melting of the RNA template, resulting in better read through of secondary structure in the mRNA. The RNase H activity has been removed from engineered versions of MMLV RT (56), and this is the enzyme that is currently most widely used for cDNA applications.

Potential enzymes must be evaluated with the specific application in mind. For example, cDNA synthesis performed for the purpose of cloning must yield full-length product. In contrast, cDNA synthesis is often performed to make labeled cDNA probes that can then be applied to fixed arrays and evaluated for gene expression. Full-length probes are not required for this application, and, in fact, shorter products may be desirable. These applications place premiums on different polymerase properties, and it is likely that no single polymerase will be best for all applications.

CONCLUSION

DNA polymerases have proven to be versatile tools in the molecular biology laboratory. They have been used to prepare DNA for cloning, to label DNA for detection, to amplify DNA, to de-

tect specific sequences, and to determine the sequence of genomes up to the size of the complete human genome. Among these applications, uses have emerged for polymerases with and without exonucleases. Similarly, uses have emerged for highly thermostable polymerases and for those that are not. Future applications are expected to take further advantage of this remarkable and versatile enzyme, perhaps in re-sequencing the places where the genomes of individuals differ or in further manipulating DNA for use in genetic investigations and alterations or therapy. There is little doubt that new and unanticipated applications of this enzyme will emerge as new polymerases and mutations of polymerases are created and characterized.

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