Multiple Amino Acid Substitutions Allow DNA Polymerases to Synthesize RNA*

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DNA and RNA polymerase exhibit similarities in structures and catalytic mechanisms, suggesting that both classes of enzymes are evolutionarily related. To probe the biochemical and structure-function relationship between the two classes of polymerases, a large library (200,000 members) of mutant Thermus aquaticus DNA polymerase I (Taq pol I) was created containing random substitutions within a portion of the dNTP binding site (motif A; amino acids 605–617), and a fraction of all selected active Taq pol I (291 of 8000) was tested for the ability to incorporate successive ribonucleotides; 23 unique mutants that added rNTPs into a growing polynucleotide chain were identified and sequenced. These mutants, each containing one to four substitutions, incorporate ribonucleotides at a rate approaching 103-fold greater than that of wild type Taq pol I. Several mutants added successive ribonucleotides and can catalyze the synthesis of RNA. Sequence analysis of these mutants demonstrates that at least two amino acid residues are involved in excluding ribonucleotides from the active site. Interestingly, wild type DNA polymerases from several distinct families selectively discriminate against rUTP. This study suggests that current DNA and RNA polymerases could have evolved by divergent evolution from an ancestor that shared a common mechanism for polynucleotide synthesis.

Both DNA and RNA polymerase can catalyze chain elongation reaction guided by single-stranded DNA templates to generate polynucleotide products (1). The order of nucleotide addition proceeds in a 5' → 3' direction via metal-mediated phosphodiester bond and release of pyrophosphate (2). In addition, both DNA and RNA polymerases resemble in morphology a cupped human right hand and bind DNA template and the incoming nucleotide within the active site cleft (3, 4). DNA polymerases differ from RNA polymerases in utilizing 2'-deoxyribonucleotides (dTTP, dCTP, dGTP, and dATP) rather than ribonucleotides (rUTP, rCTP, rGTP, and rATP). A detailed analysis of the polymerase active site is crucial to understanding how these polymerases distinguish between dNTPs and rNTPs, as well as to provide insights on how the two types of polymerases evolve to adopt similar mechanisms.

Despite the similarity in protein structure and function, there is almost no sequence identity between DNA and RNA polymerases. For example, nearly all of the over 40 prokaryotic and eubacteria DNA pol Is sequenced (including Thermus aquaticus pol I, Chlamydia trachomatis pol I, and Escherichia coli pol I) contain the DYSQIELR sequence within the dNTP binding site (motif A; Ref. 5), yet RNA pols only have in common the catalytically essential aspartic acid residue (6). If evolution proceeded from an “RNA world” containing RNA synthesizing enzymes to a “DNA world” with genomes replicated by DNA synthesizing enzymes (7, 8), it might be possible to gain insights into this process by substituting random sequences within the active site of a polymerase.

Analyses of high resolution x-ray crystal structures suggest that a single side chain prevents diverse polymerases from incorporating ribonucleotides (9–11). Rational approaches involving site-directed mutagenesis of this residue result in polymerases with unique properties. Assuming that current DNA and RNA polymerases evolved from a common ancestor and share a basic mechanism, it should be possible to evolve one of these enzymes into the other, following extensive rounds of mutagenesis and stringent selection protocols. To explore this concept, we randomly mutated a portion of the active site of a eubacteria DNA polymerase, Taq pol I, selected for functioning mutants, and tested 291 mutant enzymes for the ability to synthesize RNA. Twenty-three different mutant polymerases containing substitutions in predominantly one of two amino acids were identified that incorporated ribonucleotides at a rate approaching 103-fold greater than that of WT Taq DNA polymerase. We show that the active site within several families of WT DNA polymerases is especially evolved to exclude rUTP.

EXPERIMENTAL PROCEDURES

Screen for Ribonucleotide Incorporation Activity—The construction of the random Taq pol I library (containing 200,000 individual clones) and genetic selection protocol that yields functional mutants in vivo has been described in detail in a previous publication (5). The 350 colonies from the 8000 that complemented pol I temperature sensitive phenotype at 37 °C were isolated and grown in nutrient broth individually overnight at 30 °C. Each culture was grown to A595 of 0.3 at 30 °C in 10 ml, and Taq pol I expression was induced with 0.5 mM isopropyl-1-thio-

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The abbreviations used are: pol I, DNA polymerase I; Taq, T. aquaticus; WT, wild type; PAGE, polyacrylamide gel electrophoresis; MMLV, Moloney murine leukemia virus; RT, reverse transcriptase.
9β-D-galactopyranoside and incubations continued for 4 h. Taq pols were partially purified (50 μl total volume) using a modified protocol from Refs. 14 and 15 that allows efficient (>50%) purification of Taq pol I while removing endogenous polymerase and nuclease activities. Biochemical analysis of DNA-dependent DNA polymerase activity of these 50-μl-20 μg DNA sample was performed at 72 °C (primers or double-stranded DNA, or DNA corresponding to the 3′ terminus of T. aquaticus polA gene as described (16). Polymerizations were conducted in vitro at 55 °C for 5–30 min in 10–40-μl reaction mixtures containing 5 μM primer-template, 50 mM KCl, 10 mM Tris-HCl (pH 8), 0.1% Triton-X, 2.5 mM MgCl₂, all four rNTPs (50–500 μM each), and either WT or mutant Taq pol I (2–20 nM) as initiated with the addition of enzyme and stopped with the addition of sequencing loading buffer (Amersham Biosciences, Inc.).

RESULTS

Barriers for WT Taq Pol I to Incorporate Ribonucleotides—We measured the capacity of several subclones of DNA polymerases to incorporate ribonucleotide triphosphates relative to dNTPs. DNA polymerases from the thermophile eubacterium T. aquaticus, from hyperthermophile archaea Thermococcus litoralis, from prokaryote E. coli, and from retrovirus MMLV were analyzed. These DNA polymerases represent three major subclasses (6): prokaryotic pol I family (Taq pol I and E. coli pol I), mammalian pol α family (Vent pol, and RT MMLV RT). Incorporation of individual ribonucleotides by WT Taq pol I requires greater amounts of polymerase and at least 1000-fold higher concentrations of each rNTP relative to the corresponding dNTP (Fig. 1). It is important to note that polynucleotide products containing a 3′ ribonucleotide migrate slower within polyacrylamide gels relative to 3′ deoxyribonucleotide products. This property is useful to monitor the nature of products, as well as to ensure the purity of dNTP and rNTP substrates. Hyperbolic curve fit of steady state rates plotted as a function of nucleotide concentration yields Michaelis-Menten parameters V_{max} and K_{m}, and the initial slope of this plot reflects the catalytic efficiency of each enzyme. WT Taq pol I incorporates dGTP, dATP, and dCTP up to 30,000 times more efficiently (k_{cat}/K_{m}) than the respective ribonucleotides (Tables I and III). The low efficiency of rNTP incorporation is largely due to a 1000-fold greater K_{m} for rG, rC, and rA relative to the respective deoxynucleotides. Other DNA polymerases from pol I, pol α, and RT families also incorporate ribonucleotides inefficiently (also largely because of high K_{m} for rNTPs relative to dNTPs; Table I).

DNA polymerases are especially adept at incorporating dTTP over rUTP. Although Taq pol I incorporates dTTP and dUTP opposite template dA residue with approximately equal efficiencies, the WT enzyme incorporates dTTP 10^6-fold more efficiently than rUTP (Table I). Selection against rUTP by Taq pol I is 100-fold more stringent than the other three ribonucleotides (Tables I and II). This property is useful to monitor the nature of products, as well as to ensure the purity of dNTP and rNTP substrates. Hyperbolic curve fit of steady state rates plotted as a function of nucleotide concentration yields Michaelis-Menten parameters V_{max} and K_{m}, and the initial slope of this plot reflects the catalytic efficiency of each enzyme. WT Taq pol I incorporates dGTP, dATP, and dCTP up to 30,000 times more efficiently (k_{cat}/K_{m}) than the respective ribonucleotides (Tables I and III). The low efficiency of rNTP incorporation is largely due to a 1000-fold greater K_{m} for rG, rC, and rA relative to the respective deoxynucleotides. Other DNA polymerases from pol I, pol α, and RT families also incorporate ribonucleotides inefficiently (also largely because of high K_{m} for rNTPs relative to dNTPs; Table I).

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Evolution of the Polymerase Active Site

Fig. 1. Efficiency of dGTP and rGTP incorporation by WT. WT Taq pol I (0.3 fmol/μl for dNTP reactions and 3 fmol/μl for rNTP reactions) was incubated with 26-mer/47-mer (primer-template; 5 nM) with increasing concentration of either dGTP or rGTP for 10 min at 55 °C in 10-μl reactions. Product yield was quantified by phosphoimagery. The V_\text{max}/K_m values obtained upon a hyperbolic curve fit of the plots reflects the efficiency of nucleotide incorporation. The k_cat values were determined by dividing V_\text{max} by the enzyme concentration. Incorporation of rGMP relative to dGMP results in a product with a slower electrophoretic migration.

Table I

| Protein  | Nucleotide | V_\text{max} (rel) | K_m | V_\text{max}/K_m | Discrimination^a |
|----------|------------|-------------------|-----|-----------------|-----------------|
| Taq pol  | dTTP\(^b\) | 1.0               | 0.005 | 200             | 1.2             |
|          | dUTP       | 1.1               | 0.007 | 160             |                 |
|          | rUTP       | 0.03              | 240  | 0.00013         | 1,500,000       |
|          | dCTP       | 1.0               | 0.012 | 24              |                 |
|          | rCTP       | 0.06              | 59   | 0.001           | 24,000          |
| Klenow (3'-5' exo-) | dTTP | 1.0 | 0.016 | 63 | |
|          | dUTP       | 1.1               | 0.028 | 39             | 1.6             |
|          | rUTP       | 0.030             | 60   | 0.00050         | 130,000         |
|          | dCTP       | 1.0               | 0.040 | 25             |                 |
|          | rCTP       | 0.20              | 25   | 0.008           | 3,100           |
| Klenow (5'-3' exo-) | dTTP | 1.0 | 0.039 | 28 | |
|          | dUTP       | 1.04              | 0.055 | 19             | 1.4             |
|          | rUTP       | 0.079             | 31   | 0.0025          | 10,000          |
|          | dCTP       | 1.0               | 1.1   | 0.90            |                 |
|          | rCTP       | 0.079             | 180  | 0.00044         | 2,000           |
| Vent     | dTTP       | 1.0               | 0.054 | 19             |                 |
|          | dUTP       | 0.62              | 0.21  | 3.0             | 6.3             |
|          | rUTP       | 0.035             | 39   | 0.00090         | 21,000          |
|          | dCTP       | 1.0               | 0.21  | 5.0             |                 |
|          | rCTP       | 0.054             | 13   | 0.0042          | 1,100           |

^a Discrimination represents the efficiency (V_\text{max}/K_m) of dNTP incorporation divided by efficiency of the corresponding rNTP. For dUTP, discrimination represents efficiency of dTTP incorporation divided by that of dUTP. V_\text{max} (rel) represents steady state rates relative to that of dTTP or dCTP incorporation.

^b Primer/template sequence used with each nucleotide is defined under “Experimental Procedures.”

ues (k_cat(K_m) in steady state experiments and k_cat(K_d) in single turnover assays) in the two studies are consistent. These data indicate that several families of DNA polymerases have evolved a sophisticated mechanism to exclude ribonucleotides, especially uracil, from its catalytic site.

Testing a Large Library of Taq pol I Mutants for rNTP Incorporation—Assuming that DNA and RNA polymerases are related mechanistically and in structure-function, it should be
possible to distinguish residues responsible for dNTP versus rNTP substrate specificity following extensive mutagenesis of the polymerase active site and genetic selection of active mutants. We previously created a Taq pol I library composed of 8,000 active members containing substitutions within active site (motif A) residues. To determine whether this plasticity within the catalytic site architecture can confer altered substrate specificity, we further analyzed all 291 selected mutants. To determine whether this plasticity within the catalytic site architecture can confer altered substrate specificity, we further analyzed all 291 selected mutants. To determine whether this plasticity within the catalytic site architecture can confer altered substrate specificity, we further analyzed all 291 selected mutants. To determine whether this plasticity within the catalytic site architecture can confer altered substrate specificity, we further analyzed all 291 selected mutants. To determine whether this plasticity within the catalytic site architecture can confer altered substrate specificity, we further analyzed all 291 selected mutants.

### Table II

| No. of amino acid changes | Mutant | 605 L | L | V | A | L | D | Y | S | Q | 614 I | 615 E | 617 R |
|--------------------------|--------|-------|---|---|---|---|---|---|---|---|-------|-------|-------|
| 1                        | 53     | D     |   | K | M | P |   |   |   |   |       |       |       |
| 2                        | 94     | D     |   | V | N | K |   |   |   |   |       |       |       |
| 164                      | 187    | M     |   | L | Q |   |   |   |   |   | I     |       |       |
| 198                      | 205    | Q     |   |   |   |   |   |   |   |   |       |       |       |
| 221                      | 230    | V     | R | F | I |   |   |   |   |   |       |       |       |
| 230                      | 232    | D     | G | V | D |   |   |   |   |   |       |       |       |
| 240                      | 246    | D     | D | V |   |   |   |   |   |   |       |       |       |
| 299                      | 300    | V     | T | F | W |   |   |   |   |   |       |       |       |
| 300                      | 302    | V     |   | F | T | D |   |   |   |   |       |       |       |
| 4                        | 48     | M     | R | K | D | M |   |   |   |   |       |       |       |

* Underlining denotes mutants exhibit WT activity.

a These two mutants differ in nucleotide sequence.

### Efficient rNTP Incorporation by Mutant Taq pol Is—

To characterize the efficiency of dNTP relative to rNTP incorporation by WT and mutant enzymes, the steady state $k_{cat}$ and $K_m$ values of nucleotide incorporation were determined. The plots of nucleotide incorporation rates as a function of nucleotide concentration exhibited typical Michaelis-Menten saturation kinetics for all reactions containing either dNTPs or rNTPs with either mutant or WT enzymes. The $k_{cat}/K_m$ value obtained from fitting a hyperbolic curve directly measures the efficiency of nucleotide incorporation. Mutant homogenous enzymes containing specific substitutions at position 614 and 615 incorporating rNTPs differ in nucleotide sequence. One of these mutants exhibit WT activity.

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than the in vivo ribonucleotide concentration (approximately 1000 μM each), suggest these enzymes should be able to incorporate ribonucleotides at physiologic conditions.

To determine whether these polymerases can function as RNA polymerases by incorporating multiple ribonucleotides sequentially, we incubated purified WT Taq pol I, a mutant containing a substitution at Ile-614, and a mutant containing a substitution at Glu-615 in the presence of increasing amounts of all four rNTPs (Fig. 2). Although the WT enzyme inefficiently incorporates and extends ribonucleotides, both rNTP utilizing mutant enzymes polymerize multiple ribonucleotides, even at rNTP concentrations below that found in cells. Interestingly, the strong pause sites produced at runs of template dAs is exactly what one would predict from the kinetic data (Tables I and III). From the primer/template sequence used with each nucleotide is defined in the "Methods" section.

There are two major conclusions from this study. First, DNA polymerases selectively exclude rUTP. Second, amino acid substitutions at two different positions within the DNA polymerase catalytic site can result in enzymes that incorporate ribonucleotides and synthesize RNA. These findings were not predicted by analysis of high resolution x-ray crystal structures. These observations link DNA and RNA polymerization mechanisms and may provide insights into the evolution of DNA polymerases during the postulated progression from the RNA into DNA world (7, 8).

**DISCUSSION**

There are two major conclusions from this study. First, DNA polymerases selectively exclude rUTP. Second, amino acid substitutions at two different positions within the DNA polymerase catalytic site can result in enzymes that incorporate ribonucleotides and synthesize RNA. These findings were not predicted by analysis of high resolution x-ray crystal structures. These observations link DNA and RNA polymerization mechanisms and may provide insights into the evolution of DNA polymerases during the postulated progression from the RNA into DNA world (7, 8).

**TABLE III**

| Protein                  | Nucleotide | kcat | kcat/Km | kcat | kcat/Km |
|--------------------------|------------|------|---------|------|---------|
| Wild type                | G          | 0.020| 0.021   | 1.0  | 0.0026  |
|                          | A          | 0.012| 0.070   | 0.17 | 0.0016  |
|                          | C          | 0.013| 0.042   | 0.31 | 0.00083 |
|                          | T/U        | 0.013| 0.0050  | 2.6  | 0.00043 |
| Mutant 53 (I614K)        | G          | 0.079| 0.37    | 0.21 | 0.086   |
|                          | A          | 0.035| 0.11    | 0.32 | 0.025   |
|                          | C          | 0.079| 0.083   | 0.95 | 0.067   |
|                          | T/U        | 0.046| 0.031   | 1.5  | 0.096   |
| Mutant 94 (A608S, I614N) | G          | 0.058| 0.086   | 0.067| 0.0065  |
|                          | A          | 0.012| 0.15    | 0.080| 0.0065  |
|                          | C          | 0.058| 0.089   | 0.065| 0.0075  |
|                          | T/U        | 0.075| 0.225   | 9.2  | 0.0056  |
| Mutant 265 (I614N, L616I)| G          | 0.015| 0.0071  | 2.1  | 0.012   |
|                          | A          | 0.014| 0.048   | 0.29 | 0.0080  |
|                          | C          | 0.015| 0.034   | 0.44 | 0.0073  |
|                          | T/U        | 0.018| 0.016   | 1.0  | 0.017   |
| Mutant 346 (A608D, E615D)| G          | 0.0020| 0.12    | 0.017| 0.0056  |
|                          | A          | 0.0040| 0.20    | 0.020| 0.0060  |
|                          | C          | 0.0020| 0.29    | 0.0069| 0.0036  |
|                          | T/U        | 0.0087| 0.018   | 0.48 | 0.0048  |

* dNTP/rNTP discrimination equals efficiency of dNTP incorporation (kcat/Km) relative to rNTP incorporation.

**Methods** section.
Incubations in the presence of Mn\(^{2+}\) (0.5 mM; 2.5 mM MgCl\(_2\)) were conducted in identical conditions with 50 \(\mu\)M of each rNTP. Control (lane C) contains extension products synthesized with Mn\(^{2+}\) and subsequently incubated with 0.25 N NaOH for 10 min at 95 °C. DNA ladder products resulted from incubation of thermossequenase (mutant Taq pol I; Ref. 28) in the presence of ddNTP/ddNTP mix.

2 \times 10^6 base pair eubacteria genome (20), assuming the rUTP concentration is 10-fold greater than that of dTTP, WT Taq pol I should introduce approximately 10 rUMP into genomic DNA per replication cycle, and these substitutions would presumably be corrected by DNA repair. Other polymerases from prokaryotic pol I, eukaryotic pol, and RT families also discriminate against ribouracil (Table I). This DNA polymerase property of specifically discriminating against ribouracil might have resulted from natural selection.

Cells have evolved mechanisms to exclude uracil from DNA. When formed by deamination of cytidines (21), uracil is a potent promutagen inducing G-C\(\rightarrow\)A-T transition mutations (22). Uracil lesions are also formed by the incorporation of dUTP by DNA polymerases because dUTP can readily substitute for dTTP (Table I and Ref. 23). Removal of uracil residues in DNA is accomplished by uracil-DNA glycosylase, and repair synthesis involves the generation of single-stranded breaks, a potential source of mutagenesis (24, 25). In cells, rUTP is present in millimolar concentrations, well in excess of dTTP and dUTP concentrations (on average, 40 and 0.2 \(\mu\)M, respectively; Refs. 19 and 23) and thus can be a potent source of damage (e.g. single-stranded breaks) if introduced into DNA in amounts that exceed the capacity of the cell for DNA repair.

Analysis of a high resolution crystal structure of Taq pol I bound to DNA and dNTP (9) suggested that a single residue can sterically exclude the 2'-OH of an incoming rNTP (Fig. 4). This glutamic acid residue is conserved in over 35 prokaryotic and eubacteria pol I sequences, including that of \(E.\ coli\) pol I (GenBank\textsuperscript{TM}, Ref. 5). Polymerases in the pol and RT families contain a planar ringed amino acid (usually Tyr or Phe) at this locus; within the HIV-1 RT structure bound with DNA and dNTP the homologous Tyr-115 residue is positioned adjacent to the incoming nucleotide and likely also prevents incorporation of ribonucleotides by sterically interfering with 2'-OH group (11, 26). Modeling the selected substitutions into WT Taq pol I structure, followed by energy minimization suggests there are at least two mechanisms by which the steric interference conferred by Taq pol I Glu-615 with the 2'-OH group of the incoming ribonucleotide can be alleviated. First, modeling studies in Taq pol I suggests alterations that reduce the length of the Glu-615 side chain should permit ribonucleotide binding (Fig. 4 and Table III). Joyce and co-workers (12) demonstrated that E710A substitution within \(E.\ coli\) pol I (Klenow), at a site identical to Taq pol I Glu-615 residue, permits ribonucleotide incorporation. We find substitutions to aspartic acid for Glu-615 facilitate ribonucleotide incorporation. So far, we have not yet detected a Glu \(\rightarrow\) Ala substitution in the Taq pol
I motif A active mutant library, as well as within an extensive motif A mutant library of E. coli pol I, presumably because of reduced catalytic activity of mutants not containing either Asp or Glu at this site (5). Second, we find that diverse substitutions at the adjacent residue Ile-614 allow ribonucleotide incorporation and that evolved enzymes containing substitutions at position 614 utilize rNTPs at efficiencies equivalent to enzymes containing E615D substitutions (Fig. 3 and Table III). Analysis of Taq pol I structure model bound with DNA and a rNTP shows the ribose ring of the rNTP packs closely against Ile-614. Residue Ile-614 is located at a junction of a highly conserved β strand and α helix and is highly mutable. Energy minimizations of models of substitutions at this residue, which confers the ability to incorporate rNTPs, suggest that these substitutions cause this junction to be located further from the incoming nucleotide, thus allowing 2′-OH to fit.

In summary, we find that motif A within the DNA polymerase active site is highly plastic and can tolerate numerous substitutions while preserving physiologic DNA polymerase activity, and we have used this flexibility in structure to evolve a set of enzymes with altered substrate specificity. Mutant polymerases that can synthesize both DNA and RNA may be useful for biotechnology and allow automated coupled polymerase chain reaction amplification and transcription by cycling nucleotide triphosphates within the reaction mixture. These polymerases could also be used for DNA sequence analysis, following specific incorporation of a ribonucleotide during polymerase chain reaction and subsequent alkali cleavage at the position of ribosubstitution (27). In addition, these polymerases allow one to introduce nucleotide analogs containing adducts (e.g. fluorophores) attached to the 2′ ribose ring. The plasticity of the DNA polymerase active site should facilitate evolution within the laboratory of other polymerases for the incorporation of specific nucleotide analogs that are of mechanistic or medical importance.

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