We show that archaeabacterial DNA polymerases are strongly inhibited by the presence of small amounts of uracil-containing DNA. Inhibition appears to be competitive, with the DNA polymerase exhibiting ~6500-fold greater affinity for binding the inhibitor than a DNA.

9

Expected. Each is associated with a 3'-5' proofreading exonuclease activity, and none have a 5'-3' proofreading exonuclease activity. Archaea is a third kingdom distinct from eu-

karyotes (6, 7) and is thought to be evolutionarily closer to the thermophiles) or 37°C (pol II, Thermus aquaticus DNA polymerase) were incubated for 10 min at 70°C (pol I, Thermus thermophilus DNA polymerase) and were incubated for 10 min at 70°C (pol II, E. coli DNA polymerase and were incubated for 10 min at 70°C (pol I, E. coli DNA polymerase). Sequenase reactions contained 20 mM Tris-

HCl (pH 8.75), 100 mM KCl, 100 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin (supplied as a 10 x reaction buffer by New England Biolabs). Reactions for Pwo DNA polymerase were carried out in 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM (NH4)2SO4, and 2 mM MgSO4 (supplied as a 10 x reaction buffer by Boehringer Mannheim). Sequenase reactions contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 25 mM NaCl. E. coli pol II, Taq, Tth, Tt, Tae, Ultima, Dtol, and T5 DNA polymerase reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 5 mM MgCl2. E. coli pol I, reactions contained 50 mM potassium phosphate (pH 7.5) and 5 mM MgCl2. Polymerase α was in 40 mM Tris-HCl (pH 7.5) and 6 mM MgCl2. T4 DNA polymerase reactions contained 33 mM Tris-acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, and 0.1 mM bovine serum albumin. Unless stated otherwise, reactions contained 0.2 mM dNTPs, 0.4 units of Taq DNA polymerase, and 2 PM oligonucleotide primer. The DNA template for DNA polymerase activity was 1.4 nM (DNA circles) and 1.0 nM (DNA strands) in the usual manner.

The abbreviations used are: pol, polymerase; UDG, uracil DNA glycosylase.

MATERIALS AND METHODS

Oligonucleotides—Oligonucleotides were obtained from Life Technologies, Inc. (Table I). All of the oligonucleotides were determined to be substantially free of secondary structure by analysis with the Oligo Primer Analysis Program, Version 5.0 for Windows (National Biosciences, Inc., Plymouth, MN). Oligonucleotides were isolated with 

E. coli

DNA polymerase I, and T4 DNA polymerase were purchased from Life Technologies, Inc. DNA polymerases from bacteriophage T5 (9), Desulfurococcus strain Tok12-S1 (Dtol), Thermotoga neapolitana (Tne), and Thermus thermophilus (Tth) were cloned and purified at Life Technologies, Inc. Vent (Thermococcus litoralis), Vent exo (--), Deep Vent (Pyrococcus species GB-D), and 9°N DNA polymerases were from New England Biolabs, Inc., Beverly, MA. Pyrococcus polymerase was from Boehringer Mannheim. Pyrococcus fuscosus (Pfu) DNA polymerase was from Stratagene, Inc., La Jolla, CA. E. coli DNA pol II was a gift from Myron F. Goodman. Human DNA pol α was a gift from Dan Herendeen and Tom Kelly. Sequenase was from U. S. Biochemical Corp. Thermus flavus (Tfl) DNA polymerase was from Epicentre Technologies Corp., Madison, WI. Ultima DNA polymerase (derived from Thermotoga maritima, Tne) was from Perkin-Elmer.

Assays for DNA Polymerase Activity and Inhibition—Reactions for Vent, Vent exo (--), 9°N, and Pfu DNA polymerases were carried out in 20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin (supplied as a 10 x reaction buffer by New England Biolabs). Reactions for Pwo DNA polymerase were carried out in 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM (NH4)2SO4, and 2 mM MgSO4 (supplied as a 10 x reaction buffer by Boehringer Mannheim). Sequenase reactions contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 25 mM NaCl. E. coli pol II, Taq, Tth, Tt, Tae, Ultima, Dtol, and T5 DNA polymerase reactions contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 5 mM MgCl2. E. coli pol I, reactions contained 50 mM potassium phosphate (pH 7.5) and 5 mM MgCl2. Polymerase α was in 40 mM Tris-HCl (pH 7.5) and 6 mM MgCl2. T4 DNA polymerase reactions contained 33 mM Tris-acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, and 0.1 mM bovine serum albumin. Unless stated otherwise, reactions contained 0.2 mM dNTPs, 0.4 units of Taq DNA polymerase, and 2 PM oligonucleotide primer. The DNA template for DNA polymerase activity was 1.4 nM (DNA circles) and 1.0 nM (DNA strands) in the usual manner.

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‡ To whom correspondence should be addressed: Life Technologies, Inc., SG II, Lab 15, 8717 Grovemont Circle, P. O. Box 6009, Gaithersburg, MD 20884-9980. Tel: 301-670-7764; Fax: 301-921-2116.

1 The abbreviations used are: pol, polymerase; UDG, uracil DNA glycosylase.

2 R. Lasken and D. Chatterjee, unpublished results.

3 A. Rashtchian and R. Lasken, unpublished result.
inhibits Vent exo (−) DNA polymerase from utilizing a primed M13 single-stranded DNA substrate (Fig. 1). From 2 to 5 pmol of the dUrd-85-mer effectively blocked synthesis by −2 pmol of DNA polymerase, suggesting exceptionally tight binding of the enzyme to uracil-containing DNA. A control 85-mer with the same sequence but containing dThd instead of dUrd (dThd-85-mer, Table I) did not block the reaction. The dUrd-85-mer gave a similar level of inhibition to Vent exo (−) DNA polymerase, indicating that the 3′→5′ exonuclease activity of the enzyme does not affect the level of inhibition (data not shown). With DNAase I-activated salmon testis DNA as the substrate, 16 other DNA polymerases were tested for inhibition by the uracil-containing 85-mer (Table II). All five of the archaeobacterial DNA polymerases were inhibited at levels similar to those of Vent DNA polymerase. None of the other DNA polymerases was inhibited including five thermostable eubacterial enzymes derived from Thermus and Thermotoga spp. Human DNA pol α, E. coli pol II, and bacteriophage T4 DNA polymerase were not inhibited in spite of sharing sequence similarities with the archaeobacterial enzymes (5). A 42-mer with a sequence unrelated to the dUrd-85-mer and containing 10 dUrd residues (dUrd-42-mer, Table I) also was strongly inhibitory, suggesting that the presence of dUrd in any DNA sequence is sufficient for inhibition. A 71-mer containing a single dUrd substituted for thymine at position 23 (single-dUrd-71-mer, Table I) was also strongly inhibitory for Vent exo (−) DNA polymerase. The single-dUrd-71-mer inhibited 60% of the activity, while the dUrd-85-mer (which has 22 dUrd residues; see Table I) inhibited 94% of the activity. Globin mRNA had no inhibitory effect. Therefore, uracil in RNA does not have the same effect as dUrd in DNA. Inhibition from the dUrd-85-mer was not reduced when it was annealed with a complementary 85-mer (not containing dUrd) (Fig. 2). In this experiment, the dUrd-85-mer was annealed to an 8-fold excess of the complementary 85-mer before use as an inhibitor to DNA synthesis. The double- and single-stranded forms of the dUrd-85-mer had nearly identical levels of inhibition.

Relief of Inhibition by Treatment with Uracil DNA Glycosylase—As a further control to demonstrate the direct role of uracil in inhibition, the dUrd-85-mer was pretreated with UDG for removal of uracil bases from DNA. Pretreatment with UDG acted to relieve the inhibitory effect of the dUrd-85-mer (Fig. 3). UDG pretreatment of the dThd-85-mer control oligonucleotide (which is not an inhibitor; see Fig. 1) had no effect on the rate of DNA synthesis (data not shown).

Competitive Inhibition of DNA Polymerase by a Uracil-containing Oligonucleotide—To estimate the effect of uracil on Vent exo (−) DNA polymerase, the K_{app} was measured for DNase I-activated DNA substrate in the presence or absence of a fixed amount of dUrd-85-mer (Fig. 4). The increase in the slope for the Lineweaver-Burk plot in the presence of inhibitor is characteristic of competitive inhibition and indicates that the

### RESULTS

Inhibition of Archaeobacterial DNA Polymerase Activity by a Uracil-containing Oligonucleotide—In the course of studies using dUrd-containing primers as a method for cloning polymerase chain reaction amplified DNA (14, 15), we observed that when archaeobacterial DNA polymerases were used, reactions were consistently inhibited. Using an 85-base oligonucleotide containing dUrd (dUrd-85-mer, Table I) we studied the effect of dUrd-containing DNA on archaeobacterial DNA polymerases.

### METHODS

UDG Treatment of Oligonucleotides—145 pmol of the dUrd-85-mer were incubated at 37 °C for 15 min in the presence or absence of 1 unit of UDG (Life Technologies, Inc.) in a 29 μl reaction containing 20 μM Tris-HCl (pH 8.5) and 50 mM KCl. Then 5 pmol of the oligonucleotide were added to 200 μl of Vent exo (−) DNA polymerase reaction containing 100 μM activated salmon testis DNA substrate as described under "Assays for DNA Polymerase Activity and Inhibition." At the time points indicated, 20-μl aliquots of the reaction mixture were added to 5 μl of 500 mM EDTA.

Kinetic Gel Assay for dUTP Incorporation—The assay was carried out essentially as described (10, 11). The primer-template was designed with a single template A 16 nucleotides from the 3′-primer terminus (Table I). The primer and template oligonucleotides were purified by polyacrylamide gel electrophoresis. The primer was end-labeled using γ-32P-ATP (3000 Ci/mmol; Amersham Corp.) and polynucleotide kinase (Life Technologies, Inc.), annealed to a 2-fold excess of template by heating to 90°C for 2 min, and then left at room temperature for 1 h.

The primer-template was separated from unutilized oligonucleotide by electrophoresis; 3 ml of 500 mM EDTA. This large volume of stop solution gave a 6-fold dilution of the reaction that seemed to improve resolution under "Assays for DNA Polymerase Activity and Inhibition." At the time points indicated, 20-μl aliquots of the reaction mixture were added to 5 μl of 500 mM EDTA.

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Utilization of dUTP by Vent DNA Polymerase—A kinetic gel assay (10, 11) was used to determine whether uracil was inhibitory when it was present as the deoxynucleoside triphosphate, dUTP. This experiment allowed the direct measurement of the initial rate of incorporation as distinguished from any subsequent inhibition from the nascent dUrd-containing product which was predicted to be an inhibitor just like the dUrd-85-mer. Vent DNA polymerase was used because Vent is a well characterized representative of archaeabacterial DNA polymerases (16), and the exo(-) form was used because enzymes lacking a 3'→5' exonuclease activity require a simpler analysis (12). A primer-template (Table I) was designed with a single template A target site, designated position N, 16 nucleotides downstream from the 3' primer terminus. In a reaction containing dATP, dCTP, and dGTP but lacking dTTP, the DNA polymerase rapidly extended the primer by 15 nucleotides but was strongly blocked at the target A. The result is a strong band on a DNA sequencing gel one nucleotide before the target A at position N - 1 (Fig. 5A, arrow). Only a small proportion of the primers were extended past the A due to misincorporated dAMP, dCMP, and dGMP (12). The addition of dTTP or dUTP relieved the block as indicated by the loss of the band at N - 1 and the appearance of longer products. The relative rate for dUMP and dTMP incorporation was determined by comparing the concentration of nucleotide required to relieve the block. The relative velocity of incorporation is expressed as the ratio

\[ \frac{\text{Activity of dUMP}}{\text{Activity of dTMP}} \]
of the summed band intensities for position $N$ divided by the band intensity at $N$ (Fig. 5B). dUMP had an incorporation rate characteristic of a normal nucleotide, being only about 40% less than for dTMP. Therefore, the extreme inhibition from uracil-containing DNA (Fig. 1) is not observed when uracil is present as a deoxynucleoside triphosphate.

The DNA polymerase is also not inhibited immediately after incorporation of dUMP onto the primer terminus, as indicated by the rapid extension of primers past the target A site. If dUMP had been incorporated rapidly but then served as a poor substrate for subsequent extension, this would be revealed by the accumulation of product at the target position N or other downstream positions. No accumulation of these products is observed (Fig. 5A). As a further demonstration of this point, a time course of the reaction was carried out with saturating dUTP or dTTP, and the accumulation of full-length runoff products was measured. The full-length products accumulated at the same rate whether dUTP or dTTP was used (Fig. 6). This result requires that dUMP be rapidly incorporated and that subsequent extension of the primer is not blocked. Therefore, uracil at a 3' terminus does not trap the DNA polymerase in the same way that the uracil-containing 85-mer did (Fig. 1). To be certain that uracil was really the predominant base incorporated at the target A site, some of the runoff products were treated with UDG. More than 90% of these were cut (data not shown).

**DISCUSSION**

The goal of this study was to identify the nature of the inhibitory effect of uracil-containing DNA and the extent of the effect among DNA polymerases from different organisms. Inhibition of DNA polymerase apparently resulted from an extremely tight, nonproductive binding of the inhibitor. A kinetic analysis indicated a $-6500$-fold greater affinity of the DNA polymerase for the inhibitor over the activated DNA substrate (Fig. 4). Levels of activated DNA and the dUrd-85-mer were expressed as concentrations of nucleotides. No attempt was made to control for the difference in the length of the inhibitor (85 nucleotides) and the gapped DNA substrate (average length, several hundred nucleotides). While the measured $K_i$ is specific for this inhibitor, other uracil-containing oligonucleotides (Table I) and also an alternative DNA substrate, primed M13 single-stranded DNA, suggested $K_i$ values of similar magnitude. Although every dUrd-containing oligonucleotide tested was inhibitory, the $K_i$ may prove to be influenced by local sequence context as well as oligonucleotide length. The percentage of inhibition for a given concentration of inhibitor decreased with increasing activated DNA substrate consistent with a simple model of competitive inhibition. The extreme preference for binding inhibitor is consistent with the close stoichiometry between inhibitory levels of the dUrd-85-mer and DNA polymerase. Approximately 2 pmol of Vent exo (-) DNA polymerase were almost completely inhibited by 2-5 pmol of the dUrd-85-mer (Fig. 1). The direct role of uracil in the binding was shown by the relief of inhibition by treatment with
UDG (Fig. 3). Furthermore, an oligonucleotide of the same sequence but containing dThd in place of dUrd was not inhibitory. Even the presence of a single uracil in an oligonucleotide was sufficient for severe inhibition (Table II). Globin mRNA had no inhibitory effect. Therefore, uracil in RNA does not have the same effect as dUrd in DNA, although this may simply reflect differences in DNA polymerase affinity between RNA and DNA in general.

We also investigated the effect of uracil in the deoxynucleoside triphosphate, dUTP. A previous study (17) concluded that DNA polymerases tested were inhibited at similar levels. To date, the archaeobacterial DNA polymerases were inhibited. Human pol α, E. coli pol II, and bacteriophage T4 DNA polymerase were of particular interest because they are reportedly related to the archaeal polymerases (5). However, no inhibition was detected. The inhibitory effect is not entirely correlated with the thermostability of the DNA polymerases, since the thermophilic eubacterial enzymes derived from Thermus and Thermotoga spp. were not inhibited. However, we do note that the archaeobacteria involved can generally live at higher temperatures than Thermus and Thermotoga spp., and may be adapted for more extreme thermostability. Furthermore, it remains possible that the inhibition observed relates to some adaptation involving life at extreme temperatures that is absent in the evolutionarily divergent eubacteria. One possibility is that the strong binding serves a biological role involving the recognition and repair of uracils in vivo. In E. coli, uracil glycosylase recognizes uracils in DNA and excises them (18).

Further research on archaeobacteria may reveal whether the binding observed here is involved in this type of repair system. We have searched for primary amino acid sequence homology or conserved motifs in the sequences of known DNA glycosylases and archaeobacterial DNA polymerases. There are no obvious conserved motifs in the primary sequence. Although the crystal structure of UDG is known (19, 20), no archaeobacterial DNA polymerase structure is available yet.

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