Uracil in deoxyribonucleotide polymers reduces their template-primer activity for *E. coli* DNA polymerase I

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SUMMARY The physical and biochemical properties of two pairs of synthetic DNA template-primers were investigated. The copolymer poly(dA-dU).poly(dA-dU) and the homopolymer duplex poly(dA).poly(dU) were characterized by a lower T_m and by a higher buoyant density value than the respective thymine polynucleotides poly(dA-dT).poly(dA-dT) and poly(dA).poly(dT). The polymerizing and the primer terminus adding reactions of a homogenous *E. coli* DNA polymerase I preparation, as measured by incorporation of [3H]dAMP into the acid-insoluble fraction, were significantly poorer with uracil-containing template-primers than with thymine templates. Moreover, the uracil-containing polynucleotides inhibited the polymerizing activity of DNA polymerase I to a greater extent than the thymine polynucleotides, when the enzymatic activity was investigated with a dATP/dTTP/dUTP-free incorporation system making use of poly(dI-dC).poly(dI-dC) as the template-primer.

INTRODUCTION

The base uracil is not a conventional component of DNA. However, there are exceptions, as in bacteriophage PBS1, having uracil in its DNA substituting for thymine (1). Deoxyuridine triphosphate (dUTP) is a good substrate in vitro for prokaryotic (2) and eukaryotic (3) DNA polymerases. Although dUTP may be a normal substrate of thymidylate synthesis (4) the intracellular pool of dUTP is minimal. This is conceivably based on the activity of the enzyme dUTPase, which effectively hydrolyzes dUTP to dUMP and PP_i, thus preventing its misincorporation in semiconservative replication into DNA (4-6). If there is any uracil in DNA, either introduced by incorporation or by deamination of DNA cytosine (7), it will be excised by the enzyme uracil-DNA glycosylase, which specifically hydrolyzes the glycosidic bond between the sugar moiety and the base of DNA-dUMP (8). Uracil-DNA glycosylase is widely distributed (9-11) but not ubiquitous;

Drosophila melanogaster lacks this enzyme during a certain stage of development, but instead it has a nuclease that acts specifically upon DNA containing uracil (12).

In addition to PBS1 phages, significant amounts of uracil are found in the DNA of *E. coli* mutants deficient in both uracil-DNA glycosylase (ung) and dUTPase (dut) activities (13). Although dut ung mutants grew exponentially, their growth rates were slower than the growth rate of the wild type. The authors concluded that a plausible reason was the presence of uracil in DNA.

We have examined the effect of the replacement of thymine by uracil in synthetic DNA templates on their physical properties as well as on their template-primer activities for *E. coli* DNA polymerase I in vitro. The results indicated that the polymerizing and primer terminus-adding activities of the enzyme were poor with uracil-containing DNA templates, compared with corresponding thymine templates.

MATERIALS AND METHODS

Materials

Reagent chemicals were supplied by E. Merck (Darmstadt, West Germany) and by the Sigma Chemical Co. (St. Louis, Missouri). Nucleotides, nucleosides and bases were purchased from the Calbiochem-Behring Corp. (La Jolla, California). [Methyl-³H]-thymidine 5'-triphosphate (44 Ci/mmol), [5-³H]deoxyuridine 5'-triphosphate (11 Ci/mmol), [5-³H]deoxycytidine 5'-triphosphate (21 Ci/mmol), and [8-³H]deoxyadenosine 5'-triphosphate (22 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, England).

E. coli DNA polymerase I (EC 2.7.7.7.) was isolated from *E. coli* infected with a lambda (pol A) transducing phage carrying the wild-type gene coding for *E. coli* DNA polymerase I (14,15). This preparation has been purified to homogeneity by a modification of a method of Rhodes and coworkers (16). The enzyme was available in two different lots having specific activities of 21,750 and 11,000 units/mg of protein (New England Nuclear, Boston, Massachusetts).

Poly(dI-dC)·poly(dI-dC) was purchased from Boehringer-

Mannheim GmbH (Mannheim, West Germany). Other poly- and oligo-nucleotides were obtained from P. L. Biochemical Inc. (Milwaukee, Wisconsin).

DNA Polymerase Reaction

The basic reaction mixture consisted of Tris-HCl buffer (50 mM, pH 8.0), DTT (2.0 mM), MgCl_2 (10 mM), $[^3\text{H}]\text{dATP}$ (10 μM ; 0.1 Ci/mmol), dTTP (10 μM), 10 μM of the appropriate template-primer (calculated on the basis of molar extinction at 260 nm), and 10 units/ml of E. coli DNA polymerase I. Incubation was performed in 50 μl aliquots for 5 or 10 minutes at 37 °C. Many variations of this routine procedure were tested, as specified later. The reaction was stopped and polynucleotides precipitated, along with 10 μg of carrier DNA, with 10 % TCA. The precipitate was collected on a Whatman GF/C glass fiber filter, rinsed, dried and counted.

Thermal Melting of Template-Primers

The thermal melting properties of the dialyzed template-primers were analyzed with a continuously recording spectrophotometer (Varian Cary 118C) equipped with a warmable cell housing. In addition to the alternating copolymers and DNA duplicates with equimolar contents of dAMP and either dTMP or dUMP, we prepared corresponding 2:1 poly(dT)·p(dA)₁₀ and 2:1 poly(dU)·p(dA)₁₀ polymers by mixing appropriate amounts of the mother compounds in 0.1 N NaCl, pH 7.0. The other polymers were also dissolved in 0.1 N NaCl, pH 7.0; the temperature was raised at a rate of 1 °C/3 min.

Acid Hydrolysis of Template-Primers and Base Analysis

In order to check the base composition of the template-primers, 1 abs. unit (at 260 nm) was hydrolyzed with 98 % formic acid and the bases were resolved chromatographically (17).

Isopycnic Analysis of Template-Primers in CsCl Equilibrium

Gradients

The analysis was performed in neutral CsCl (10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA) according to Birnie (18). The centrifugation conditions were: an MSE Superspeed 75 ultracentrifuge, Titanium Swing-Out Rotor (6x5ml), 30,000 rpm, centrifugation time 90 hours at 25 °C. Fractions of 0.2 ml were collected and their optical density at 260 nm was analyzed. The density of CsCl in every fraction was determined with an Atago

Fig. 1. Thermal melting curves of synthetic DNA template-primers. (A) 2:1 poly(dU)·p(dA)₁₀ (●) and 2:1 poly(dT)·p(dA)₁₀ (○). (B) Poly(dA)·poly(dU) (●) and poly(dA)·poly(dT) (○). (C) Poly(dA-dU)·poly(dA-dU) (●) and poly(dA-dT)·poly(dA-dT) (○).

accuracy refractometer.

Rate-Zonal Centrifugation in Sucrose Gradients

Chain length estimation of the template-primers was performed by zonal sedimentation in concentration gradients of 5 to 20 % (w/v) sucrose in 0.7 M NaCl, 1 mM EDTA plus either 0.3 M NaOH or 0.3 M potassium phosphate, pH 7.4, in the above-mentioned rotor. The centrifugation time was 15 hours at 5 °C, at a speed of 40,000 rpm.

Estimation of Uracil-DNA Glycosylase and dUTPase Activities

The test enzyme *E. coli* DNA polymerase I preparation was assayed for uracil-DNA glycosylase and dUTPase activities as described in detail elsewhere (6,19); activities were nil.

RESULTS

Thermal Melting of Template-Primers

Figure 1 demonstrates the thermal denaturation curves of different polynucleotides. The T_m values of the uracil-containing polynucleotides were lower than those of corresponding thymine template-primers: (i) poly(dU)·p(dA)₁₀ 17.0 °C, poly(dT)·p(dA)₁₀ 30.0 °C ($\Delta T_m=13.0$ °C), (ii) poly(dA)·poly(dU) 56.0 °C and poly(dA)·poly(dT) 68.5 °C ($\Delta T_m=12.5$ °C), (iii) poly(dA-dU)·poly(dA-dU) 54.5 °C and poly(dA-dT)·poly(dA-dT) 61.5 °C ($\Delta T_m=7.0$ °C).

Fig. 2. CsCl equilibrium density gradient profiles of synthetic DNA template-primers. Densities at 25 °C (g/cm^3) are marked at the top. (A) Poly(dA)·poly(dT). (B) Poly(dA-dT)·poly(dA-dT). (C) Poly(dA-dU)·poly(dA-dU). (D) Poly(dA)·poly(dU).

Buoyant Density of Template-Primers

Figure 2 illustrates the profiles of isopycnic equilibrium density analysis with CsCl for different polynucleotides. The results indicated that the template-primers were homogenous and no odd-species of DNA analogues were present in the preparations

Chemical Composition of Template-Primers

According to semiquantitative base analysis, the relative purine/pyrimidine ratios of the template-primers were:

poly(dA)·poly(dU) 1.0 (reference); poly(dA)·poly(dT) 0.8;
poly(dA-dU)·poly(dA-dU) 0.9; poly(dA-dT)·poly(dA-dT) 1.0.

Sedimentation in Sucrose Gradients

The sedimentation coefficients of polynucleotide template-primers were calculated on the basis of rate-zonal centrifugation (20). (i) Alkaline polynucleotides, single stranded; poly(dA)·poly(dT) $S_{20}^0, w=6$; poly(dA)·poly(dU) $S_{20}^0, w=4$; poly(dA-dT)·poly(dA-dT) $S_{20}^0, w=8$; poly(dA-dU)·poly(dA-dU) $S_{20}^0, w=4$. (ii) Neutral polynucleotides, double stranded; poly(dA-dT)·poly(dA-dT) $S_{20}^0, w=10$; poly(dA-dU)·poly(dA-dU) $S_{20}^0, w=6$.

Polymerase Reactions

The template-primer activity of uracil-containing polynucleotides was inferior to that of the corresponding thymine template-primers. The overall differences between the two types of template-primers were: the incorporation of $[^3\text{H}]\text{dAMP}$ into poly(dA-dT)·poly(dA-dT) was 24.5 ± 12.6 picomoles/assay (mean $\pm$ SD,

Fig. 3. Incorporation of radioactive dAMP into the acid-insoluble fraction by E. coli DNA polymerase I in various conditions with poly(dA-dT)·poly(dA-dT) (—o—) and with poly(dA-dU)·poly(dA-dU) (—●—). The routine procedure was varied as indicated. (n=3).

n=11, results from two experiments combined), or 5.5-fold compared with poly(dA-dU)·poly(dA-dU) (4.5 ± 3.4 picomoles/assay), and into poly(dA)·poly(dT) 58.3 ± 4.9 picomoles/assay (n=6, results from one experiment), or 2.3-fold compared with poly(dA)·poly(dU) (24.9 ± 3.2 picomoles/assay). Both differences were statistically highly significant ($p < 0.001$; Student's t-test). Analogous differences were observed in modified conditions when reaction time, enzyme concentration, temperature or pH was varied (Figure 3). The results were similar if $[^3\text{H}]$ dTTP instead of $[^3\text{H}]$ dATP was used as tracer (results not shown).

Figure 4 illustrates the effect of substrate (dNTP) concentration on the enzymatic addition of deoxyribonucleotide

Fig. 4. Incorporation of radioactive dAMP into the acid-insoluble fraction by E. coli DNA polymerase I with poly(dA-dT)·poly(dA-dT) (—o—) and with poly(dA-dU)·poly(dA-dU) (—●—). (A) Standard reaction conditions; substrate (dATP/dTTP) concentrations were varied. (B) dTTP was omitted from the standard reaction mixture; dATP concentration was varied. (n=3).

Table 1. Template-primer activity of poly(dA-dU). poly(dA-dU) and poly(dA-dT).poly(dA-dT) for E. coli DNA polymerase I. Effect of replacement of dTTP by dUTP in the reaction mixture. Effect of 5 mM uracil.

Uracil-DNA glycosyl- ase inhi- bition	Incorporation of radioactive dAMP (picomoles/assay)			
	Into poly(dA-dU). poly(dA-dU)		Into poly(dA-dT). poly(dA-dT)	
	With dTTP	With dUTP	With dTTP	With dUTP
None	5.5	5.2	39.0	44.1
5 mM Ura	6.7	5.5	40.1	43.4

monophosphates when only one, and both of the triphosphates were present in the reaction mixture. No obvious differences were observed in the incorporation if only [^3H]dATP without cold dTTP was present in the reaction mixture. On the other hand, poly(dA-dU).poly(dA-dU) was a much poorer template-primer than poly(dA-dT).poly(dA-dT) in the large range of dNTP concentrations tested, if both complementary dNTPs were present in the reaction.

No remarkable changes in the incorporation of [^3H]dAMP was noted when dTTP in the standard reaction mixture was replaced by dUTP (Table 1). Moreover, Table 1 demonstrates that 5 mM of uracil (a potent inhibitor of uracil-DNA glycosylase, ref. 7) in the reaction mixture only slightly, if at all, increased the incorporation of [^3H]dAMP.

Inhibition of DNA polymerase I by Template-Primers

The template-primer concentration curves in Figure 5 illustrate that the uracil-containing template-primers were not only poor templates but that they inhibited the enzymatic polymerization of thymine-containing template-primers. This inhibition was independent of non-specific contaminants in the polynucleotide preparations, since a rigorous repurification of the template-primers with CsCl ultracentrifugation (see above) and several phenol extractions, followed by a 3-day dialysis, did not remove this inhibition.

The nature of template-primer inhibition was further evaluated using poly(dI-dC).poly(dI-dC) as a polymerizing template-

Fig. 5. Incorporation of radioactive dAMP into the acid-insoluble fraction by *E. coli* DNA polymerase I. Template-primer concentrations were varied. (A) Poly(dA).poly(dT) (—○—), poly(dA).poly(dU) (—●—), and both of the template-primers (—◐—). (B) Poly(dA-dT).poly(dA-dT) (—○—), poly(dA-dU).poly(dA-dU) (—●—), and both of the template-primers (—◐—). (n=3).

Fig. 6. Lineweaver-Burk plots of *E. coli* DNA polymerase I catalyzed DNA synthesis reactions. $V = [^3\text{H}]\text{dCMP}$ (A&B) or $[^3\text{H}]\text{dAMP}$ incorporation (C) (picomoles/10 min). The template-primer concentrations were varied; otherwise routine conditions. (A) Poly(dI-dC).poly(dI-dC) as a template-primer alone (—x—), and together with 1 μM of poly(dA-dT).poly(dA-dT) (—○—) or with 1 μM of poly(dA-dU).poly(dA-dU) (—●—). (B) Poly(dI-dC).poly(dI-dC) as a template-primer alone (—x—), and together with 5 μM of poly(dA-dT).poly(dA-dT) (—○—) or with 5 μM of poly(dA-dU).poly(dA-dU) (—●—). (C) Poly(dA-dT).poly(dA-dT) as a template-primer alone (—○—) and together with a fixed concentration (3 μM) of poly(dA-dU).poly(dA-dU) (—●—). (n=3).

primer and dGTP and dCTP as substrates. Figure 6 demonstrates that poly(dA-dT)·poly(dA-dT) as well as poly(dA-dU)·poly(dA-dU) were inhibitors of *E. coli* DNA polymerase I, the latter polynucleotide a much stronger inhibitor. Not even the highest poly(dI-dC)·poly(dI-dC) concentration, i.e., 10 μ M, was enough to restore the enzymatic activity, when inhibited by 1 μ M poly(dA-dT)·poly(dA-dT) or poly(dA-dU)·poly(dA-dU). The effect of a fixed poly(dA-dU)·poly(dA-dU) concentration (3 μ M) on the polymerase activity with varying poly(dA-dT)·poly(dA-dT) concentrations demonstrated a similar pattern in the Lineweaver-Burk plot (Figure 6).

DISCUSSION

DNA polymerase I can catalyze three different modes of polymerization: gap filling, nick translation, and strand displacement. In each of these three modes of polymerase I action, the basic reaction is the same: addition of a deoxyribonucleotidyl unit to a 3'-OH terminus with resultant pairing of the added nucleotides to the complementary nucleotides on the template strand (for review, see ref. 21). The results of the present investigation demonstrated that if thymine in synthetic polynucleotide templates was replaced by uracil, the net incorporation of nucleotides by DNA polymerase I was greatly reduced. Although the exact biological role of polymerase I is not known, the enzyme is required for both the repair and the replication of DNA in vivo (21). The poor template-primer activity of uracil-containing DNA, as demonstrated in this investigation, could be responsible for the reduced growth rate of *E. coli* ung dut mutants containing uracil in their DNA (13).

Although the alternating copolymer poly(dA-dT)·poly(dA-dT) and the homopolymer duplex poly(dA)·poly(dT) are well characterized in the literature (see ref. 22), very little data are available concerning the corresponding uracil derivatives. The buoyant density values in neutral CsCl for poly(dA-dT)·poly(dA-dT), 1.675 g/cm³, and for our reference calf thymus DNA, 1.700 g/cm³, were in good agreement with the reported values of 1.672-1.678-1.679 g/cm³ for poly(dA-dT)·poly(dA-dT) and 1.699 g/cm³ for calf thymus DNA (see ref. 23). In this

study the value 1.655 g/cm^3 was repeatedly obtained for poly(dA)·poly(dT) after 72 hour and 90 hour centrifugations. This value is slightly higher than the literature values of 1.635 – 1.638 – 1.647 g/cm^3 (23). The reason is not obvious; our centrifugation time may have been too short for less dense molecules, however. The buoyant density values for uracil-containing template-primers poly(dA-dU)·poly(dA-dU), 1.723 g/cm^3 , and poly(dA)·poly(dU), 1.695 g/cm^3 , were 43 and 40 mg/cm^3 higher than the values for corresponding thymine templates. A similar density difference has been recorded with poly(dA-dT)·poly(dA-dT)-primer-synthesized poly(dA-dU)·poly(dA-dU); 43 mg/cm^3 (24). The buoyant density of poly(dA-dU)·poly(dA-dU) obtained in the present investigation was similar to those of PBS1 and PBS2 phages (1.722 g/cm^3), where uracil replaces thymine (23). Furthermore, the replication of PBS2 with dUTP gives a product with a density equal to the primer (24).

The stabilizing effect of some substituents at the 5-carbon position, such as methyl or bromine in homopolymer helices, is known (25). The substitution of uracil for thymine decreased the thermal stability of the template-primers. The T_m values of 68.5°C for poly(dA)·poly(dT) and 61.5°C for poly(dA-dT)·poly(dA-dT) obtained in this work are in a full agreement with the T_m values (68.5°C and 61.0°C , respectively) reported in the literature (26).

The DNA of *B. subtilis* phage PBS2 contains uracil instead of thymine, and has, in comparison to DNAs of comparable %G:C, a T_m that is lower by 5°C (see ref. 24). The T_m of poly(dA-dU)·poly(dA-dU) (prepared enzymatically with dUTP and a primer) is reportedly 4.5°C lower than that of poly(dA-dT)·poly(dA-dT) (24). A quite similar T_m difference between poly(dA-dT)·poly(dA-dT) and poly(dA-dU)·poly(dA-dU), i.e., 7°C , was recorded in the present investigation. Moreover, our T_m of 54.5°C for poly(dA-dU)·poly(dA-dU) was exactly the same as the value in the literature for the same polymer obtained in 0.111 M K^+ at pH 6.8 (27).

Sági and Ötvös (28) found a direct relationship between the template-primer efficiency and the thermal stability of

DNA copolymers when either *E. coli* DNA polymerase I large fragment or *E. coli* RNA polymerase was used for catalysis. According to their data the lower the thermal stability the greater the template-primer efficiency value in their series of copolymers containing 5-carbon-alkyl-substituted uracil derivatives. Uracil with no 5-carbon substituent seems to be an exception to the rule; when present in polymers, it decreases the template-primer activity as well as the T_m .

Estimation of the primer activity of a template-primer requires information about free 3'-OH termini. Based on the sedimentation values of the template-primers studied in this work it can be roughly estimated (29) that the number of free termini in the uracil-containing template-primers was 5-fold compared with equimolar (dNMP) concentrations of thymine-containing template-primers. An equal incorporation of dAMP in poly(dA-dT)·poly(dA-dT) and poly(dA-dU)·poly(dA-dU) was noted when dTTP was omitted from the reaction mixture. The primer terminus adding activity of *E. coli* DNA polymerase I with poly(dA-dU)·poly(dA-dU) was then only 20 % of that anticipated on the basis of free 3'-OH termini and the priming activity of poly(dA-dT)·poly(dA-dT). This means that the priming terminus adding activity was deteriorated to a similar extent as the polymerizing activity, when thymine in the template-primer was replaced by uracil.

We do not have direct conformational information on the 3'-OH priming ends of these polynucleotides. It can be concluded from the present results, however, that dUMP in the 3'-OH terminus does not adversely affect polymerization. That is to say, dTTP in the reaction mixture could be replaced by dUTP without losing the activity of any type of template-primer. It could be the complementary dUMP in the template strand and not the primer 3'-OH dUMP, which impairs polymerization. On the other hand, demonstration of the inhibitory action of uracil-containing template-primers did not require direct contact of the enzyme with the incoming dTTP; the inhibition was also readily demonstrable with poly(dI-dC)·poly(dI-dC) as a template-primer, when the triphosphate substrates were dGTP and dCTP.

The mechanism by which uracil in polynucleotides impairs

their template-primer activity involves the reaction between the catalytic enzyme and the template-primer. This inhibition was strong, and, at least under certain conditions, appeared to be non-competitive in nature.

On the basis of previous data (28), which we have also confirmed (Vilpo, unpublished), and on the basis of the results of the present study, it can be concluded that uracil-containing synthetic DNA analogues are poor template-primers for DNA and RNA polymerases. If uracil-dependent inhibition takes place in vivo, then this kind of disadvantage could partially explain, why uracil is so strictly excluded from DNA in most species.

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