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Temperature-Sensitive DNA Polymerase Induced by a Bacteriophage T5 Mutant: Relationship between Polymerase and Exonuclease Activities†

Robert K. Fujimura* and Barbara C. Roop

ABSTRACT: DNA polymerase induced by bacteriophage T5*ts*53, a mutant with temperature-sensitive polymerase, was purified to about 95% purity as judged by dodecyl sulfate gel electrophoresis. The 3′ → 5′ exonuclease associated with the polymerase had higher activity than that associated with the parent wild-type enzyme. It was more stable to heat than the polymerase, and it degraded primer-template even in the presence of 4 dNTP's at higher temperature. However, the evidence presented shows that the inhibition of DNA synthesis by higher temperature was primarily due to defects in polymerase function rather than to overactive exonuclease. The presence of primer-template DNA stabilized the polymerase to heat. Purified *ts*53 polymerase was also shown to discriminate against incorporation of BrdUMP, especially at higher temperature. This is in agreement with observations made in vivo with *ts*53-infected bacteria.

Analysis of DNA synthesized in vivo in *Escherichia coli polA*$^-$ infected with T5*ts*53, a mutant with temperature-sensitive DNA polymerase (Dewaard et al., 1965), showed residual T5 DNA synthesis at nonpermissive temperatures which was due to phage-induced protein(s) (Fujimura, 1971a). This raised the question whether this synthesis is due to the phage-induced polymerase already identified or to another polymerase. Another difference observed at that time was that the repair-type synthesis at nonpermissive temperatures incorporated hardly any BrdUMP,[1] while replication at the permissive temperature did incorporate BrdUMP (Fujimura, 1971b). So another question raised was whether discrimination against BrdUMP incorporation at higher temperature was due to the polymerase.

To answer the above questions, all the detectable polymerases in *E. coli polA*$^-$ and *polA*$^-$, *polB*$^-$ mutants infected with T5*ts*53 were analyzed. There was only one phage-induced polymerase fraction (Fujimura, 1973a,b); it had activity to both denatured DNA and nicked DNA as primer-templates. Activities to two types of primer-templates were characterized further with wild-type T5 DNA polymerase purified to homogeneity and shown to be an intrinsic property of one DNA polymerase protein (Fujimura and Roop, 1976).

In preliminary work with purified temperature-sensitive polymerases (*ts*53 and *ts*5E), we have observed enhanced exonuclease activity at higher temperatures, which degraded primer-templates even in presence of 4 dNTPs (Fujimura, 1974). The purified wild-type polymerase was shown to have exonuclease associated with it (Steuart et al., 1968a) and to be 3′ → 5′ exonuclease (Das and Fujimura, 1976). This raised a possibility that inhibition of synthesis at higher temperatures with *ts*53 or *ts*5E polymerase may be due to higher 3′ → 5′ exonuclease activity.

This report presents evidence that inhibition of DNA synthesis at higher temperatures with *ts*53 polymerase is primarily caused by a defect in polymerase function of the enzyme (not by overactive exonuclease). It further shows that the purified *ts*53 polymerase does discriminate against BrdUMP incorporation at higher temperatures. A possible reason is also given for residual synthesis at higher temperatures in vivo.

Materials and Methods

Organisms. Bacteriophage T5*ts*53 was obtained from the late F. Lanni. Wild-type T5 phage T5$^+$, which is the parent strain of the mutant, was obtained from Y. Lanni. T5*rev* is a revertant strain picked from T5*ts*53 stock. Host bacteria for these phages are *E. coli* R15 *polA*$^-$ obtained from S. Kondo (Kato and Kondo, 1970).

Enzyme Assays. Both polymerase and nuclease assays were carried out as described previously (Fujimura and Roop, 1976)

† From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received April 5, 1976.* Research sponsored by the United States Energy Research and Development Administration under contract with the Union Carbide Corporation.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; BrdUMP, bromodeoxyuridine monophosphate; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Preparation of T5*ts*53 Polymerase (from 13.4 g of Cells).

Steps	Protein		Act. at 25 °C with Denatured T7 DNA		
	mg/ml	Total mg	Spec Act. (units/μg)	Total Units	Yield (%)
Phase separation	7.7	554	6	3.32×10^6	100
DEAE-Cellulose	0.56	142	19	2.70×10^6	81
Phosphocellulose	0.93	1.86	763	1.42×10^6	43
Sephadex G-200	0.18	0.47	1250	5.88×10^5	18

in the buffered salts: 67 mM Tris-HCl (pH 8.6), 6.7 mM $MgCl_2$, and 17 mM dithiothreitol. The amounts of DNA (in nucleotide units) and enzyme are specified in the figures and tables. For polymerase assays, 33 μM each of dCTP, dATP, dGTP, and dTTP (50 nCi of [*methyl*-^{3}H]dTTP per nmol) was used unless specified otherwise. The purified DNA polymerase preparations were diluted about tenfold with the enzyme dilution buffer containing serum albumin before use (Steuart et al., 1968a). One unit was defined as 1 pmol of [^{3}H]dTMP incorporated at 25 °C for 20 min under these conditions using alkali-denatured, intact T7 DNA.

[*methyl*-^{3}H]dTTP was obtained from Schwarz/Mann. [5-^{3}H]dCTP, [6-^{3}H]BrdUTP, and [α-^{32}P]dATP were obtained from New England Nuclear. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals. In some cases the purity of the dNTPs was analyzed by converting them to their corresponding monophosphates by use of snake-venom phosphodiesterase according to the method of Finamore and Warner (1963) (but scaled down to 50 μl). The product was analyzed by thin-layer electrophoresis at pH 3.5 as described previously (Fujimura, 1970).

T5-Induced DNA Polymerase Preparations. All T5 DNA polymerases were prepared from *E. coli* R15 infected with appropriate T5 phages as described for wild-type (Fujimura and Roop, 1976). For the temperature-sensitive polymerase preparations, the cells were infected with *ts*53 and incubated at 30 °C for about 50 min before being harvested. Preparation of temperature-sensitive polymerase is summarized in Table I.

For assays of the wild-type T5 DNA polymerase, we have routinely used 60 mM $(NH_4)_2SO_4$ when denatured DNA was used as a primer-template (Fujimura and Roop, 1976). However, as will be shown subsequently, the salt causes inhibition of the temperature-sensitive polymerase activity. Therefore $(NH_4)_2SO_4$ was not added to any reaction mixture in the current studies unless specified.

The specific activities of the temperature-sensitive DNA polymerase preparation and the T5$^+$ polymerase preparation used for comparison were about equal at 25 °C (1670 and 1690 units/μg, respectively, with the same preparation of denatured T7 DNA).

DNA Preparations. Most of the assays were carried out with fragmented, denatured, ^{32}P-labeled T7 DNA. Procedures for preparation of T7 DNA were the same as described previously (Fujimura and Roop, 1976) except for modifications described below. The ^{32}P-labeled T7 DNA was treated with pancreatic DNase at 2.7 ng/ml. The nicked DNA was then denatured directly in a dialysis tube by treatment with 0.2 N NaOH for 5 min. The solution was neutralized by titration with 1 M Tris-HCl (pH 4.1) and dialyzed against about 100 volumes of 0.02 M Tris-HCl (pH 8.1) containing 0.05 M NaCl and 1 mM EDTA. The DNA thus prepared was boiled for 5 min just before use (method 1). The average size of such DNA was determined by sucrose gradient centrifugation in neutral pH to be about one-fourth that of intact T7 DNA.

In some cases where higher activity was desired, T7 DNA was nicked with 13 ng/ml pancreatic DNase and denatured by boiling for 5 min (method 2).

DNA was labeled at both ends, starting from unlabeled, fragmented, denatured T7 DNA prepared by method 1. The 3′ ends of the DNA were labeled by incorporation of [^{3}H]dTTP using wild-type T5 DNA polymerase. To a reaction mixture (final volume, 2 ml) containing 33 μM each of 4 dNTPs, 25 μCi of [*methyl*-^{3}H]dTTP, and 30 mM $(NH_4)_2SO_4$, 160 μl of the enzyme dilution buffer and 1500 units of DNA polymerase were added. The reactions were carried out at 37 °C for 20 min and stopped by making the solution 10 mM in EDTA. The product was separated from the precursors using an 0.8 cm^2 × 10 cm Sephadex G-100 column equilibrated with 0.02 M Tris-HCl (pH 8.1) containing 0.05 M NaCl and 1 mM EDTA (Steuart et al., 1968a).

To label the 5′ end of DNA, the DNA from above was dephosphorylated with 0.4 unit of *E. coli* alkaline phosphatase (Worthington) per 1.2-ml solution and incubated at 37 °C for 30 min. The phosphorylation of DNA was carried out as described by Frenkel and Richardson (1971) with [γ-^{32}P]ATP (New England Nuclear), which was adjusted to 1.4×10^6 cpm/nmol, and polynucleotide kinase obtained from Terra-Marine Bio Research. After phosphorylation DNA was separated from the precursors by Sephadex G-100 chromatography as above, and the sample was further dialyzed against 1 l. of 0.01 M Tris-HCl (pH 8.6)–0.02 M NaCl.

Other Methods. Polyacrylamide gel electrophoresis runs were carried out and results were analyzed as described previously (Fujimura and Roop, 1976).

Protein concentration was determined by the method of Lowry et al. (1951) after protein was precipitated with trichloroacetic acid and resuspended in 0.1 ml of water.

Results

Exonuclease Activity Which Increases by Mutation is Associated with Purified DNA Polymerase. Temperature-sensitive DNA polymerase (*ts*53) purified by our methods appears to be about 95% pure when analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 1a). So for some reason it is less pure than a wild-type T5 DNA polymerase preparation. The mutant polymerase had the same mobility as a wild-type polymerase (data not shown). Gel electrophoresis of native protein showed that enhanced exonuclease activity at higher temperatures had the same mobility as the polymerase activity which was detectable only at a lower temperature and was superimposable on the main protein band

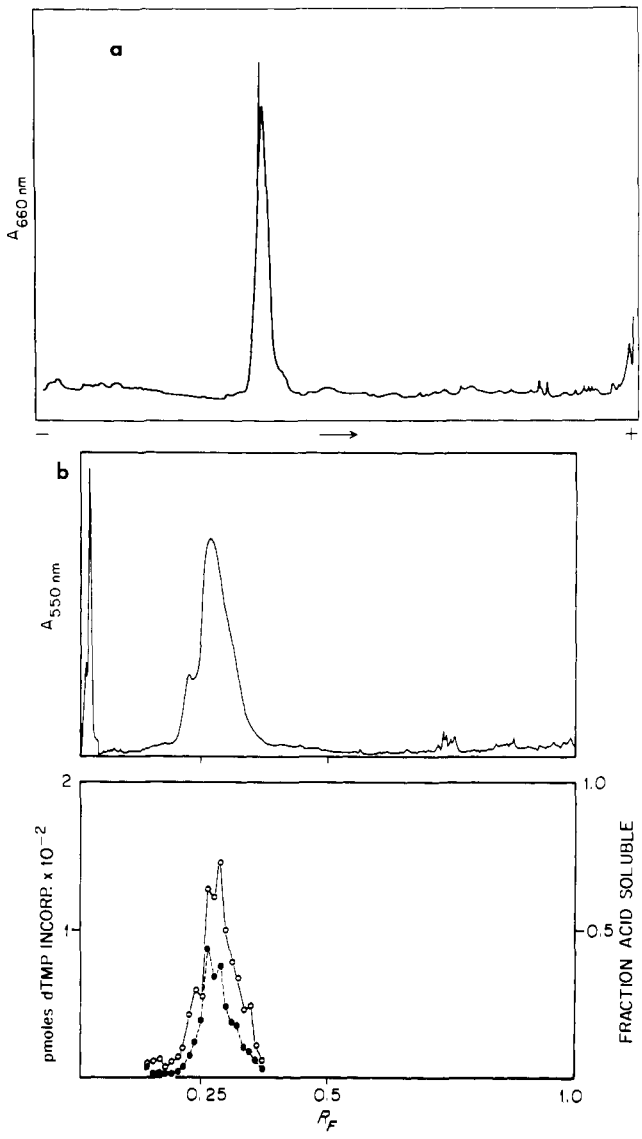

FIGURE 1: Polyacrylamide gel electrophoresis of temperature-sensitive T5 DNA polymerase. Electrophoresis runs were carried out and analyzed as described previously (Fujimura and Roop, 1976). (a) Denatured protein. About 9 μg of ts53 polymerase was used in 5% gel. (b) Native protein. About 7 μg of ts53 polymerase was used in 7.5% gel. Eluents from each gel slice were assayed for polymerase activity at 25 °C (○), and for nuclease activity at 43 °C (●) each for 40 min under the conditions described in Methods. Thirty-two nanomoles of fragmented-denatured ^{32}P-labeled T7 DNA produced by method 2 was used with 30 μl of eluent in each sample.

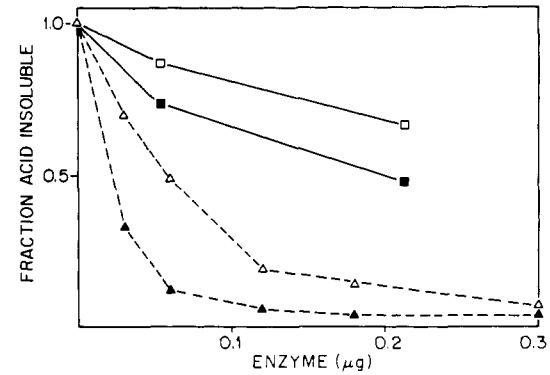

FIGURE 2: Degradation of terminally labeled DNA. Nicked and then denatured DNA (7 nmol) was treated with various amounts of *ts*53 and T5*rev* polymerase at 43 °C for 10 min in a 300-μl nuclease reaction mixture. Hydrolysis of the ^{32}P-labeled 5′ terminal (△) and the ^{3}H-labeled 3′ terminal (▲) of DNA treated with *ts*53 polymerase, and the ^{32}P-labeled 5′ terminal (□) and the ^{3}H-labeled 3′ terminal (■) of DNA treated with T5*rev* polymerase.

TABLE II: Comparison of Polymerase and Nuclease Activities at Different Temperatures.[a]

Temp (°C)	4 dNTPs	Initial Rate (pmol of Nucleotide/min) Degradation	Polymerization
	Temperature-Sensitive Polymerase		
25	−	7	
	+	*b*	120
37	−	74	
	+	68	77
43	−	120	
	+	76	28
	Wild-Type Polymerase		
25	−	4	
	+	*b*	189
37	−	26	
	+	*b*	642
43	−	83	
	+	*b*	1060

[a] Compositions of polymerase and nuclease reaction mixtures are given in Methods. Amounts of enzyme and DNA used were 0.26 μg and 32 nmol, respectively. [b] Amount too small to be detected by the technique used.

(Figure 1b). There appeared to be two protein bands. We feel the minor one was probably an extended form of the polymerase molecule due to instability of the conformation of the mutant polymerase protein, as will be suggested by subsequent data.

Fragmented, denatured T7 DNA labeled at both ends was used to test the direction of hydrolysis by exonucleolytic activity present at higher temperature with temperature-sensitive polymerase. The results showed (Figure 2) that the 3′ end was degraded faster. With revertant polymerase, which has temperature-stable polymerase activity (data not shown), the exonuclease activity was decreased. There was no detectable hydrolytic activity with micrococcal nuclease treated DNA (DNA with a 3′ terminal phosphate), but when such DNA was dephosphorylated there was activity (data not shown). The 3′ → 5′ exonuclease of T4 DNA polymerase has such a property (Nossal and Hershfield, 1971). Thus, the exonuclease activity enhanced at 43 °C by the temperature-sensitive mutation was 3′ → 5′ exonuclease, the same as that of wild-type (Das and Fujimura, 1976), and there was no detectable 5′ → 3′ exonuclease.

There was no detectable endonuclease activity when ϕX174 RFI DNA was treated with the DNA polymerase preparation and analyzed by alkaline sucrose gradient centrifugation (data not shown).

Inhibition of Polymerization Reaction at Higher Temperature is Due Primarily to a Defect in Polymerase Rather Than to Overactive Exonuclease. As shown in Table II, the exonuclease activity in the temperature-sensitive polymerase was higher than that in the parent wild-type DNA polymerase at all the temperatures assayed. However, the increase in the rate of hydrolysis with increase in temperature from 37 to 43 °C in the absence of 4 dNTPs was greater with wild-type than

TABLE III: Turnover of Nucleotides During Synthesis.[a]

Temp (°C)	Nucleoside Monophosphate (pmol (300 μl)$^{-1}$ (40 min)$^{-1}$)		
	Nucleoside Triphosphate		Soluble Primer-Template
	Incorporated	Turnover	
	Wild-Type Polymerase		
25	1860	600	*b*
43	11300	4700	1200
	Temperature-Sensitive Polymerase		
25	2130	560	*b*
43	60	390	5100

[a] Turnover experiments were carried out by modification of methods of Hershfield and Nossal (1972) described in detail elsewhere (Das and Fujimura, 1976). Polymerase reactions were carried out in the polymerase reaction mixture described in Methods by use of 35 nmol (360 cpm/nmol) of fragmented denatured T7 DNA prepared by method 1. Amounts of polymerases were adjusted to give about the same amount of incorporation of [^{3}H]dTTP at 25 °C. From each reaction product, 100 μl was used to determine the amounts of [^{3}H]dTMP incorporated into the acid-insoluble fraction and ^{32}P-labeled primer-template solubilized. When the fraction of DNA solubilized was less than 2%, it was not detectable in significant number by the method used. From 20- and 40-μl aliquots of the reaction products, [^{3}H]dTTP and [^{3}H]dTMP were separated by thin-layer chromatography on poly(ethylenimine)cellulose (Randerath and Randerath, 1967) developed in 1 M LiCl. The controls were subjected to all the processes of the samples at both temperatures without the enzymes, and background activities were subtracted from that of the samples. Values obtained for dTMP were converted to 4 dNTPs using a base composition of T7 DNA. [b] Amount too small to be detected by the technique used.

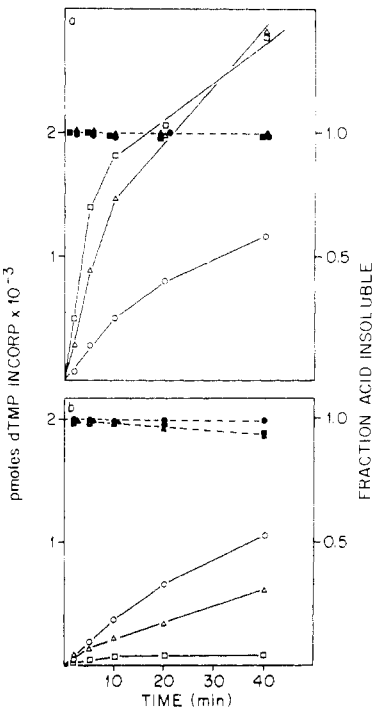

FIGURE 3: Temperature dependence of enzymatic activities in synthesis conditions. Time course of synthesis was determined at various temperatures with an excess of DNA. At zero time, the reaction tubes were transferred to the desired temperature bath as enzymes were added, and at each time point a 300-μl aliquot of reaction mixture was taken out. There were 32 nmol of fragmented, denatured T7 DNA and 0.26 μg of enzyme per 300 μl in the polymerase reaction mixture. Amounts of dTMP incorporated at 25 (○), 37 (△), and 43 °C (□), and concomitant amounts of fragmented, denatured T7 DNA remaining insoluble in acid at 25 (●), 37 (▲), and 43 °C (■) are shown. (a) Wild-type enzyme; (b) temperature-sensitive enzyme.

with *ts*53 polymerase. In synthesis conditions, degradation of primer-template was clearly detectable only with the temperature-sensitive polymerase, but there was only a slight increase between 37 and 43 °C (1.1-fold), accompanied by a greater decrease in polymerization rate (40% that at 37 °C).

T5 DNA polymerase catalyzes conversion of dNTP to dNMP in the presence of primer-templates (Das and Fujimura, 1976), as was shown with T4 DNA polymerase (Hershfield and Nossal, 1972; Muzyczka et al., 1972). According to these authors, dNTP must first be incorporated at the primer end before it is hydrolyzed to dNMP (probably by 3′ → 5′ exonuclease).

Thus the functional polymerase is essential for such a turnover reaction. If the defect in temperature-sensitive polymerase is in polymerase function, one would also expect less turnover of dNTP to dNMP under synthesis conditions at high temperature. The results were as expected (Table III). At 25 °C the amount of dNTP incorporated into DNA and the amount turned over were the same for wild-type and temperature-sensitive polymerase. With wild-type, at 43 °C there was close to eightfold increase in turnover; yet the amount incorporated increased about sixfold. With temperature-sensitive polymerase, there was no increase in turnover with increasing temperature, yet there was hardly any incorporation into DNA. The combined amounts of nucleoside monophosphates formed by turnover and degradation of primer-template were about the same for the temperature-sensitive and wild-type polymerases (5490 and 5900 pmol, respectively). Thus, a simplest explanation for inhibition of synthesis is that polymerase function is defective with the temperature-sensitive polymerase.

Inhibition of temperature-sensitive polymerase activity by higher temperature was almost instantaneous (Figure 3). The reactions at higher temperatures were carried out by addition of enzyme to reaction mixtures as they were transferred from 0 °C to a higher temperature bath. Yet the effect was noticeable by 2 min (Figure 3b). The time course of synthesis was biphasic, as is clearly shown for wild-type enzyme (Figure 3a); the nature of the biphasic time course is reported in a separate communication (Das and Fujimura, manuscript in preparation). With temperature-sensitive mutant polymerase, both early and late phases of synthesis were clearly inhibited (Figure 3b). Temperature-shift experiments also showed that the temperature effect is almost instantaneous (Figure 4). When the temperature was shifted from 25 to 43 °C in the middle of a time course, the synthesis stopped almost instantly and degradation of newly synthesized strand as well as preexisting DNA occurred. Fragmented denatured DNA was present in rate-limiting quantity. Apparently not all the DNA added was equally active as primer-template for the polymerase, but more were sensitive to the exonuclease activity. Thus concomitant hydrolysis of both preexisting and newly synthesized DNA occurred. Then after 10 min, when the temperature was lowered back to 25 °C, the synthesis started again almost instantly, with the rate almost the same as in the beginning. Thus the temperature effect was apparently reversible in the presence of DNA. However, if the enzyme was incubated alone at 43 °C, the polymerase activity was inactivated irreversibly at quite a fast rate (Table IV). The enzyme was incubated for various

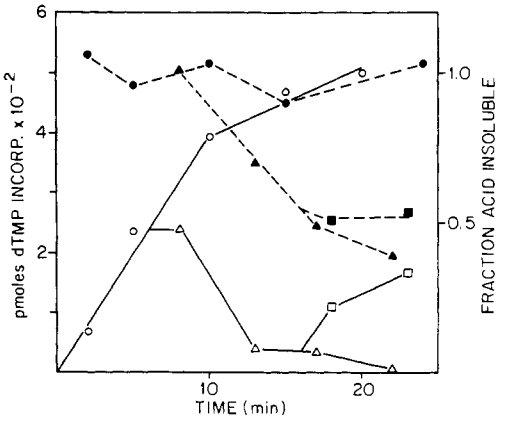

FIGURE 4: Effect of temperature shift during the course of the reaction. The reaction was started at 25 °C, and at 6 min an aliquot of the mixture was transferred to 43 °C. Then at 16 min an aliquot was transferred back to 25 °C. Fragmented-denatured T7 DNA (4.6 nmol/300 μl) was the rate-limiting substrate. The enzyme concentration was 0.54 μg/300 μl. At various times 300-μl aliquots were taken out. The time course of synthesis is shown at 25 °C (○), after the shift to 43 °C (△), and after the shift back to 25 °C (□). Fractions of primer-template DNA remaining insoluble in acid at these temperatures are shown by ●, ▲, and ■.

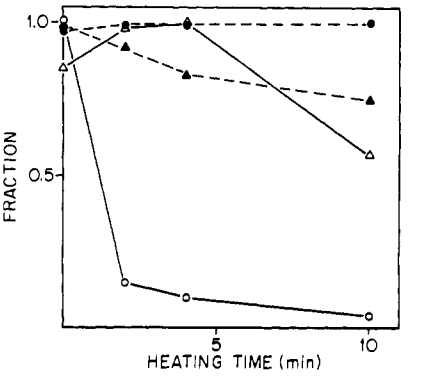

FIGURE 5: Effect of DNA-enzyme complex on the stability of enzyme to heat. In one set of reactions, DNA-enzyme complex was formed by preincubating for 2 min at 25 °C. The complex was then heated for various times at 50 °C in the polymerase reaction mixture described in Materials and Methods, the temperature was shifted to 25 °C, 4 dNTPs were added, and the mixture was incubated for 20 min (△). Primer-template was T7 DNA prepared by method 2. The concentrations of DNA and temperature-sensitive enzyme were the same as in Figure 3. In the second set of reactions, the enzyme in the enzyme dilution buffer was heated as described above; then DNA and 4 dNTPs were added and assayed at 25 °C for 20 min (○). Fractions of DNA which remained insoluble in acid for each type of reaction are shown by ▲ and ●, respectively. Each experimental point is an average of duplicates.

TABLE IV: Effect of Preincubation of Temperature-Sensitive Polymerase at 43 °C on Polymerization and Exonuclease Activity.[a]

Length of Preincubation at 43 °C (min)	Ratio of Initial Rate	
	Polymerase, 25 °C	Exonuclease, 43 °C
0	1.0	1.0
10	0.25	0.58
30	0.07	0.32

[a] Compositions of polymerase and nuclease reaction mixtures were given in Materials and Methods. Amounts of polymerase and fragmented denatured T7 DNA per 300 μl were 0.54 μg and 4.5 nmol, respectively. After preincubation at 43 °C for various lengths of time in the absence of 4 dNTPs, the time course of the polymerase reaction was determined at 25 °C by addition of 4dNTPs and the nuclease reaction at 43 °C. With nonpreincubated enzyme, the rate of nucleotide incorporation at 25 °C was 90 pmol/min, and the rate of nucleotides hydrolyzed at 43 °C was 144 pmol/min.

lengths of time at 43 °C and immediately transferred to 25 °C for polymerase assay. Within 10 min, 75% of the polymerase activity was irreversibly lost. The exonuclease activity was also being inactivated, but at slower rate (only about 40% within 10 min).

The results in Figure 4 suggest that the enzyme is more stable to heat in the presence of DNA. This was further investigated by direct tests of stability to heat in the presence of DNA. DNA-enzyme complex was formed in absence of 4 dNTPs and preincubated at 50 °C for various lengths of time; then 4 dNTPs were added and reactions were carried out at 25 °C for 20 min. Even after 10 min of heating, 60% of the polymerase activity remained (Figure 5). The primer-template was degraded during 50 °C incubation, as indicated by the fact that larger fractions were degraded with increasing heating time. When the enzyme was heated alone at 50 °C and later assayed at 25 °C, only about 10% of the polymerase activity remained after 4 min of heating. Thus, primer-template stabilizes the conformation of the enzyme for polymerase function.

The effect of salt suggested that the conformation of temperature-sensitive polymerase is less stable than that of wild-type. During the purification, the polymerase activity was assayed with alkaline denatured DNA, and it was noticed that $(NH_4)_2SO_4$ addition inhibited the reaction, unlike the case with wild-type polymerase. The effect of salt on temperature-sensitive and wild-type polymerases was compared under more controlled conditions with fragmented, denatured T7 DNA in excess. With the wild type polymerase, greater than 1.5-fold stimulation of the polymerase activity was observed in presence of 20 to 70 mM $(NH_4)_2SO_4$, but for the temperature-sensitive polymerase there was inhibition of the polymerase activity at the whole range studied (about 50% inhibition at 70 mM).

To test whether the enzyme at higher temperature would favor the exonuclease mode of binding at the primer site and thus inhibit the polymerase activity, additional enzyme was added after the initial synthesis had stopped. The volume of the second aliquot was negligible compared with the volume of the total reaction mixture, so that the effect on the temperature and concentration of other reagents would be minimal. As shown in Figure 6, when the reaction was initiated at 40 °C with DNA concentration limiting, the reaction came rapidly to a halt followed by degradation of the product. The amount of the enzyme was then increased twofold. The rapid incorporation of nucleotides again occurred, followed by degradation of the product. An additional net increase in nucleotide incorporation accompanied by decay was clearly observed every time this experiment was repeated. If the initial reaction had stopped because the exonuclease mode of binding at the primer site was favored even though the polymerase was functional, there should have been no additional net incorporation of nucleotides when fresh enzyme was added. A simple explanation is that the polymerase function is not heat stable, but the exonuclease function is more stable, causing hydrolysis of DNA.

Temperature-Sensitive Polymerase (ts53) Discriminates against Incorporation of BrdUMP at Higher Temperature. Previously in vivo studies with *ts*53-infected cells have shown

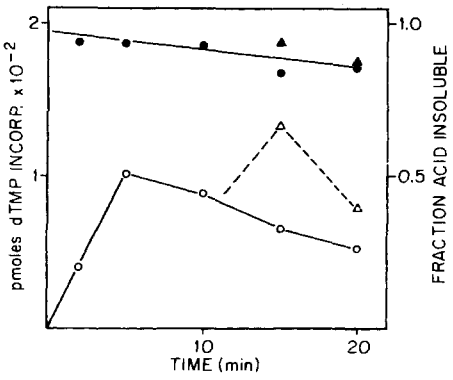

FIGURE 6: Effect of additional amounts of enzyme during a time course. With DNA concentration as the rate-limiting factor (as in Figure 4), a time course of reaction was carried out at 40 °C. At 11 min the amount of enzyme was increased twofold in one aliquot of reaction mixture (2 μl of the enzyme stock per 750 μl of the reaction mixture). A 300-μl aliquot was taken out at each time point. Amount of dTMP incorporated before (○) and after the additional amount of the enzyme (△). Acid-insoluble fractions of primer-template remaining before (●) and after (▲) enzyme addition.

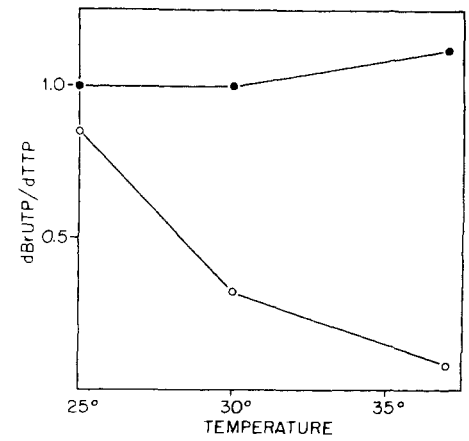

FIGURE 7: Effect of temperature on BrdUMP and dTMP incorporation. Two groups of 100-μl reaction mixtures were prepared in buffered salts consisting of 67 mM Tris-HCl (pH 8.6), 10 mM $MgCl_2$, and 10 mM dithiothreitol. Both groups contained 7 nmol of fragmented, denatured T7 DNA and 100 μM each of dCTP, dATP, and dGTP. One group contained 100 μM dTTP (150 nCi/nmol of [*methyl*-^{3}H]dTTP), and another group contained 100 μM BrdUTP (150 nCi/nmol of [6-^{3}H]BrdUTP). Both groups of reaction mixtures were used to assay with about 0.2 μg of $T5^+$ or *ts*53 polymerase, each with about the same specific activity at 25 °C. The reaction tubes containing each combination were incubated at 25, 30, and 37 °C for 20 min, and acid-insoluble radioactivity was measured in duplicate, as described in Materials and Methods. Ratios of [^{3}H]BrdUMP and [^{3}H]dTMP incorporated were determined at each temperature for $T5^+$ (●) and *ts*53 (○), and the ratio for $T5^+$ polymerase at 25 °C was taken as 1.0.

that, at high temperatures, there is no detectable amount of incorporation of BrdUMP into T5 DNA even though there is residual repair-type synthesis (Fujimura, 1971b). One of the possibilities raised in those studies was that *ts*53 polymerase discriminates against incorporation of BrdUMP at higher temperature. Thus incorporation of dTMP and BrdUMP was compared between wild-type and temperature-sensitive polymerases. The reactions were carried out with fragmented, denatured T7 DNA as primer-template in reaction mixtures with normal deoxyribonucleoside triphosphates and in another set of mixtures with BrdUTP substituting for dTTP. The reactions were carried out at various temperatures for 3, 6, and 20 min. As the temperature was raised to 37 °C, the amount synthesized with wild-type enzyme increased about threefold over that at 25 °C for both dTTP- and BrdUTP-containing mixtures. With temperature-sensitive polymerase, dTMP incorporation at 30 °C was about the same as at 25 °C, decreasing at 37 °C to about 30% of that at 25 °C; but BrdUMP incorporation was already decreased at 30 °C to 50% of that at 25 °C, and there was hardly any incorporation at 37 °C. The results of the experiments are summarized as the ratio of BrdUMP incorporated to dTMP incorporated after 20 min (Figure 7). The ratios of the amount of [^{3}H]BrdUMP incorporated to the amount of [^{3}H]dTTP incorporated were taken at each temperature and for each polymerase, and the ratio for wild-type at 25 °C was used as the reference. At 25 °C the ratio with temperature-sensitive polymerase was consistently slightly lower than that with wild type. At 37 °C the ratio was less than 10% that of the wild type. Similar results were obtained when [α-^{32}P]dATP was used as a label in both normal nucleotides and BrdUTP-containing reaction mixtures. However, with [^{3}H]dCTP as a label there was no obvious difference. One of the possibilities raised was that [^{3}H]dCTP has a small amount of contaminant; therefore the purity of [^{3}H]dCTP was tested. Only about 3% of the total radioactivity could be dTTP or dUTP. Base analysis of labeled nucleotides incorporated into DNA showed only [^{3}H]dCMP labels. Thus for some unexplainable reason, the amount of [^{3}H]dCMP incorporated was about the same for the normal nucleotide mixtures and BrdUTP-containing mixtures. However, the buoyant density in alkaline CsCl of DNA synthesized in the presence of BrdUTP with [^{3}H]dCTP as a label was less at 37 °C than at 25 °C (data not shown). The sample synthesized at 25 °C was as dense as the one labeled with [^{3}H]BrdUTP.

As was the case with normal nucleotides (Table III), turnover from BrdUTP to BrdUMP was less with *ts*53 polymerase at both 25 and 37 °C than with wild-type at 25 °C (data not shown). Thus the discrimination of BrdUMP incorporation was due to the polymerase function and not the exonuclease function.

Discussion

The data presented show that there are three differences between *ts*53 DNA polymerase and its wild-type parent polymerase.

First, the 3′ → 5′ exonuclease associated with the DNA polymerase is higher in *ts*53 polymerase preparations, and it is very likely caused by a single mutation that caused inhibition of DNA synthesis at higher temperature.

Second, the polymerase function of *ts*53 polymerase is defective at higher temperature, and this is the primary cause of inhibition of DNA synthesis. This defect is probably caused by instability in the conformation of protein which differentially inactivates the polymerase function over the exonuclease function. The data imply that the active sites for two enzymatic activities overlap, but are not identical. There are at least two possible explanations for differential stabilities of two enzyme activities. One is that the conformation around the active site is more critical for polymerase function than for exonuclease function. Thus at higher temperature, binding to DNA protects the protein conformation sufficiently to permit only exonuclease to function, but the distortion is so small that the enzyme reverts back to the form which permits polymerase function as soon as the temperature is lowered. Another possibility is that the conformation of the active site for the polymerase function remains intact at the higher temperature, but the exonuclease mode of binding to primer-templates is favored,

while at lower temperature the polymerase mode is favored. We feel the first explanation is favored by the experimental results of Figure 6, which shows that the fresh enzyme added during the time course of reaction at a high temperature functions as polymerase.

The third difference observed in *ts*53 polymerase is that the mutant polymerase discriminates against incorporation of BrdUMP, and the degree of discrimination increases with increasing temperature. In vivo studies with *ts*53-infected cells showed that, even at 25 °C, BrdUMP substitution of dTMP was lower than in wild-type infected cells (Fujimura, 1970), and at nonpermissive temperatures there was no detectable amount of incorporation of BrdUMP into DNA even though there was residual T5 DNA synthesis (Fujimura, 1971b). The results obtained in the present investigation for in vitro synthesis (as shown in Figure 7) were consistent with these observations. Discrimination against base analogue by *ts*53 polymerase is similar to the phenomenon observed with the mutator–antimutator group of T4 DNA polymerase mutants (Muzyczka et al., 1972; Hershfield, 1973). There are at least two types of antimutator strains. One type is caused by a relatively high level of 3′ → 5′ exonuclease which preferentially degrades misincorporated nucleotides (Muzyczka et al., 1972). The other type is caused by a defect in polymerase function which discriminates more against the incorporation of a wrong base. The *ts*53 polymerase is similar to this second group. Incorporation of base analogue was inhibited more without a concomitant increase in turnover of nucleoside triphosphates, and there was no preferential degradation of DNA containing BrdUrd (data not shown). Attempts were made to incorporate dCTP to poly(dA)·oligo(dT) with wild-type and temperature-sensitive DNA polymerase, but no significant amount of incorporation was observed with either polymerase.

In vitro DNA synthesis under our conditions of study is repair-type synthesis since it requires primer–template with a 3′-OH end, such as denatured DNA in "hooked" structure or with oligonucleotides (Steuart et al., 1968b), nicked DNA (Fujimura and Roop, 1976), or gapped DNA (data not shown). Figure 5 shows that DNA polymerase in complex with primer–template is more stable to heat than the enzyme by itself. Thus, the phage-protein-dependent, repair-type synthesis observed in vivo in *ts*53-infected bacteria at nonpermissive temperatures (Fujimura, 1971a) could be accounted for by the temperature-sensitive polymerase stabilized by complexing with nicked or gapped DNA. No other phage-induced DNA polymerase was found during the purification of the temperature-sensitive polymerase. There may be additional factors present which stabilize repair-type synthesis. Earlier preparations of *ts*53 polymerase were more stable for synthesis at 43 °C with nicked DNA as primer–template (Fujimura, 1973b). More recent preparations are more sensitive to temperature even though both preparations are of about the same purity as assayed by sodium dodecyl sulfate gel electrophoresis.

The purified T5 DNA polymerase appears to be more versatile than T4 DNA polymerase. Unlike T4 DNA polymerase (Masamune and Richardson, 1971), it is capable of synthesis from single-strand breaks with concomitant strand displacement (Fujimura and Roop, 1976). However, it does require many additional protein factors for replicative-type DNA synthesis as has been shown with many other organisms. Hendrickson and McCorquodale (1972) have isolated amber mutants of T5 for at least six different genes that are essential for DNA replication and several others that affect the rate and extent of DNA replication. Among these gene products there may be those that stimulate strand displacement, those that control processiveness of DNA polymerase during chain elongation, and those that affect the balance of polymerase–exonuclease activities.

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