EVIDENCE FOR TEMPLATE-SPECIFIC SITES IN DNA POLYMERASES *

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SUMMARY. Using rabbit hemoglobin messenger RNA as template, E. coli polymerase I produces poly (dT), poly (dA)·(dT) and antimessenger DNA products. Mild heating of the enzyme causes a differential loss in activity as indicated by three rates of inactivation for the three types of synthesis. Heat inactivation studies have also been carried out with DNA polymerases from oncogenic RNA viruses and mammalian sources using various homopolymer-oligomer pairs as primer-templates. In general, for any given enzyme these synthetic primer-templates reveal different extents of inactivation of the polymerase. These findings may be interpreted to suggest a) that the binding of DNA polymerase to various primer-templates produces conformational changes in the enzyme which are dependent on the type of template bound, or b) that many, if not all, DNA polymerases have different subsites for different templates.

In a previous paper we showed that a number of DNA polymerases can utilize natural and synthetic RNAs as templates, in which case sigmoidal saturation kinetics rather than the usual hyperbolic curves are observed for DNA synthesis with respect to polymerase concentration (1). Sigmoid kinetics may be taken as as evidence for a cooperative phenomenon when trivial artifacts are properly eliminated. We suggested from those results that a polymerase dimer (or oligomer) is formed on the primer-template in order for synthesis to proceed. We imagine the sequence to involve the binding of one monomer, with no synthesis occurring, followed by the more facile addition of a second (or third, etc.) monomer after which synthesis can proceed. We have also published evidence (1) for allosteric binding of deoxynucleoside triphosphates to the primer-template-polymerase oligomer complex, the first deoxynucleoside triphosphate acting as a modifier and the second, as the precursor.

These results, together with the fact that E. coli DNA polymerase I yields three different products with hemoglobin mRNA as the template, suggested that the synthetic center of DNA polymerases may be more complex than first imagined. In order to probe what might be called the fine structure of the active center, we have carried out heat inactivation studies on DNA polymerases from various sources. We observe that, for all the DNA polymerases studied, the decrease in the rate of DNA synthesis (when the enzyme is rate-limiting) due to heat inac-

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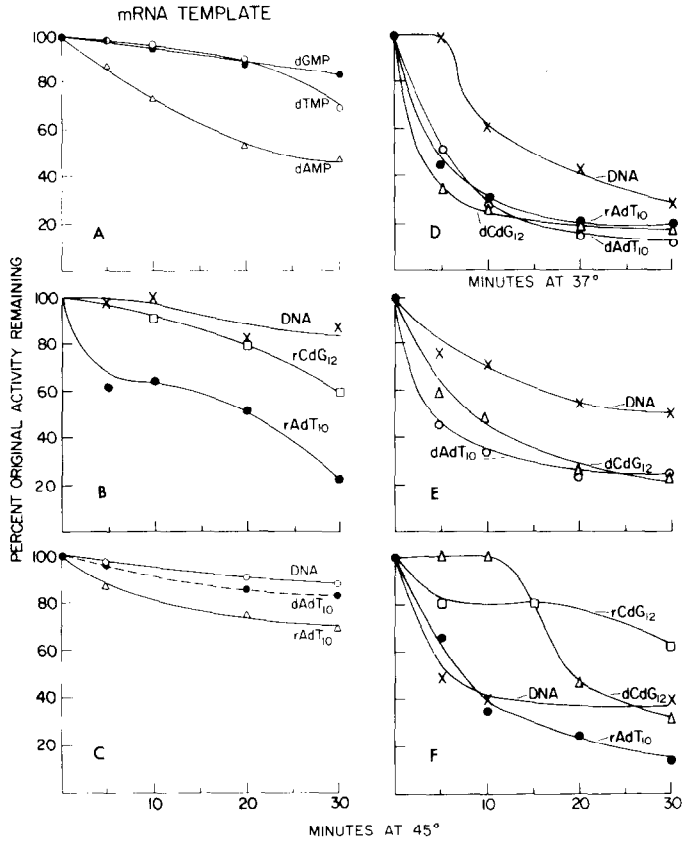


Legend for Fig. 1. Heat inactivation for various homopolymer-oligomer pairs (P-L Biochemicals, Inc.), hemoglobin mRNA (G.D. Searle Co.) and calf thymus DNA (5). Three DNA polymerases were used: E. coli polymerase I donated by Dr. L. Loeb (6) in Figs. 1A, 1C; Mason-Pfizer monkey tumor virus reverse transcriptase,, Fig. 1B; AMV reverse transcriptase, Fig. 1F (the latter two enzymes were donated by Dr. R.C. Gallo; 3.5S DNA polymerase from calf thymus chromatin (donated by Dr. L.C. Chang), Fig. 1E; 6-8S polymerase prepared from chronic myelocytic leukemia white cells according to McCaffrey et al (4), Fig. 1D. Heating was carried out at the times and temperatures shown. Reaction conditions in each case were chosen to give optional synthesis. Aliquots of 50 µl were withdrawn from the heated polymerase solution (0.05 µg/ml) in 0.05M Tris, pH 7.8-50 µg/ml albumin and added to an ice-cold reaction assay mixture (total final volume 0.1 ml) containing the template (8 µM, except for DNA which was 75 µM, calculated as nucleotide; 0.3 µM of oligo dT_{10} was annealed to globin mRNA) and 50 µg/ml albumin in buffer. Three buffers were used: Fig. 1A, 0.05M, Tris pH 7.8, 0.5mM $MnCl_2$ 0.025M KCl, and 10mM mercaptoethanol; Figs. 1B, 1C, 1D, 1F: 0.05M Tris pH 7.8, 2mM $MgCl_2$, and 10mM mercaptoethanol; Fig. 1E, 0.05M glycyl-glycine, pH 8.6, 8mM $MgCl_2$ and 1mM mercaptoethanol. The assay mixtures containing DNA or homopolymers (except poly (dA)·(dT_{10}) as templates were incubated for 30 minutes at 37°; those containing poly (dA)·(dT_{10}) were incubated for 5 minutes; with rabbit globin mRNA, incubation times were 30, 15 and 15 minutes for dGMP, TMP and dAMP incorporation, respectively. The specific activity of the labeled substrates was adjusted to 500 cpm/pmole. The concentration of the substrates was 8 µM in all cases. When more than one was used, the concentrations were equal. Reactions were stopped by 5% TCA-0.01M pyrophosphate, collected on GF/B filters and counted.

The 100% incorporation values in pmoles are: Fig 1A - dGMP, 13; TMP, 70; dAMP, 46. Fig. 1B - DNA 16.5; $rCdG_{12}$, 4.7; $rAdT_{10}$, 24.1.
Fig. 1C - DNA, 43; $dAdT_{10}$, 25.8; $rAdT_{10}$, 21.5.
Fig. 1D - DNA, 0.25; $rAdT_{10}$, 0.5; $dCdG_{12}$, 1.65; $dAdT_{10}$, 2.1.
Fig. 1E - DNA 25.6; $dCdG_{12}$, 13.1; $dAdT_{10}$, 2.1.
Fig. 1F - $rCdG_{12}$, 2.1; $dCdG_{12}$, 0.55; DNA, 0.22; $rAdT_{10}$, 2.2.

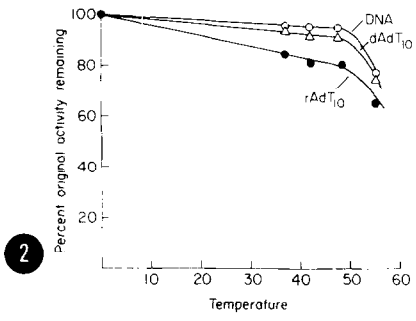
tivation of the enzyme is different for different templates.

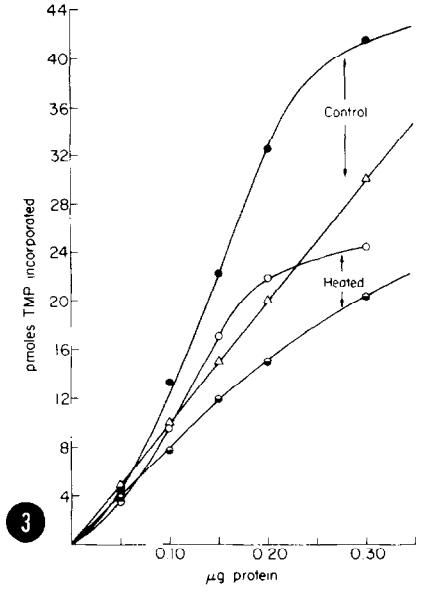
Figure 1A shows the gradual inactivation of E. coli DNA polymerase I produced by varying the time of pre-incubation at 45°C, using rabbit hemoglobin mRNA as the template. Three products are formed on this single template: poly dT, (using TTP as the only precursor); poly (dA)·(dT), (using dATP and TTP as the precursors); and DNA complementary to the mRNA, using H^3-dGTP with the other three unlabeled triphosphates. The analysis of these products and the reaction conditions have been described previously (2). The polymerase preparation is homogeneous and the three synthetic activities reside within one molecule (2). It can be seen from the figure that there are three different rates of inactivation of the DNA polymerase, corresponding to each of the three DNA products. While the difference between dGMP and TMP incorporation is small, it is reproducible. Assays were done in triplicate (error ± 2%) and the average value is shown. In Fig 1C is shown the effect of heat on DNA polymerase I using DNA, poly (dA)·(dT_{10}) and poly (rA)·(dT_{10}) as primer-templates. The rate of heat inactivation is significantly different for each of the primer-templates.

Differential heat inactivation with different primer-templates also occurs with other enzymes, namely, two oncornavirus DNA polymerases: one from Mason-Pfizer tumor monkey virus (Fig 1B), and another from avian myeloblastosis virus (Fig 1F). Two mammalian enzymes, the 3.5S DNA polymerase from calf thymus chromatin (3), Fig 1D, and the 6-8S polymerase from human leukocytes obtained from a chronic lymphocytic leukemia patient, Fig 1E, show the same effect. In almost all cases, poly (rA)·(dT_{10}) elicits the reaction most sensitive to heat inactivation of the enzyme.

Heat inactivation of E. coli polymerase I was also studied by heating for a fixed time interval at several different temperatures (Fig 2). Comparison of these results with those obtained by heating for different times at a fixed temperature (Fig 1C) should give the same order of inactivation with different primer-templates, in the absence of secondary complicating reactions. The results bear out this expectation. Again, although the differences are small, they are reproducible in each case. The primer-template poly (rA)·(dT_{10}) elicits the greatest difference in both cases. The curve for poly (dC)·(dG_{12}), not included in the figure, falls between the curves for DNA and poly (dA)·(dT_{10}).

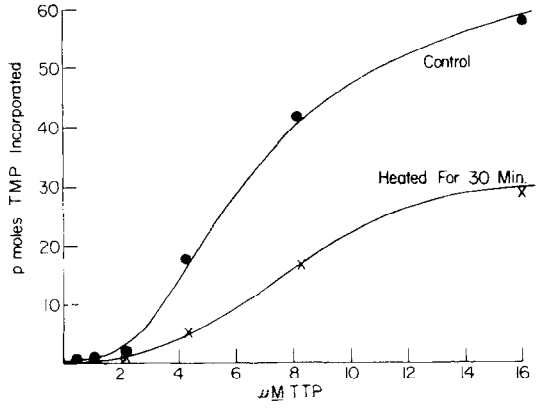
We had previously shown that E. coli DNA polymerase I exhibits cooperative phenomena (as evidenced by sigmoid saturation kinetics) with respect to both enzyme concentration and triphosphate concentration, using an RNA- but not a DNA-like template. We were interested to see if both cooperative effects persist after partial heat inactivation of the enzyme. Figs. 3 and 4 show that the sigmoid character of both types of curve persists after partial heat inactivation, using poly (rA)·(dT_{10}) as primer-template. With poly (dA)·(dT_{10}) as primer-template, the enzyme saturation curve remains hyperbolic (Fig 3). All the curves are displaced to lower incorporation values after partial heat inactivation; both the initial slopes as well as the final maximum rates are lower. The lat-





Legend for Fig. 2. Heat inactivation at various temperatures for five minutes using E. coli polymerase I. Assays and processing of samples as described in legend for Fig. 1. The 100% values in pmoles are the same as in Fig. 1C.

Legend for Fig. 3. Kinetics of synthesis by E. coli polymerase I as a function of polymerase concentration, using unheated polymerase (control) or polymerase heated for 30 min. at 45°. The primer-template is poly (rA)•(dT_{10}) (before heating ●——● and after heating ○——○)) or poly (dA)•(dT_{10}) (before heating Δ——Δ and after heating ⊖——⊖). Assay and processing of samples as described in legend for Fig. 1.



Legend for Fig. 4. Kinetics of synthesis by E. coli polymerase I (specific activity 50,000 nmoles/mg (1)) as a function of TTP concentration, using unheated (control) or heated (30 min. at 45°) polymerase. Poly (rA)•(dT_{10}) is the primer-template. Assays and processing of samples as described in legend for Fig. 1.

ter observation shows that partial heat inactivation affects all polymerase molecules rather than simply inactivating a certain fraction while the rest remain unaltered. The decreased initial slopes in Fig. 3 are consistent with a weakened binding constant of the enzyme to the primer-template. The lower maximum velocity is consistent with a decreased binding constant between the enzyme and the precursor and/or a change in the active center producing a decrease in the efficiency of catalysis; the former possibility is supported by the decreased initial slope in Fig. 4. A more definitive conclusion cannot be given because the possible involvement of other variables, such as metal ions, has not been investigated as yet.

In conclusion, we have found that inactivation of DNA polymerases produced by heating depends upon the type of primer-template used to measure that inactivation.* The data indicate that the apparent binding constants of polymerase to both primer-template and deoxynucleoside triphosphates are probably decreased by partial heat inactivation and there may be a change in the catalytic site as well. A decrease in the apparent binding constant between the polymerase and the primer-template could be explained by either an actual decrease in binding or by a decreased ability of the enzyme to undergo a conformational change induced by the primer-template to produce the active form. The differential heat inactivation observed with different primer-templates when the enzyme concentration is rate-limiting indicates either that the active site on the enzyme differs with different templates - an unlikely possibility - or that the template binding site is actually a collection of subsites with different specificities, which can be differentially inactivated. The results can be interpreted in either of two ways. The binding of the enzyme to the template may result in a conformational change of the enzyme which, in turn, results in the active form of the enzyme. We suggest that the ability to undergo this conformational change depends on the primer-template inducing it and the state of the enzyme. Partial heat inactivation could alter the enzyme such that it could no longer undergo a conformational change to the active form. An alternative interpretation of our results is that there exist subsites within the active center of the polymerase molecule and that they recognize specific bases and the type of sugar-phosphate backbone of the primer-template. Heat inactivation of the enzyme may be affecting these specific subsites in some fashion and cause the observed differences in the utilization of specific primer-templates. Clearly, however, not all of the results are explained by these interpretations, suggesting other subtleties within the active centers of DNA polymerases which await further clarification.

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* Purified mouse mammary tumor virus reverse transcriptase, upon storage, has been found to lose DNA-polymerizing ability at different rates depending upon the primer-template used to measure activity (A. Dion and A. Vaidya, personal communication).

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