

## POLYDEOXYTHYMIDYLATE AS TEMPLATE FOR COMPLEMENTARY ENZYMATIC SYNTHESIS

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Received 30 September 1971

### 1. Introduction

Calf thymus DNA polymerase [1] catalyzes the polymerization of dATP in the presence of polydeoxythymidylate,  $dT_n$ . The polydeoxyadenylate segment is attached by phosphodiester linkage to the 3'-end either of the  $dT_n$  or of an added oligodeoxyadenylate initiator [2]. In contrast, an absolute requirement for exogenous initiation is characteristic of *Micrococcus luteus* DNA polymerase [3]. For both enzymes the reaction does not proceed beyond 1-fold synthesis. However, various extents of reaction (1-fold and less) have been reported [2-4]. One explanation for early conclusion of the reaction is formation of a stable triple-stranded structure involving two strands of  $dT_n$  and one of  $dA_n$ .

The triple-stranded complex  $dA_n \cdot 2dT_n$  has been observed on physical mixing of the single strands in the proportion 1  $dA_n$  to 2  $dT_n$  at high sodium ion concentration where  $n$  was in the range 3,000-6,000 nucleotide units [5]. The product was characterized by its two-step thermal hyperchromicity profile at 260 nm, for which the  $T_m$  values in 0.2 M  $Na^+$  are 43° and 74.5°, by equilibrium gradient centrifugation in cesium sulfate, and by a thermal transition that occurs at 284 nm for triple- to double-stranded and not for double- to single-stranded. The molecular structure of the polymerase-catalyzed, tripled-stranded product may have two quite different forms. One structure exists because half of the  $dT_n$  strands have

completed 1-fold synthesis and are associated with the remaining half of the  $dT_n$  in a triple-stranded structure. Alternatively, 0.5-fold synthesis has occurred on all of the  $dT_n$  strands, and the single-stranded portion of the  $dT_n$  has looped back upon the double-stranded structure to give the triple-stranded product.

This work is concerned with determination of the structures and finding how the experimental conditions influence their formation. All syntheses with a  $dT_n$  template were run without initiator to aid identification in that the newly-synthesized segment cannot depart from the template.

### 2. Materials and methods

Terminal deoxynucleotidyltransferase [1] was used [6] to polymerize dTTP onto tritium labeled  $dT_4$  [7], resulting in  $dT_n$  samples with average  $n$  values of 110, 170, and 460 as determined by radioisotopic dilution [6] and by agarose column chromatography [8]. Similarly, dATP and  $dA_6$  gave  $dA_{240}$ .

Calf thymus DNA polymerase [1], 756 units/mg (1 unit = 1 nmole of dTTP polymerized per mg of protein per hr at 37° using  $dT_6A_{100}$ ,  $dT_6$ , and dTTP-2- $^{14}C$ ), was titrated in a series of reactions at 15° in 40 mM potassium phosphate buffer (pH 7.0), conditions under which the polymerase does not recycle after completing one round of 1-fold synthesis. The

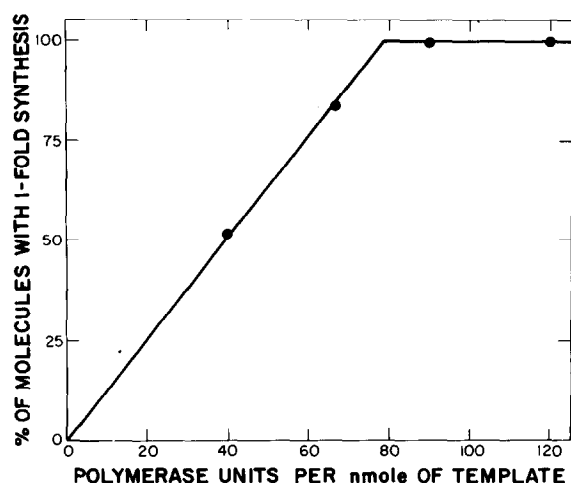


Fig. 1. Titration of calf thymus DNA polymerase with  $dA_{240}$  at  $15^\circ$ . Each reaction contained  $4.5 \mu M$   $dA_{240}$ ,  $4.8 \mu M$   $dT_{10}$ ,  $2.5 mM$   $dTTP-2-^{14}C$  (1,600 dpm/nmole),  $8 mM$   $MgCl_2$ ,  $1 mM$  2-mercaptoethanol, and  $45 mM$  potassium phosphate buffer (pH 7.0) plus calf thymus DNA polymerase as indicated in the legend of the abscissa, all in  $0.10 ml$  volume.

reactions employed  $dA_{240}$ ,  $dT_{10}$ , and  $dTTP-2-^{14}C$  and were allowed to proceed until no further synthesis was measurable using the filter paper disc method [9]. By varying the amount of polymerase for each reaction the equivalence point was determined (fig. 1) to be 78.5 units per nmole of template. If one polymerase molecule binds to one template molecule, a molecular weight of 103,800 daltons can be calculated — in agreement with the value of 110,000 for the same enzyme activity determined by Sephadex G-200 column chromatography [10].

A series of reactions using  $dT_{110}$ ,  $dT_{170}$ , or  $dT_{460}$  were run for a minimum of 24 hr at  $37^\circ$  in 2.8 ml total volume containing  $5 \mu M$  template, a 5-fold excess of  $dATP-8-^{14}C$  over that required for 1-fold synthesis,  $8 mM$   $MgCl_2$ ,  $1 mM$  2-mercaptoethanol,  $40 mM$  potassium phosphate buffer (pH 7.0), and calf thymus DNA polymerase in equivalence ratios to template (R) of 4, 2, 1, 0.5, 0.3 and 0.2. The reactions were monitored for completion of synthesis by filter paper disc method [9]. Protein was removed

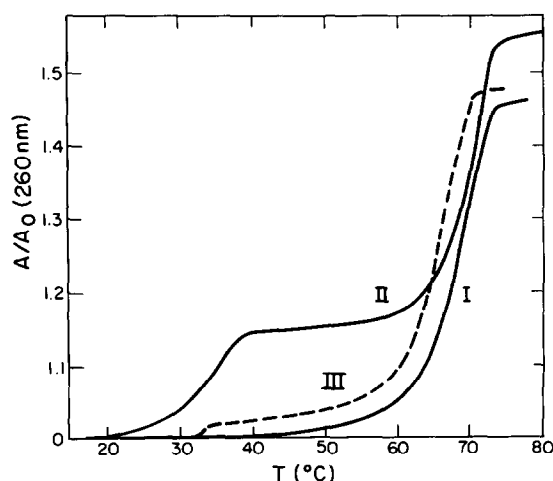


Fig. 2. Melting profiles of polydeoxythymidylate-polydeoxyadenylate complexes. The temperatures on the abscissa are corrected and were measured in the solution as it was simultaneously heated and analyzed spectrophotometrically at 260 nm [13]. The solutions contained  $0.1 M$  NaCl and  $0.05 M$   $Na_2HPO_4$  (pH 7.0, adjusted with  $H_3PO_4$ ) in a total volume of  $1.0 ml$ . Solution I also contained  $0.59 \mu M$   $d(T_{110}-A_{110})$ . The polymer in solution II was from the  $15^\circ$  reaction; solution III was from the prematurely stopped reaction.

by phenol extraction [11], and agarose column chromatography in  $5 mM$  NaCl [9] separated all polynucleotide material from low molecular weight substances. Each isolated polymer was analyzed for phosphorus content [12], UV absorption, radioactivity, and thermal hyperchromicity [13], fig. 2. Two further reactions were run as above except that R values were 1 and 0.33, the potassium phosphate buffer (pH 7.0) was  $0.15 mM$ , and 3 days were needed for completion.

Having determined that all these reactions had gone completely 1-fold, two identical reactions were run at  $37^\circ$  and  $R = 2$ , stopping one when it had reached an estimated 0.5-fold synthesis and letting the other proceed to completion. Finally, one reaction at  $15^\circ$  was run at  $R = 0.3$ . By filter paper disc analysis it appeared that termination of synthesis occurred at about 0.35-fold synthesis.

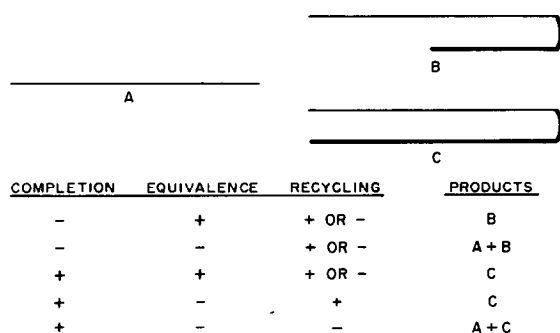


Fig. 3. Relationship of conditions to products in uninitiated calf thymus DNA polymerase reactions utilizing  $dT_n$  (A) as template with dATP. The dA units are represented by the heavy lined portions of the 0.5-fold synthesis product B and the 1-fold C. Lack of completion refers to purposeful or accidental premature termination of reaction either abruptly or by depletion of an essential ingredient. Equivalence and recycling refer to the polymerase in quantity and incubation temperature used.

### 3. Results and discussion

All reactions at  $37^\circ$  that were allowed to proceed to completion gave products with specific radioactivity indicating a dA/dT ratio of  $1.0 \pm 0.1$ , with agarose column chromatography showing molecular weight approximately double that of the template used, and with a single-step melting profile (curve I, fig. 2). By these criteria the products have the structure  $dT_n-dA_n$ . The product from the reaction using insufficient polymerase at  $15^\circ$ , a temperature that inhibits recycling, gave a dA/dT ratio of 0.38 and the melting profile of curve II, fig. 2. Such results suggest that the product is the mixture  $dT_n-dA_n + dT_n$ . This same profile was obtained from an equimolar physical mixture of  $dT_n$  with the material that gave curve I. The product from the reaction that was prematurely stopped showed (curve III, fig. 2) only a trace of a low temperature transition followed by a sharp melting at about  $3^\circ$  below that of curve I. The analytical value for dA/dT was 0.47. The structure of this product can be written  $dT_n-dA_{0.47n}$ ; the lowered  $T_m$  of the large hyperchromicity transition is due to the shortened continuous length of dA · dT hydrogen bonding.

The conclusions of this study are given in fig. 3. There are four types of products or product mix-

tures: B, in which the reaction was prematurely terminated, shown here arbitrarily as approximately 0.5-fold synthesis; C, in which 1-fold synthesis was completed on all template strands; A + B, in which both premature termination and insufficient polymerase left some unreacted A along with B; and A + C, in which insufficient polymerase under non-recycling conditions gave 1-fold synthesis on only part of the original A. All of these cases have been encountered in this work, and in each case the experimental conditions seem to account for the nature of the product according to the logic of fig. 3.

Cases B and A + C both have the potential for existing in a triple-stranded structure. Looping back of the single-stranded portion of B would form a structure in the polarity arrangement with  $T_1$  antiparallel to both A and  $T_2$ , where  $T_1$  and A represent the  $dT_n$  and  $dA_n$  segments held together by Watson-Crick [14] hydrogen bonding, and  $T_2$  is another segment of  $dT_n$  functioning as the third strand. The preferred structure of Miles [15] for  $rA_n \cdot 2rU_n$  has  $U_1$  and  $U_2$  both antiparallel to A. Construction with CPK atomic models of each of the  $T_1$  and  $T_2$  both antiparallel to A and the  $T_1$  antiparallel to both A and  $T_2$  structures showed that the former or Miles structure was superior in both steric freedom and separation of phosphate groups. Only the A + C product can readily assume the Miles structure; it gave the two-step melting profile (curve II, fig. 2). The intramolecular triple-stranded structure from B is not of the Miles polarity; the melting profile is (curve III, fig. 2) almost entirely the single-step, double- to single-stranded transition.

### Acknowledgement

This work was performed under the auspices of the U.S. Atomic Energy Commission.

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