DNA COMPLEMENTARY TO RABBIT GLOBIN mRNA MADE BY E. COLI POLYMERASE I *

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SUMMARY. E. coli DNA polymerase I has been used to synthesize DNA complementary to rabbit globin mRNA. In addition to the heteropolymeric DNA, poly (dT) and poly (dA)·(dT) are also synthesized. The extent of synthesis of these three products decreases at different rates upon heat inactivation of the polymerase, suggesting that the sites of synthesis on the enzyme are either entirely or partially separate. The use of polymerase I for copying RNAs makes possible the ready availability of complementary DNAs.

INTRODUCTION

Many DNA polymerases possess the ability to use various RNAs, both synthetic and natural, as templates in a reaction which produces DNA products. Some of these DNA polymerases have been termed reverse transcriptases. However, at present, the precise attributes of a reverse transcriptase, as opposed to a DNA polymerase, are unknown, for in many cases both activities can be elicited from the same molecule by altering the reaction conditions. Even with a single template, more than one type of product can be obtained. We have studied this situation using a well-characterized enzyme, E. coli DNA polymerase I, and a relatively simple natural RNA template, rabbit globin mRNA.

We show that three products can be formed: poly (dT), poly (dA)·(dT) and complementary DNA copies of the globin mRNA. The first two are synthesized more rapidly than the last; the production of all these products is low in the presence of Mg^{++} but is markedly increased in the presence of $MnCl_2$ and KCl. This may help explain why some investigators using natural RNAs have obtained DNA products (1,2) while others (3) report that only homopolymers are produced with the E. coli enzyme.

Taking advantage of the fact that this single template yields three different products depending on the conditions, we have carried out heat inactivation studies of the enzyme with respect to the synthesis of each of the three products. There are different rates of inactivation for each of the three products formed, suggesting that these synthetic activities reside in different subsites of the active center. Differential inactivation rates have also been observed with different templates, for a number of DNA polymerases (8). The separation of different activities within the molecule may prove to be generally true of DNA polymerases and this provides a basis for studying the differential inhibition of DNA synthesis by potential chemotherapeutic agents.

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RESULTS

Conditions of the Reaction. (a) Metal ions: Table 1 shows the effect of varying the KCl and $MnCl_2$ concentrations on heteropolymer synthesis (indicated by the incorporation of H^3-dGMP in the presence of the other three unlabeled triphosphates). Synthesis is maximal between 0.025 and 0.05M KCl in 0.5mM $MnCl_2$ and between 0.05 and 0.5mM $MnCl_2$ in 0.05M KCl. Table 2 shows that the synthesis of all three products is promoted by KCl and $MnCl_2$ at these concentrations, as compared to 2mM $MgCl_2$. Poly (dT) synthesis is indicated by the incorporation of H^3-TMP as the sole substrate, and synthesis of poly (dA)·(dT) by the incorporation of TMP + H^3-dAMP.

(b) Concentration of mRNA and amount of product formed: In an assay mixture containing 0.05 µg of E. coli polymerase I (4), the enzyme is nearly saturated by 0.2 µg of mRNA; increasing the mRNA to 2 µg leads only to a two-fold increase in incorporation. It can be seen from Table 2 that 5 pmoles of dGMP were incorporated in the presence of 650 pmoles of mRNA nucleotide. This corresponds to a transcription of about 2.5% of the mRNA in 30 minutes. The same incorporation was obtained in 45 minutes; at 90 minutes incorporation was down by 20%. This suggests nucleolytic action and we surmise that the low yield of complementary DNA may, in part, be due to this. The extent of synthesis varied somewhat for different mRNA preparations. The homopolymers are formed in amounts up to 8 times larger than the heteropolymeric product.

(c) Oligo dT_{10} requirement: The reaction was found to be strongly dependent on the presence of oligo dT_{10}. In its absence, incorporation was reduced by about 90% for both heteropolymer (Table 2) and homopolymer synthesis.

The Products of the Reaction. (a) Sucrose gradient velocity sedimentation: The three different products were synthesized in the presence of different substrates, as described in Table 2. All products were run on neutral as well as alkaline sucrose gradients. In all cases, the free DNA product (alkaline gradient) had an S value of 3-3.5, as determined by 4S and 16S RNA markers. The native hybrid products (neutral gradient) had S values ranging between 7 and 8, Fig. 1. These probably represent the template-primer-product complex, which would be expected to sediment somewhat slower than the mRNA alone (S=10) because of the double-stranded region. In each case, there appeared a large molecular weight product which was almost completely eliminated by alkali, Fig 1. These high molecular weight products, probably aggregates involving the template, were not investigated further.

(b) Cesium sulfate density gradient centrifugation: Aliquots of the above products were dissolved in cesium sulfate and sedimented to equilibrium. The results are shown in Fig 2. In the case of the poly (dT) product (Fig 2A), there is a peak of material banding at a density of 1.417, which is close to the density of poly (dT) ($\rho = 1.424$) (5). The material in the denser region ($\rho \approx 1.48$) apparently contains fragments of the mRNA template associated with the newly synthesized poly (dT) strand, since alkaline treatment removes this material.

TABLE 1

Effects of KCl and $MnCl_2$ on the incorporation of dGMP

M KCl	pmoles dGMP Incorp.	mM $MnCl_2$	pmoles dGMP Incorp.
0.00	0.8	0.000	0.8
0.010	2.0	0.025	2.4
0.025	5.0	0.050	5.0
0.050	5.1	0.100	4.9
0.075	3.6	0.250	5.0
0.100	1.6	0.500	5.0

Assays were carried out in 0.1ml solution containing: 50mM Tris (pH 7.8), 10mM mercaptoethanol. The concentration of the deoxynucleoside triphosphates (Schwarz/Mann) was 4 μM; the specific activity of the H^3-dGTP was 500 cpm/pmole. 0.05 μg of E. coli polymerase I (4) and 0.2 μg of globin mRNA hybridized with 0.01 μg of dT_{10} were used in each assay. The mRNA was a generous gift of Dr. Arthur Bank. Similar results were obtained with mRNA purchased from G.D. Searle (England). In the KCl reaction the $MnCl_2$ was used at 0.5mM; in the $MnCl_2$ reaction the KCl was used at 0.05M. Reactions were carried out for 30 minutes at 37° and were stopped with 5% TCA containing 0.01M pyrophosphate; precipitates were collected on Whatman GF/B filters and counted.

The thymidylate-deoxyadenylate polymer is shown in Fig 2B. A large proportion of the product bands at a density centered around 1.410. This value is close to the value reported for poly (dA)·(dT) ($\rho \approx 1.419$) (6). Since there is no incorporation of dAMP in the absence of TTP (Table 2), we conclude that dAMP is not incorporated by end addition to either the mRNA or to the oligo dT_{10} primer. We assume, therefore, that the product is most likely the homopolymer poly (dA)·(dT), formed by synthesis of poly (dT) on the poly A region of the template, followed by displacement and synthesis of poly (dA) on the newly-formed poly (dT). The material banding at a density of 1.57 contains the product still associated with the mRNA template; alkaline treatment removes it.

The heteropolymeric product is shown in Fig 2C. Here the label was H^3-dGTP. There is no free product banding at low density as in the previous cases. Most of the material bands in the density range 1.54-1.64 which indicates that there is significant mRNA associated with the synthesized DNA strand. On alkaline digestion, the product moves to the expected density of single-stranded DNA ($\rho \approx 1.44$).

(c) Hybridization of the DNA Product to mRNA. In order to show that the DNA product was complementary to the mRNA, back-hybridization experiments

TABLE 2

Homopolymer and Heteropolymer Synthesis with Rabbit Globin mRNA

Substrate	Product	pmole incorporation		
		2mM $MgCl_2$	0.5mM $MnCl_2$ - 0.025M KCl	After Heating[a]
H^3-TTP	poly (dT)	5.0	40	12
H^3-dATP	-	-	< 0.05	-
TTP + H^3-dATP	poly (dA)·(dT)	2.5	12	1.2
H^3-dGTP + dCTP, dATP, TTP	heteropolymer	1.0	5[b]	3.5
" minus dATP			0.2	
" " dCTP			0.2	
" " TTP			0.15	
" " oligo dT			0.5	
" " RNA			0.15	
" plus RNase pretreatment[c]			0.15	

Assays were carried out in 0.1ml solution containing: 50mM Tris (pH 7.8), 50mM mercaptoethanol, 100 µg/ml of actinomycin D and 8 µM each of the indicated triphosphates. 0.05 µg of E. coli polymerase I (4) and 0.2 µg of hemoglobin mRNA hybridized with 0.01 µg of dT_{10} were used in each assay (except for those using H^3-dGTP, where 0.25 µg of enzyme was used). The specific activity of the labeled substrates was 500 cpm/pmole. Incubations were for 30 minutes at 37°.

a) The enzyme was pre-heated for 30 minutes at 45°. Assay carried out in 0.5mM $MnCl_2$-0.025M KCl.
b) In the absence of KCl the incorporation is 0.8 pmoles (see Table 1).
c) RNase (100 µg/ml, 0.05M Tris, pH 7.8) pretreatment of mRNA was for 5 minutes at 37°.

were performed. The basis for determining hybrid formation was the resistance to the Neurospora single-strand specific (S_1) nuclease (7). The product of the reaction, as described in Table 3, was treated with alkali and annealed with 0.4 µg of mRNA for varying times. Hybrid formation was complete in 30 minutes, at which time nearly 100% of the input counts were resistant to the S_1 nuclease (Table 3). The specificity of hybridization is shown by the fact that neither 28S ribosomal nor EMC RNAs hybridize to the DNA product. The 20% resistance to S_1 nuclease in these cases is due to self-annealing of the DNA product, as shown by the control lacking RNA (Table 3).

TABLE 3

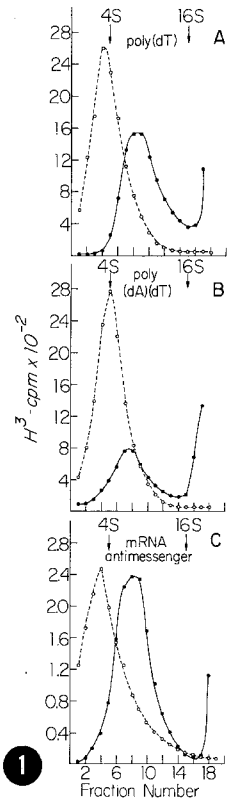
Back-Hybridization of the DNA Product to mRNA

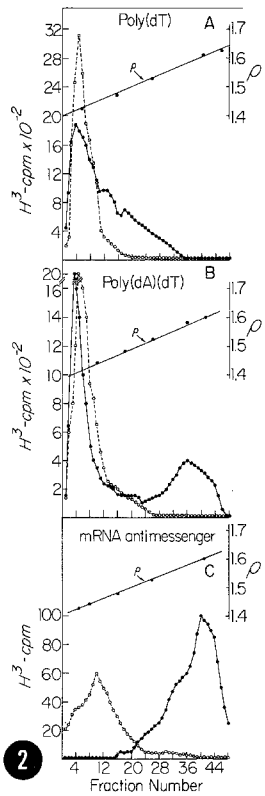
Sample	% Resistant to S_1 nuclease
1. Product H^3-DNA alone (without RNA)	20
2. Product H^3-DNA + mRNA annealed for 30, 60 or 90 minutes	95 ± 5
3. Same as 2 above except for 120 minutes	80
4. Product H^3-DNA annealed for 90 minutes with *E. coli* 28S ribosomal RNA	20
5. Same as 4 except with EMC RNA	20

Synthesis of H^3-DNA was carried out as described in Table 2 except that the reaction volume was ten-fold higher. The reaction mixture was extracted with phenol-cresol; 100 µg of denatured calf thymus DNA was added to the aqueous layer and was precipitated with 2 volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 40 µl of 0.5N NaOH. The solution was drawn up in a capillary, sealed and heated to 100° for 20 minutes. The contents were neutralized with concentrated HCl; the final volume was about 50 µl. 5 µl aliquots were used for each assay. A control (100%) was put through the procedure without mRNA and with no subsequent nuclease treatment. Sample 1 was the same except that it was treated with the nuclease. The hybridization was carried out as follows. To 5 µl aliquots was added 5 µl of 6XSSC containing 0.4 µg mRNA, and 2 µg of RNA for samples 4 and 5. After thorough mixing on a sheet of parafilm, the mixture was drawn into a 50 µl capillary tube and sealed at both ends. The tubes were then immersed in a waterbath at 69°C for the desired time, and then chilled in ice. The contents of each tube were then poured into a tube containing 0.5ml of S_1 nuclease assay mixture (consisting of 0.01M Na-Acetate buffer, pH 4.5, 0.3M NaCl, 0.003M $ZnCl_2$, 10 µg albumin/ml and heat denatured calf thymus DNA at 10 µg/ml). The reaction was incubated for 2 hrs at 50°C after addition of 1 unit of S_1 Neurospora nuclease, and acid-insoluble counts were determined by TCA precipitation.

Heat Inactivation. Heat inactivation of *E. coli* polymerase I and several other DNA polymerases shows that the apparent extent of inactivation of the enzyme varies with the template (8). In the present study, we have shown that with the single template, hemoglobin mRNA, the enzyme has three inactivation rates (Table 2), i.e. the extent of synthesis of each product is affected differently. This fact has practical advantages: relatively purer antimessenger is obtained with the heated enzyme (Table 2). The inactivation data in Table 2 were for 30 minutes. The entire time curve for this and other DNA polymerases will be published elsewhere (8).

Three Synthetic Activities are on the Same Molecule. To show that the three synthetic activities belong to one molecule, two experiments were carried out. Two samples of enzyme, one previously isolated by us (4) and another by the





Legend for Fig 1A, B, C. Sedimentation velocity of the products. Products were synthesized in 6-, 4- and 10-fold multiples of the standard reaction (Table 2) for A,B and C, respectively. The product was extracted with phenol-cresol. 50 µl was applied in A and B and 100 µl in C. The same was done for the alkaline gradients except that the aliquots were first heated to 100° for 5 minutes in 0.1N NaOH. A 5-20% (W/V) sucrose gradient was used; for the alkaline run the gradient also contained 0.1N NaOH. The run was for 18 hours at 27,000 rpm at 23° in a Spinco SW 50.1 rotor. Twenty equal fractions were collected from the top, precipitated with TCA, collected on GF/B filters and counted. The markers were 4S t-RNA and 16S E. coli ribosomal RNA. ●——● , neut o----o , alkaline.

Legend for Fig 2A, B, C. Cesium sulfate gradient of products. The products were obtained as in Fig 1 and the same amounts were dissolved in the cesium sulfate. In the case of the alkaline treated material the products were first neutralized with HCl. ●——● , neutral; o----o , alkaline.

Kornberg procedure (donated by Dr. L. Loeb) (2), were sedimented in a glycerol velocity gradient (SW 50.1, 45,000 rpm, 15 hrs). The three synthetic activities were assayed across both gradients and all were found in a single peak. In the second experiment, the three activities in the peak were assayed in the presence of anti-serum against pure polymerase I (donated by Dr. L. Grossman). All activities were reduced by 90-95%.

DISCUSSION. We have shown that E. coli DNA polymerase I uses rabbit hemoglobin

mRNA as a template to produce poly (dT), poly (dA)•(dT) and anti-mRNA DNA. The extent of homopolymer synthesis is greater than that of the anti-messenger. Heat inactivation of the polymerase shows the greatest decrease for poly (dA)•(dT) synthesis, followed by poly (dT) and anti-messenger. This suggests distinct areas of the enzyme for each type of synthesis; the areas may be separate or may overlap. Although the yield of product is lower using the heated enzyme, the proportion of anti-messenger is greater. We plan to examine in the manner described here the products formed from the same mRNA using the reverse transcriptases of the oncogenic RNA viruses.

The size of the anti-messenger formed (S=3.5) is significantly smaller than that of the template (S=10). Other investigators using viral reverse transcriptases (9,10,11) have obtained larger DNA products. The small size may be due to destruction of the product by the nucleolytic action of polymerase I or to incomplete transcription of the RNA or both. It has been shown (12) that globin mRNA has poly A-rich stretches 50-70 bases long containing about 70% A and that the 3'-terminus contains 5 or 6 A residues (13). The exact disposition of the A-rich stretches is unknown. The oligo dT primer probably couples to the short poly A stretch at the 3'-end as well as to the A-rich regions, some of which must be internal. Synthesis originating at the 3'-end would give complete complementary strands which might be nicked once or twice, reducing the size of the final product. Synthesis originating internally would lead directly to shorter complements. Since the heteropolymeric DNA product has been shown to hybridize completely and specifically to the template mRNA, it therefore represents complementary strand synthesis.

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