Volume 6 Number 7 1979 Nucleic Acids Research

A study on the unprimed poly (dA-dT) synthesis catalyzed by preparations of E.coli DNA polymerase I

I.A.Nazarenko, L.E.Bobko, A.G.Romashchenko, Yu.L.Khripin and R.I.Salganik

Institute of Cytology and Genetics of the Siberian Branch of the USSR Academy of Sciences, Novosibirsk State University, USSR

Received 2 April 1979

ABSTRACT

Evidence was obtained indicating that the initiation of poly (dA-dT) de novo synthesis is provided by deoxynucleoside diphosphate: oligonucleotide deoxynucleotidyl transferase (dNDP-transferase) present in preparations of E.coli DNA polymerase I and capable of catalyzing the unprimed polymerization of dNDP. dNDP-transferase synthesyzes short oligonucleotides which form template-primer complexes repeatedly replicated by DNA polymerase I. This conclusion was based on the following observations : the abolition of the lag period of poly (dA-dT) synthesis by preincubation of DNA-polymerase I preparations with dADP and dTDP; the presence of oligo (dA-dT) among the preincubation products; the suppressive effect of dithiothreitol and N-ethylmaleimide (inhibitors of dNDP-transferase) on the de novo, but not on the primed synthesis of poly (dA-dT), catalyzed by preparations of DNA-polymerase I.

INTRODUCTION

Investigation performed in Kornberg's laboratory have shown that preparations of E.coli DNA polymerase I are capable of providing the unprimed (de novo) synthesis of poly(dA-dT)[1] and poly(dG)·poly(dC)[2]. Subsequently, it has been reported that similar reactions can be catalyzed by DNA polymerases from B.subtilis [3], M.lysodeicticus [4,5], M.luteus[6], bacteriophage T4 [7], and calf thymus [8,9]. These findings have raised the question, whether the capacity to catalyze the unprimed synthesis of the polynucleotides is a property of the enzyme. An alternative possibility was that certain admixtures of oligonucleotides in the enzyme preparations serve as repeatedly replicating templates and primers for the polymerases . However, attempts to establish the presence of templates and primers in the preparations of the enzymes have not been successful [10, 11]. Recently, an enzyme capable of catalyzing the template - and primer-independent poly-

merization of deoxyribonucleoside diphosphates has been detected in preparations of E.coli DNA polymerase I [12]. This enzyme has been designated as deoxynucleoside diphosphate : oligonucleotide deoxynucleotidyl transferase (dNDP-transferase). Data have been obtained suggesting that the enzyme provides poly(dG)·poly(dC) synthesis through the formation of complementary homo-oligonucleotides which serve as templates and primers for DNA polymerase I (dNDP, substrates for this reaction, are always present in preparations of dNTP).

A reasonable assumption was that the de novo synthesis of poly(dA-dT) proceeds in a way similar to that of poly(dG)·poly(dC) [12]. The aim of this study was to verify this assumption.

MATERIALS AND METHODS

Isolation of DNA polymerase I. DNA polymerase I was extracted from E.coli MRE 600 cells as described by Jovin et al.[13], with DEAE cellulose fractionation done according to Richardson [14]. The specific activity of the enzyme was determined by the procedure of Aposhian [15].

Incubations. The incubation mixtures for the de novo synthesis contained 0.25 mM dATP, 0.25 mM dTTP (Novosibirsk, USSR), $5 \cdot 10^4$cpm ^{3}H-dTTP (10-20 Ci/mmole, Amersham), 6 mM $MgCl_2$, 60 mM potassium phosphate buffer (pH 7.5), 0.5-1 units of the enzyme in a volume of 0.1 ml. In those experiments, in which template-dependent synthesis was studied, 0.005 A_{260} units of poly(dA-dT) were added. The reaction mixtures were incubated for the times shown in the appropriate figure legends at 37°C; incubation was terminated by cooling to 0°C; 0.2 ml of bovine serum albumin (1mg/ml) and 0.3 ml of 1 M perchloric acid were then supplemented. The precipitate was collected by filtration through nitrocellulose filters (Sinpor, Czechoslovakia);radioactivity was measured in a liquid scintillation counter Mark II (Nuclear-Chicago).

Reaction conditions in assays using dNDP [16] as substrates were the same as those outlined above with the exception that 0.5 mM dADP and 0.5 mM dTDP were employed instead of dATP and ^{3}H-dTTP. Unless indicated otherwise, incubation was performed at 37°C for 2 hours.

Two dimensional thin layer chromatography (TLC). Norit A (Serva) was used for desalting samples [17] before their application to Silufol UV 254 plates (20·20 cm) (Serva). Solvent I, isopropanol/ conc.ammonia / water (70:10:20, v:v:v), was used in the first dimension; solvent II, 3.2 M ammonium sulphate in 7 M sodium phosphate (pH 6.8) / n-propanol (125:2, v:v) was used in the second dimension. Oligonucleotide spots were visualized under ultraviolet light, scraped into separate tubes and eluted with water at 37°C overnight.

Microcolumn chromatography[18]. Samples for microcolumn chromatography were diluted so that salt concentration did not exceed 1 mM. A solution that contained 0.05 A_{260} units of nucleotide material was applied to a TEAE cellulose column (30 µl). Elution was performed at a flow rate of 0.01 ml/min with a linear gradient (from 0 to 0.3 M) of NaCl in 7 M urea - 0.02 M Tris-HCl buffer (pH 7.5). Absorption was measured at 260 nm.

Dephosphorylation of nucleotide material with bacterial alkaline phosphatase. Reaction mixture contained in 0.1 ml 0.4 A_{260} units of nucleotide material, 1 M Tris-HCl buffer (pH 8.0), and 150 units of E.coli alkaline phosphatase, specific activity 4000 units/mg (the enzyme preparation was a gift of Dr. V. K. Starostina). One unit of phosphatase activity was defined as the amount of the enzyme hydrolyzing 1 nmole of dAMP per minute at 37°C. The reaction mixture was incubated at 37°C for 30 min.

Acid hydrolysis of oligonucleotides. Hydrolysis with dilute sulfuric acid was carried out as described by Shapiro and Chargaff [19]. Desalted samples were incubated in 0.1 M H_2SO_4 at 100°C for 50 min and neutralized with NaOH. The validity of this method was tested in model experiments with oligo(dT) and poly(dA-dT). After acid treatment, the size of oligo(dT) did not change, whereas poly(dA-dT) became a mixture of adenine, pTp, and pT as a result of hydrolysis. Pertinent evidence was provided by microcolumn chromatography.

RESULTS.

Involvement of dNDP-transferase in the initiation of poly

(dA-dT) synthesis. It has been previously reported that dNDP-transferase catalyzes the polymerization of dNDP in the absence of template and primer and that the enzyme occurs as an admixture in preparations of E.coli DNA polymerase I[12]. In view of these findings, we investigated whether preincubation of DNA polymerase I with dADP and dTDP affects the unprimed synthesis of (dA-dT) copolymer. It was found that such preincubation results in a significant reduction, or even abolition, of the lag period (Fig.1)

When DNA polymerase I preparation was preincubated with dADP and dTDP for 1 hour, the synthesis of (dA-dT) copolymer started much earlier after the addition of dNTP than in experiments in which preincubation was omitted. When the preincubation took 2 hours, there was no lag period. The reaction kinetics were similar when dADP and dTDP were incubated with dNDP--transferase, free of DNA polymerase[12], and DNA polymerase was added together with dATP and dTTP afterwards. This de novo reaction without a lag period occurred only when dADP and dTDP

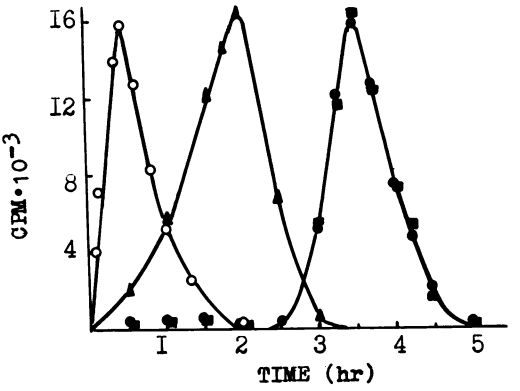

Figure 1. Kinetics of the de novo synthesis of poly(dA-dT) without and after preincubation of DNA polymerase I with dADP and dTDP. Preincubation was carried out as described in "Methods", 0.25 mM each of dATP and ^{3}H-dTTP were then added; the radioactivity of the acid-insoluble product was measured at the time intervals indicated: (-●-) no preincubation with dADP and dTDP; (-▲-) after preincubation for 1 hr; (-○-) after preincubation for 2 hrs; (-■-) after preincubation of the enzyme with either dADP or dTDP for 2 hrs, the reaction mixtures were subsequently combined and dATP and dTTP were added.

were supplemented together; in contrast, when preincubation was done separately with each dNDP, and the mixtures were pooled before the addition of dNTP's, no reduction of the lag period was observed.

It has been previously shown that poly(dA) synthesis, catalyzed by dNDP-transferase, is inhibited by dADP at concentrations exceeding 1-2 mM[12]. The experiments demonstrated an effect of the substrate concentration on primed and unprimed synthesis of (dA-dT) copolymer (Fig.2)

The rate of the template-dependent reaction increased proportionally to rising concentrations of dATP and dTTP to 12 mM (Fig.2A), whereas raising concentrations of dATP and dTTP to values exceeding 2 mM resulted in a prolongation of the lag period of unprimed synthesis(Fig.2B).Preincubation was done over a wide range of dADP and dTDP concentrations; it was realized that a concentration of 1-2 mM is optimal and that the subsequent replicative synthesis of poly(dA-dT) is inhibited at higher concentrations(Table 1)

It seems that high concentrations of dATP and dTTP, like those of dADP and dTDP, suppress the reaction catalyzed by dNDP-

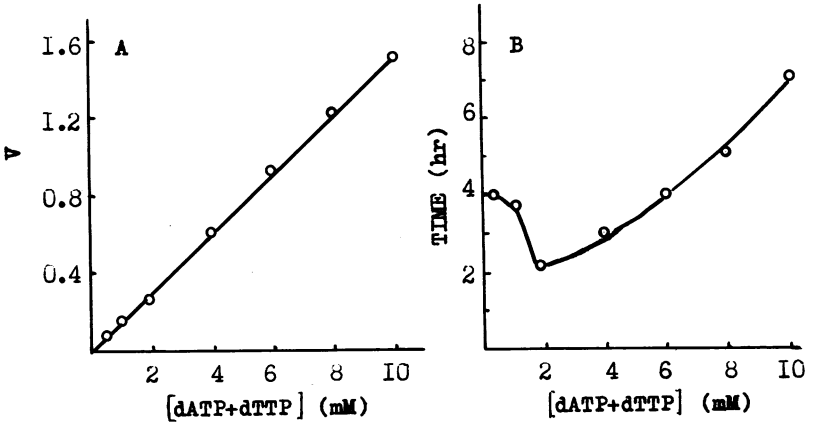

Figure 2. Effect of substrate concentration on primed and unprimed synthesis of poly(dA-dT). A - the rate of primed synthesis related to dATP and dTTP concentrations. B - the length of the lag period of the de novo synthesis related to concentrations of dATP and dTTP.

TABLE 1. Effect of dADP and dTDP concentrations in the preincubation mixture on the synthesis of poly(dA-dT).

N°	Concentration of dADP + dTDP(mM)	^{3}H-dTMP incorporation into the acid-insoluble product (cpm)	
		After 30 min	After 60 min
1	0.1	92	80
2	0.5	352	3200
3	1.0	4190	25070
4	1.5	-	27200
5	2.0	-	33250
6	2.5	-	30100
7 *	5.0	-	16000
7**	5.0	-	16150
8	10.0	115	5100

Preincubation of the enzyme was performed as described in "Methods", 0.25 mM each of dATP and dTTP were then added to the mixture and the radioactivity of the acid-insoluble product was measured.
* - 6 mM $MgCl_2$
**- the concentration of $MgCl_2$ was raised to 30 mM to ensure that the reaction was not inhibited by lack of Mg^{++}.

-transferase.

N-ethylmaleimide and dithiothreitol are inhibitors of the unprimed synthesis of poly(dA) catalyzed by dNDP-transferase[20]. While unaffecting the template-dependent reaction, catalyzed by DNA polymerase I (Fig. 3A), N-ethylmaleimide and dithiothreitol strongly inhibit the de novo synthesis of (dA-dT)copolymer (Fig. 3B). Their presence during preincubation leads to a complete suppression of the subsequent poly(dA-dT)synthesis. However, when N-ethylmaleimide or dithiothreitol was added together with dNTP, the synthesis of the polynucleotides proceeded in a manner similar to that observed without these substances (Fig. 4).

These observations indicate that dNDP-transferase is the enzyme responsible for the synthesis of templates and primers during the so-called unprimed step of poly(dA-dT) synthesis ca-

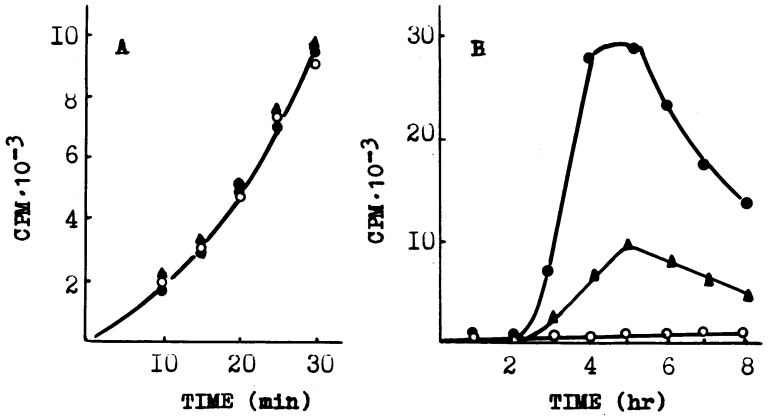

Figure 3. Effect of N-ethylmaleimide and dithiothreitol on primed and unprimed poly(dA-dT)synthesis. A - primed and B - - unprimed synthesis, (-●-) under standard conditions, (-o-) in the presence of 60 mM N-ethylmaleimide, (-▲-) in the presence of 5 mM dithiothretiol

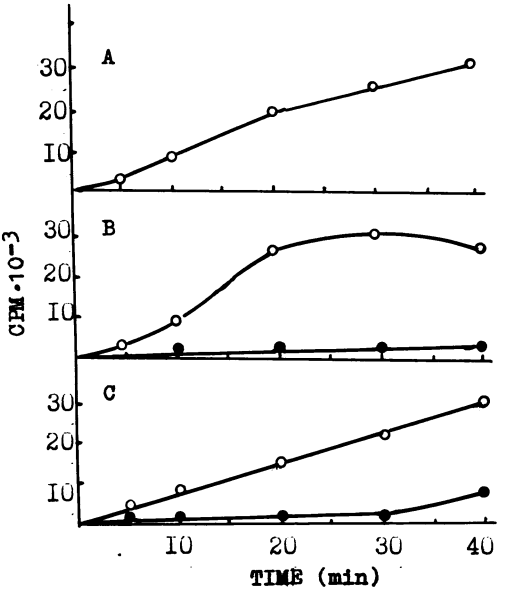

Figure 4. Effect of N-ethylmaleimide and dithiothreitol on the reaction proceeding during the preincubation of the enzyme with dADP and dTDP. Synthesis of poly(dA-dT): A - - after preincubation under standard conditions, B - in the presence of 60 mM N-ethylmaleimide in the preincubation mixture(-●-),in the reaction mixture containing dNTP(-o-), C - in the presence of 5 mM dithiothreitol during preincubation(-●-),in the reaction mixture with dNTP(-o-).

talyzed by preparations of DNA polymerase I.

Analysis of the reaction products synthesized during the incubation of DNA polymerase I preparations with dADP and dTDP.

Reaction product size. The reaction products resulting from the preincubation of DNA polymerase I with dADP and dTDP (see Fig. 1) are acid— soluble. For this reason, the amount and size of these oligonucleotides were determined by means of two-dimensional TLC on Silufol UV plates (Fig. 5). Spots 4,5,6 and 7 were identified as dADP, dAMP, dTDP and dTMP, respectively. Spots 1, 2, 3 appeared only after incubation and seem to be reaction products; they represented 23, 9 and 18% of the total UV-absorbing material, respectively. Oligonucleotides from spot 1 carried 7-9 charges (as judged by the position of the markers, Fig. 6A). The reaction products from spot 2 were oligonucleotides carrying 8 charges (Fig. 6B). Spot 3 represented oligonucleotides with 6-8 charges (Fig. 6C).

The shape of spot 4 suggested the presence of very short oligonucleotides, inseparable from dADP. They were identified as follows. After incubation of DNA polymerase I with dADP and dTDP, the reaction mixture was treated with E.coli alkaline phosphatase under conditions ensuring dephosphorylation of the

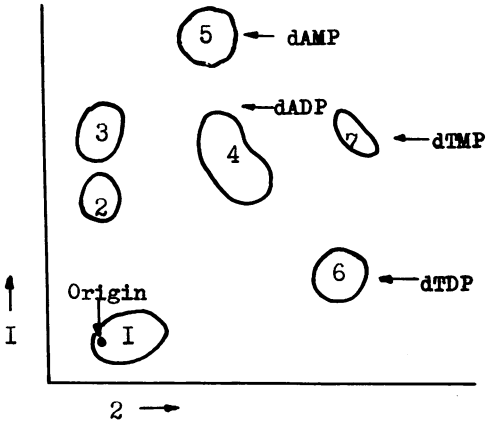

Figure 5. Two -dimensional TLC of the reaction products after incubation of dADP and dTDP with the preparation of DNA polymerase I.

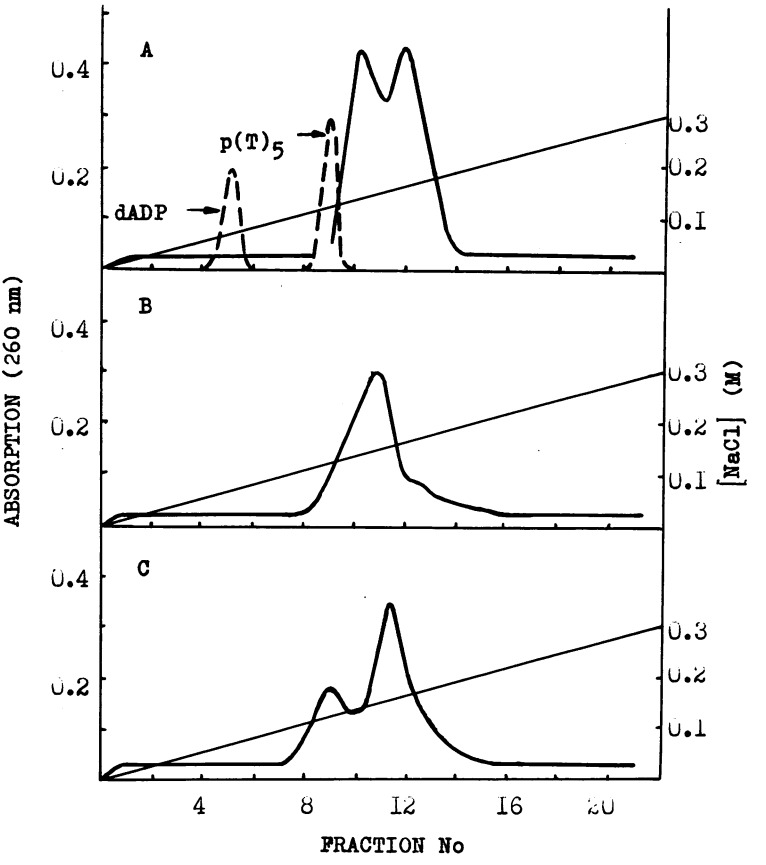

Figure 6. Microcolumn chromatography of the reaction products after incubation of dADP and dTDP with the preparation of E. coli DNA polymerase I. The reaction products were eluted from the spots (see Fig.5): A- spot 1, B- spot 2, C- spot 3.

substrates, and then subjected to microcolumn chromatography (Fig.7). Alkaline phosphatase did not show any appreciable nuclease activity, as demonstrated by incubation with oligo(dT). With the procedure described, the reaction products were found to contain di- and trinucleotides too. It was also noted that pretreatment with phosphatase shifted the peaks of hexa- deca-nucleotides to a position corresponding to shorter oligonucleotides. This was taken to mean that oligonucleotides, synthesized

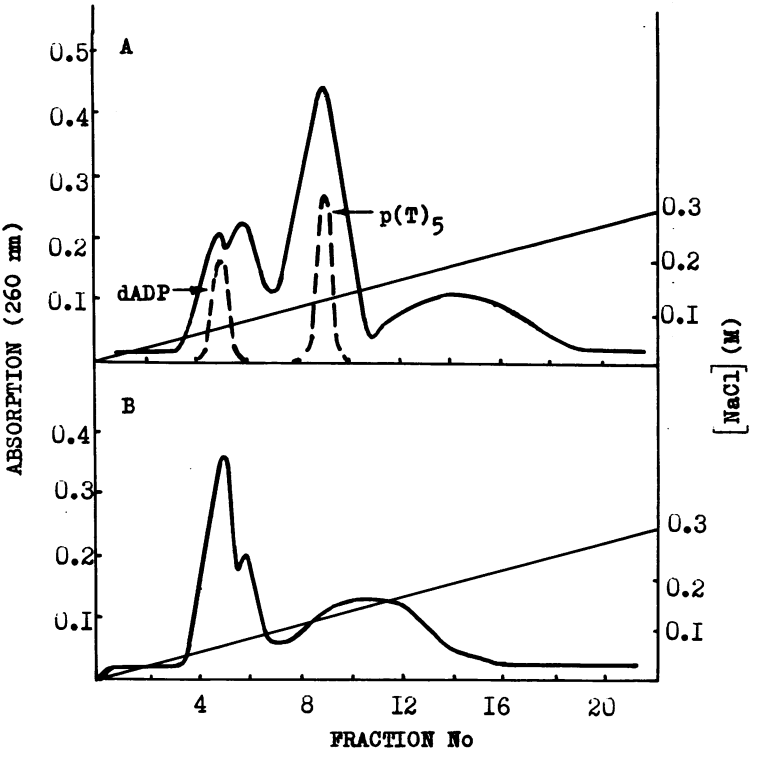

Figure 7. Microcolumn chromatography on TEAE cellulose of : A- the products of dADP and dTDP polymerization, B- the same products after treatment with alkaline phosphatase.

from dNDP, carry phosphate groups accessible to alkaline phosphatase.

Composition of reaction products. Inasmuch as oligoadenylates in solvent system 1 used in the TLC migrate faster than oligothymidilates and the material from spots 1, 2, 3 is of similar size, it was thought possible that the oligonucleotides from spot 1 are enriched with thymidine residues, while those from spots 2 and 3 are enriched with adenine residues. Conclusive evidence for this possibility came from analysis of the spectral characteristics of oligonucleotides from spots 1 and 3 (Table 2).

TABLE 2. Spectral characteristics of oligonucleotides eluted from spots 1 and 3 (see Fig. 5).

Nucleotide material	λ max	$\frac{250}{260}$	$\frac{280}{260}$	$\frac{290}{260}$
Spot 1	267	0.66	0.58	0.14
Spot 3	260	0.75	0.20	0.07
dAMP	259	0.8	0.15	0.00
dTMP	267	0.65	0.73	0.23

The presence of homo- and heterooligonucleotides in material from spots 1 and 3 was demonstrated by acid hydrolysis. The products of hydrolysis were subjected to a microcolumn chromatography : 12 % of the total UV-absorbing material did not bind to TEAE cellulose, namely adenine. The elution profiles of oligonucleotides from spot 1, obtained before acid treatment (see Fig. 6) and after it (Fig. 8), were compared. After acid treatment the ratio of peak 1 to peak 2 changed and new peaks, 3 and 4, appeared. Peak 3 represents short oligothymidilates, presumably products of acid hydrolysis of heterooligonucleotides.6-8% of the nucleotide material occurs as peak 4 with a position corresponding to that of pTp. Hence, (dA-dT)oligomer content made up 6-8 % or less of the total reaction products. After acid treatment, the bulk of UV-absorbing material from spot 3 ceases to bind to TEAE cellulose. Obviously, oligonucleotides of this spot contain mainly adenine. However, a small portion of the nucleotide material is eluted by NaCl gradient which is indicative of the presence of heterooligonucleotides.

Based on acid hydrolysis data, it may be concluded that the major components of the polymerization products of dADP and dTDP are homooligonucleotides, and that minor components are heterooligonucleotides. The latter seem to contain (dA-dT) copolymer.

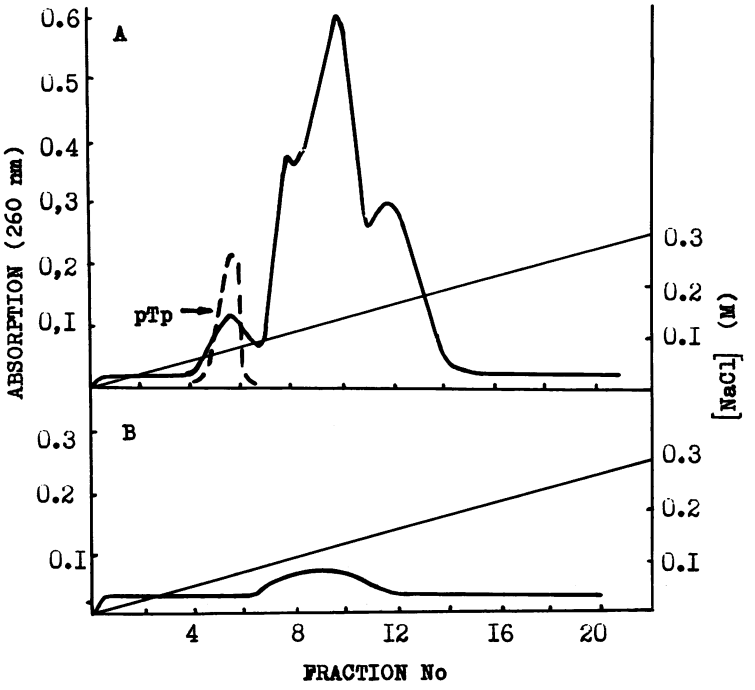

Figure 8. Microcolumn chromatography of acid-treated oligonucleotides eluted from: A- spot 1 and B- spot 3 (see Fig.5). After application of the sample, the microcolumn was washed with water: 12% of the UV-absorbing material from spot 1 (A) and 93% from spot 3 did not bind to TMAE cellulose.

DISCUSSION

The following assumptions underlie considerations concerning the mechanisms of the unprimed synthesis of polydeoxynucleotides, catalyzed by DNA polymerases : 1) The presence of contaminating endogenous templates and primers in the enzyme[1,8]; 2) the capacity of DNA polymerase *per se* to initiate the formation of templates and primers [11,21]; 3) the existence of a protein synthesizing templates and primers for DNA polymerase [12].

The results of experiments designed to isolate endogenous templates and primers from enzyme preparation were disappointing [1,10]. The second assumption is hardly tenable, because, with

increasing purity [2,14,21] and modified separation procedure[22,23], the capacity of DNA polymerase to catalyze the de novo reaction decreases or disappears, and also because template-dependent and template-independent synthesis are differently related to Mg^{++} concentration [8,24].

In a previous study, it has been shown that preparations of E.coli DNA polymerase I contain dNDP-transferase, an enzyme capable of catalyzing template- and primer-independent polymerization of dNDP[12]. It has been established that dNDP-transferase participates in the initiation of poly(dG)·poly(dC) synthesis [12]. Quite plausibly, the enzyme provides the initiation of another known type of template-independent synthesis. In fact, preincubation of DNA polymerase I preparation with dADP and dTDP results in the elimination of the lag period during the de novo synthesis of poly (dA-dT) (Fig. 1).

The capacity of N-ethylmaleimide and dithiothreitol, inhibitors of dNDP-transferase, to suppress the unprimed synthesis of poly(dA-dT), catalyzed by DNA polymerase I preparations, lends support to the idea that it is dNDP-transferase that directs the initiation of the synthesis of poly(dA-dT). A similar effect was achieved by adding high concentrations of substrates, inhibiting both the unprimed synthesis of poly (dA-dT) and activity of dNDP-transferase, to the incubation mixture. Hence, precisely dNDP- transferase specifies the synthesis of short oligonucleotides which act as templates and primers for the synthesis of the high molecular weight poly (dA-dT), catalyzed by DNA polymerase I.

The products synthesized as a result of preincubation of DNA polymerase I with dADP and dTDP are oligonucleotides of different composition and length;oligo(dA) and oligo(dT) predominate, but mixed oligonucleotides are also encountered. The oligonucleotides are short and dNTP preparations contain small amounts of dNDP, which serve as substrates for dNDP-transferase. These are the possible reasons why investigators have failed to isolate initiating oligonucleotides [10].

Despite relatively low contents of oligo(dA-dT) in the reaction products, E.coli DNA polymerase I efficiently synthesizes poly(dA-dT) and only under special conditions, in the pre-

sence of anthracycline antibiotics [25] and proflavine[26], it provides the synthesis of poly(dA)·poly(dT). The preferential synthesis of copolymer may be explained as follows: 1)short oligo (dA) and oligo(dT) chains tend to form three-stranded complexes [27] and thus cannot be utilized as templates and primers by DNA polymerase; 2) E.coli DNA polymerase I prefers poly(dA-dT) to any other template[28]. In the presence of poly(dA-dT)-binding anthracycline antibiotics, the homopolymer pair poly(dA)·poly (dT) is synthesized, but only after a prolonged,24 hour lag period[25](the corresponding lag period for poly(dA-dT) synthesis is 2-5 hours). Apparently, long oligo(dA) and oligo(dT) chains, capable of forming template-primer complexes(in contrast with the short ones lacking this capacity), arise during this long lag period and ensure replication.

There are data indicating that pH, ionic strength and buffer composition determine what kind of polydeoxynucleotide is synthesized de novo[21]. The present investigation suggests that these factors can influence both the reaction catalyzed by dNDP--transferase and the formation of template-primer complexes[29]. It cannot be excluded that these factors also affect nucleases and thereby produce a degradation of oligonucleotides with definite sequences.

Three steps can be distinguished in the so-called template--independent synthesis of polydeoxynucleotides, catalyzed by DNA polymerase I: 1) a template-independent synthesis of short oligonucleotides catalyzed by dNDP transferase; 2)the formation of template-primer complexes with the involvement of these oligonucleotides and 3)a prolonged replication of template--primer complexes by DNA polymerase I.

The template-independent synthesis of polydeoxynucleotides can be catalyzed by DNA polymerases from sources other than E.coli. It may be assumed that, whatever the source of DNA polymerase may be, the template-independent synthesis conforms to the pattern established here.

REFERENCES

1. Schachman,H.K.,Adler,I.,Lehman,J.R.,Kornberg,A.(1960) J.Biol.Chem. 235,3242-3249.
2. Radding,C.M.,Josse,J.,Kornberg,A. (1962) J.Biol.Chem. 237, 2869-2876.
3. Okazaki,T.,Kornberg,A. (1964) J.Biol.Chem.239,252-268.
4. Zimmerman,B.K. (1966) J.Biol.Chem. 241,2035-2041.
5. Grant,R.C.,Harwood,S.J.,Wells,R.D. (1968) J.Amer.Chem.Soc. 90,4474-4476.
6. Harwood,S.J.,Schendel,P.F.,Wells,R.D. (1970) J.Biol.Chem. 245,5614-5624.
7. Nossal,N.G. (1974) J.Biol.Chem. 249,5668-5676.
8. Henner,D.,Furth,J.J. (1975) Proc.Nat.Acad.Sci.USA 72,3944--3946.
9. Henner,D.,Furth,J.J. (1977) J.Biol.Chem. 252,1932-1938.
10. Radding,C.M.,Kornberg,A. (1962) J.Biol.Chem. 237,2877-2882.
11. Kornberg,A. (1965) "Evolving Genes and Proteins",Bryson,V. and Vogel,H.J.,Eds. Academic Press,Inc.,N.Y.
12. Nazarenko,I.A.,Potapov,V.A.,Romashchenko,A.G.,Salganik, R.I. (1978). FEBS Letters 86,201-204.
13. Jovin,I.M.,Englund,P.T.,Bertsch,L.L. (1969) J.Biol.Chem. 244,2996-3008.
14. Richardson,C.C.,Schilkraut,G.L.,Aposhian,H.V.,Kornberg,A. (1964) J.Biol.Chem. 239,222-232.
15. Aposhian,H.V.,Kornberg,A. (1962) J.Biol.Chem. 237,519-525.
16. Preparations of dNDP did not contain dNTP, as judged by microcolumn chromatography.
17. Spencer,J.H.,Chargaff,E. (1963) Biochim.Biophys.Acta 68, 9-17.
18. Grachev,M.A. (1973) Ultramicroanalysis of Nucleic Acids, pp.104-122,Nauka,Moscow.
19. Shapiro,H.S.,Chargaff,E. (1964) Biochim.Biophys.Acta 91,262-270.
20. Nazarenko,I.A.,Bobko,L.E.,Romashchenko,A.G.,Khripin,Yu.L., Salganik,R.I. (1979) Mol. Biol. 13, 216-227
21. Burd,J.F.,Wells,R.D. (1970) J.Mol.Biol. 53,435-459.
22. Lezius,A.G.,Henning,S.B.,Menzel,C.,Metz,E. (1967) Eur.J. Biochem. 2,90-97.

23. Yushkova,L.F.,Nechanevich,N.F.,Potapov,V.A.,Romashchenko, A.G. (1976) Biokhimia 41,420-425.
24. Travaglini,E.C.,Loeb,L.A. (1974) Biochemistry 13,3010-3017.
25. Olson,K.,Luk,D.,Harvey,C.L. (1972) Biochim.Biophys.Acta277, 269-275.
26. McCarter,J.A.,Kadahama,N.,Tsiapalis,C.(1969) Can.J.Biochem. 47,391-399.
27. Naylar,R.,Gilham,P.T. (1966) Biochemistry 5,2722-2728.
28. Englund,P.T.,Kelly,R.B.,Kornberg,A. (1969) J.Biol.Chem. 244,3045-3052.
29. Sobel H.M. (1969) Genetic Organization ed by Gasperi,E.W., Ravin,A.W. 1,p.91,New York,Academic Press.