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Biochemistry

De novo synthesis of a polymer of deoxyadenylate and deoxythymidylate by calf thymus DNA polymerase α

[poly(dA-dT)/DNA unwinding protein]

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ABSTRACT **In a reaction mixture containing calf thymus DNA polymerase α (DNA nucleotidyltransferase; deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7), calf thymus DNA unwinding protein, DNA, deoxyadenosine 5′-triphosphate and deoxythymidine 5′-triphosphate, a copolymer of deoxyadenylate and deoxythymidylate is synthesized after a lag period of 1–2 hr. In the presence of the four deoxyribonucleoside triphosphates only deoxyadenylate and deoxythymidylate are incorporated into the polymer and the rate of synthesis is decreased. The reaction variably occurs in the absence of DNA or DNA unwinding protein but with a greatly entended lag period. The optimal Mg^{2+} concentration for synthesis of the polymer of deoxyadenylate and deoxythymidylate is 1 mM, in contrast to an optimal Mg^{2+} concentration of 8 mM for DNA synthesis with activated DNA as template. Characterization of the product of *de novo* synthesis indicates that it is the alternating copolymer, poly(dA-dT).**

The unprimed synthesis of a copolymer of deoxyadenylate and deoxythymidylate by a bacterial DNA polymerase (DNA nucleotidyltransferase; deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase; EC 2.7.7.7) was first reported by Schachman *et al.* (1). Subsequently four other types of polydeoxynucleotides have been prepared in such *de novo* reactions, including poly(dA)·poly(dT), poly(dI)·poly(dC), poly(dG)·poly(dC), and poly(dI-dC) (2, 3). These polynucleotides have been extremely useful in studies of the relationship of nucleotide sequence to the properties of DNA molecules (4).

The unprimed synthesis of a copolymer or homopolymer of deoxynucleotides by eukaryotic DNA polymerase has not been previously reported. In the course of studies on the effect of DNA unwinding protein on DNA synthesis by DNA polymerase α of calf thymus, we observed the incorporation of [^{3}H]dTTP into acid-insoluble material, but only after a long lag period. Analysis of the reaction indicates that dGTP and dCTP are not required for the reaction to occur and that the product is the copolymer, poly(dA-dT).

MATERIALS AND METHODS

Materials. Unlabeled deoxyribonucleotides and $(dT)_{12-18}$ were purchased from P-L Biochemicals, [^{3}H]dTTP, [α-^{32}P]dTTP, and [α-^{32}P]dATP were obtained from New England Nuclear Corp. Staphylococcal nuclease, spleen phosphodiesterase, and calf thymus DNA were obtained from Worthington Biochemical Corp. Poly(rA) and poly(dA) were obtained from Miles Laboratories and yeast tRNA from Calbiochem.

Standard Assay for DNA Polymerase. Reaction mixtures (0.25 ml) contained: 20 mM potassium phosphate buffer (pH 7.6), 8 mM KCl, 8 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 30 μg of bovine serum albumin, dATP, dGTP, dCTP (160 μM each), activated calf thymus DNA (160 μM as deoxynucleotides), 160 μM [^{3}H]dTTP (5–50 cpm/pmol) and DNA polymerase. Activated DNA was prepared as described by Aposhian and Kornberg (5) except that 50 mM NaCl was used. DNA (0.375 mg/ml) was incubated with 0.5 μg/ml of pancreatic DNase for 3.5 min.

DNA Polymerase. DNA polymerase was purified from calf thymus as previously described (6), followed by phosphocellulose chromatography (7). The enzyme was further purified by chromatography on DEAE-cellulose, phosphocellulose, and DNA cellulose (E.-C. Wang, manuscript in preparation). The DNA polymerase preparations used in these studies had specific activities of 500–2200 units/mg of protein, where 1 unit of enzyme incorporates 1 nmol of [^{3}H]dTTP into acid-insoluble material in 1 hr with activated DNA as a template. The polymerase so obtained is the DNA polymerase α as assessed by size (6–8 S), pH optimum, and inhibition by sulfhydryl reagents (8).

Unwinding Protein. Unwinding protein was purified from calf thymus by the procedure of Herrick (9). The preparation used in these studies is homogeneous as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The unwinding protein, which has been designated UP1 by Karpel *et al.* (10), behaves as a symmetrical monomer of 24,000 daltons, which can decrease the melting temperature of double-stranded nucleic acids (11).

Nearest Neighbor Analysis. The procedure of Josse *et al.* (12) was used except that samples were desalted before application to Whatman 3 MM paper. Samples which had been digested with staphylococcal nuclease and spleen phosphodiesterase were adsorbed to Norit A (charcoal), washed with H_2O, and eluted from the Norit with 50% ethanol, 4% NH_4OH. Samples were then lyophilized, dissolved in 0.1 ml of H_2O, and applied to Whatman 3 MM paper. Recovery of radioactivity from Norit adsorption and elution was at least 95% and at least 95% of the radioactivity was recovered from the paper after electrophoresis as one of the four deoxyribonucleoside monophosphates.

RESULTS

Kinetics of synthesis of a copolymer of dA and dT

When DNA polymerase α is incubated with native calf thymus DNA in the presence of UP1, dATP, [^{3}H]dTTP, and 1 mM $MgCl_2$, [^{3}H]dTTP is incorporated into acid-insoluble material but only after a period of approximately 1 hr during which no synthesis occurs (Fig. 1). The rate of synthesis rises exponentially for approximately 3 hr and synthesis ceases after approximately 6 hr of incubation. If dATP is omitted synthesis is negligible. When UP1 is omitted synthesis begins later and the rate of synthesis rises more slowly. Both dATP and dTTP are incorporated during such expo-

Abbreviation: UP1, DNA unwinding protein.

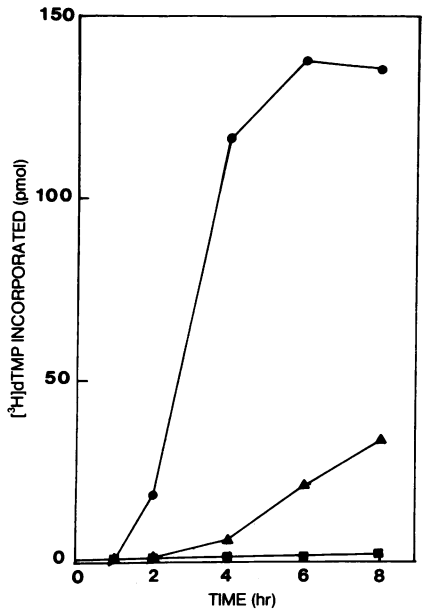

FIG. 1. Course of *de novo* synthesis of a dA- and dT-containing polymer by DNA polymerase α. Reaction mixture contained (in 0.1 ml) 5 mM potassium phosphate buffer (pH 7.2), 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10 mM NaCl, 100 μM dATP, 100 μM [^{3}H]dTTP (58 cpm/pmol), native calf thymus DNA (39 μM as deoxynucleotides), 8.8 μg calf thymus UP1, and 1.5 units of calf thymus DNA polymerase α. Incubation was at 37°. At the indicated times 10 μl aliquots were removed and incorporation of radioactivity into acid-insoluble material was determined by the filter paper assay of Yoneda and Bollum (7). ●, Complete reaction mixture; ▲, minus UP1; ■, minus dATP.

nential synthesis, but incorporation of dGTP and dCTP is not detectable when they are included in the reaction mixture.

Requirements for *de novo* synthesis

As shown in Table 1, dGTP and dCTP are not required and their presence is inhibitory to *de novo* synthesis. The optimal Mg^{2+} concentration for synthesis of the *de novo* product is 1 mM. At 8 mM Mg^{2+}, synthesis of *de novo* product at 4 hr is 25% of that observed with 1 mM Mg^{2+}. In contrast, the optimum Mg^{2+} concentration for the replication of DNA is 8 mM Mg^{2+}. At 1 mM Mg^{2+} the rate is 30% of that observed at 8 mM Mg^{2+}. In the experiments shown here *de novo* synthesis did occur in the absence of unwinding protein or native DNA. However, the lag periods in their absence have been variable, though always longer than for the complete reaction. After 8 hr of incubation, the *de novo* product was synthesized in three of eight experiments in which UP1 was omitted and in two of eight experiments in which DNA was omitted. The complete reaction mixture synthesized the *de novo* product in all experiments (20 of 20), and with several different preparations of DNA polymerase α and UP1.

As shown in Table 2, several different types of polydeoxynucleotides are capable of stimulating the synthesis of *de novo* product. Activated DNA and native DNA are both very effective in reducing the lag period before *de novo* synthesis. Lag periods are somewhat longer when denatured DNA or $(dT)_{12-18}$ are present. The presence of either poly(dA) or polyribonucleotides was without effect. Although the length of the lag period is dependent on the type of polynucleotide initially present, the doubling time for poly(dA-dT) synthesis and the extent of the reaction are approximately the same for all the polydeoxynucleotides tested. An explanation of the relative effectiveness of different forms of DNA and of the ineffectiveness of poly(dA) awaits a more detailed study of the effect of polydeoxynucleotides of different size and base composition.

Table 1. Requirements for synthesis *de novo* of a polymer of dA and dT

Conditions	[^{3}H]dTMP incorporated (pmol/10 μl aliquots) 1 hr	4 hr	6 hr
Complete	<1	116	138
+ dGTP and dCTP (100 μM each)	<1	50	100
— UP1	<1	6	21
— Native calf thymus DNA	<1	<1	16
— dATP	<1	<1	1
— DNA polymerase α	<1	<1	<1

Reaction conditions and determination of incorporated radioactivity are as described in the legend to the figure.

The product of the reaction is poly(dA-dT)

After 4 hr of synthesis the *de novo* product ranges in size from 2.4×10^4 daltons to 4.9×10^5 daltons with a modal value of 2.0×10^5 daltons, measured by alkaline sucrose gradient centrifugation (13). Analysis of the *de novo* product indicates that dATP and dTTP are incorporated in equal amounts. Nearest neighbor analysis of the *de novo* product further indicates that it consists of dA and dT in alternating sequence (Table 3). Each [^{32}P]deoxythymidylate is incorporated adjacent to deoxyadenylate and each [^{32}P]deoxyadenylate is incorporated adjacent to deoxythymidylate. The small amount of ^{32}P which is not transferred from dA to dT or dT to dA may be due to the small amount of linear, nonexponential synthesis which occurs in the reaction mixture used to produce the *de novo* product. The *de novo* product is therefore poly(dA-dT).

DISCUSSION

Poly(dA-dT) can be synthesized by calf thymus DNA polymerase α in the absence of primer. Such synthesis is stimu-

Table 2. Stimulation of the *de novo* synthesis of a polymer of dA and dT by polynucleotides

Exp.	Polynucleotide*	[^{3}H]dTMP incorporated (pmol/10 μl) 0.5 hr	1 hr	3 hr	4 hr
1	Activated calf thymus DNA	26	293	477	506
	Native calf thymus DNA	1	166	433	584
	Denatured calf thymus DNA	<1	2	305	331
	Poly(rA)	<1	<1	<1	<1
	None	<1	<1	<1	<1
2	Activated calf thymus DNA	3	81	132	144
	$(dT)_{12-18}$	<1	<1	21	93
	Poly(dA)	<1	<1	<1	<1
	Yeast tRNA	<1	<1	<1	<1
	None	<1	<1	<1	<1

Reaction conditions and determination of incorporated radioactivity are as described in the legend to the figure except that 3.0 units of DNA polymerase were used.

* All polynucleotides were present at 39 μM (as nucleotides).

Table 3. Nearest neighbor analysis of the *de novo* products of calf thymus DNA polymerase α

Substrates	Experiment	Distribution of radioactivity after digestion (%) dCMP	dAMP	dGMP	dTMP
[α-^{32}P]dTTP + dATP	1	0.1	97.9	0.6	1.4
[α-^{32}P]dTTP + dATP	2	0.2	96.2	0.7	2.9
[α-^{32}P]dATP + dTTP	3	0.4	0.2	0.5	98.9
[α-^{32}P]dATP + dTTP	4	0.2	0.2	0.4	98.7

The *de novo* product was synthesized in a complete 4 hr reaction as described in the legend to the figure except that either [α-^{32}P]-dTTP (95.5 cpm/pmol) or [α-^{32}P]dATP (69 cpm/pmol) was present. Nearest neighbor analysis was performed as described in *Materials and Methods*. 100% of radioactivity represents 1.74×10^4, 1.60×10^4, 5.17×10^4, and 8.56×10^4 cpm for experiments 1 through 4, respectively.

lated by the presence of a calf thymus DNA unwinding protein, UP1. The interaction of UP1 and DNA polymerase α in producing poly(dA-dT) is of interest in view of the multiple interactions of prokaryotic DNA unwinding proteins and DNA polymerases (14–18). Nossal has recently demonstrated synthesis of a dA- and dT-containing copolymer by phage T4 DNA polymerase which is dependent on the presence of T4 gene 32 protein (19).

The initial synthesis of poly(dA-dT) has not been completely explained even in the most intensively studied system, the reaction catalyzed by *Escherichia coli* DNA polymerase I, because initial synthesis occurs at a level too low to measure by tracer incorporation (20). One model for this reaction would be that UP1 stimulates synthesis of initial molecules of poly(dA-dT) by facilitating strand displacement at nicks in native DNA. Thus dA + dT rich sequences at nicks can be reiteratively replicated. The variable requirement for the addition of DNA may be due to the presence of DNA in amounts too low to detect in either the DNA polymerase or the UP1 preparations. The replication of poly(dA-dT) would be further stimulated by UP1-facilitated "strand slippage," to continually expose new single-stranded poly(dA-dT) template. Exponential synthesis of poly(dA-dT) (Fig. 1) may depend on the presence of low levels of endonuclease for generation of 3′-OH termini (cf. 21).

DNA from crab testis has been shown to contain a satellite DNA which consists almost exclusively of dA and dT in alternating sequence (22, 23). The ability of eukaryotic DNA polymerase to synthesize poly(dA-dT) and repeatedly replicate it provides an explanation for its origin and for the large variation in the amounts of the dA-dT satellite among various *Cancer* species (22).

Though we have not observed *de novo* synthesis of a polymer which contains dG and dC by the DNA polymerase α, it is possible that conditions may be found under which such polymers will be synthesized. The ability of eukaryotic DNA polymerase to repeatedly replicate a simple polymer containing all four deoxyribonucleotides would lend plausibility to an evolutionary scheme in which satellite DNAs arise by repeated rounds of synthesis of a DNA sequence with a very short repeat length (24).

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