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DNA REPLICATION BY NOVEL MACROMOLECULAR COMPLEXES INVOLVING DNA POLYMERASE III HOLOENZYME ACTIVITY

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SUMMARY: A fraction (P1) which showed DNA polymerase III holoenzyme activity was obtained by partial purification including Polymin P fractionation from extracts of E. coli K-12 wild type (pol A^+, pol B^+) cells. The P1 fraction was composed of three macromolecular complexes, 11 S, 18 S and 24 S, all of which possessed holoenzyme activity. The activity of the P1 fraction was maximal at about 70 mM NaCl. The synthesis of long-chain poly(dT) with a poly(dA)·oligo$(dT)_{10}$ primer was dependent on the presence of ATP, but not on the presence of spermidine, suggesting that the single-stranded DNA binding protein (SSB) was present in the fraction. The intermediate lengths of the products in the absence of ATP and NaCl also suggest the functioning of DNA polymerase III'.

INTRODUCTION: Much of the complicated mechanism of DNA replication in prokaryotic and eukaryotic cells has been elucidated (1), since the discovery and purification of DNA replicating enzymes. It has also been genetically demonstrated (2,3) that the activities of both DNA polymerase III holoenzyme (pol III holoenzyme) and exonuclease of DNA polymerase I (pol I) are essential for DNA replication in E. coli cells. On the other hand, the functionally high-ordered organization of both the enzyme and accessory proteins concerning the DNA replication (4) suggest the possibility that many of those functional protein molecules are easily associated to or dissociated from their complexes according to the functions for replication processes, which may alter their modes of action. A hypothetical mechanism of DNA replication has been briefly discussed in terms of the molecular weight and subunit constitution of DNA polymerase III' (pol III') by McHenry (5). We report here pol III holoenzyme preparations that show high sedimentation constants and other properties differing from those already reported (5-11).

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MATERIALS AND METHODS: E. coli K-12 cells grown to early mid-log phase were obtained from Miles Laboratories. Salmon sperm DNA, type III, was obtained from Sigma and activated by the procedures of Livingston et al. (12) and McHenry and Crow (13). Poly(dA) ($s_{20,w}$=6.8) and oligo(dT)$_{10}$ were purchased from Miles Laboratories. Poly(dA):oligo(dT)$_{10}$= 20:1 (molar ratio) was prepared by annealing poly(dA) and oligo(dT)$_{10}$ at 40°C for 5 min in 20 mM potassium phosphate (pH 6.8) containing 50 mM KCl. DNA concentrations are expressed as equivalents of nucleotide phosphorus.

Purification and assay of DNA polymerase III holoenzyme activity - Fraction II (polymin P treatment and ammonium sulfate precipitation) was prepared as described (14) except that the Polymin P solution was added to the cell extract (prepared from 1000 g of frozen E. coli K-12 cells) to a final concentration of 0.6%. Fraction II protein (1 g) was applied to a column (3.2 x 42 cm) of DEAE-Sepharose CL-6B and then eluted with a 2 l linear gradient of Buffer II (20 mM Tris-HCl (pH 7.2), 5 mM dithiothreitol, 0.1 mM EDTA, 20% glycerol) to Buffer II plus 0.5 M NaCl. Fractions of 10 ml each were collected. Fractions, P1 and P2 were eluted from 0.22 to 0.23 M and from 0.26 to 0.28 M, respectively. DNA-synthesizing activity on activated salmon sperm DNA (15 nmol, as nucleotide) was assayed in a 50 µl volume containing: 33 mM 4-morpholinopropane sulfonic acid - KOH (pH 7.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 40 µM each of dGTP, dCTP, dATP, [^{3}H]dTTP (110 dpm/pmol), 0.1 mg/ml bovine serum albumin, 10 µl each of DEAE-sepharose fractions. Incubation was for 10 min at 30°C. With poly(dA)•(dT)$_{10}$ template-primer (60 µM poly(dA)), the activity was measured in the same way as with the activated salmon sperm DNA, except that dGTP, dCTP and dATP were omitted and 200 µM ATP was added.

Product size measurements - For product size measurement using the P1 fraction, reaction mixtures (50 µl) were assayed on poly(dA)•(dT)$_{10}$ template-primer as described above. The reaction mixtures were incubated at 30°C for 30 min, at which time EDTA was added to 50 mM and then samples were treated by the method of Maniatis and Efstratiadis (15). The samples were electrophoresed in a 5% polyacrylamide gel containing 98% formamide for 5 h at 100 V. The gels were sliced with a razor into small pieces from which products were eluted by gel extraction buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1% sodium dodecyl sulfate), and counted in a liquid scintillation counter.

RESULTS: We attempted to purify pol III holoenzyme from extracts of wild type (pol A$^+$, pol B$^+$) cells of E. coli K-12, where Polymin P fractionation 14) between 0.35 and 0.6% was also utilized to obtain the pol III holoenzyme activities together with those of related functional proteins. After this treatment, the most of the pol I activity remaining was removed by fractionation of the pol III holoenzyme preparation through a DEAE-Sepharose column. The activities of fractions P1 and P2 on the various templates are summarized in Table 1. Both activities were entirely NEM-sensitive with all the templates used. The activity of P1 was largely dependent on the presence of ATP when assayed on poly(dA)•oligo(dT)$_{10}$. The DNA-synthesizing activities common to the core enzyme of DNA polymerase III

Table 1. Properties of DEAE-Sepharose fractions

Components	P1	P2
Salmon sperm DNA		
	pmol	pmol
Complete system	124	50
+ Ethanol(10%)	164	96
+ KCl(60 mM)	64	23
+ NEM(10 mM), - DTT	5	4
- DTT	129	41
+ araCTP(50 μM)	108	44
Poly(dA)•$(dT)_{10}$		
+ ATP, + NaCl(60 mM)	540	9
+ ATP, - NaCl	360	-
- ATP, + NaCl	63	10
+ ATP, + NEM(10 mM)	7	-

Details of DEAE-Sepharose fractions, P1 and P2 and assay conditions are described in Materials and methods. Values shown in the Table are dTMP incorporated.

(pol III core enzyme), pol III', DNA polymerase III* (pol III*) and pol III holoenzyme on the template of activated salmon sperm DNA (5,12-14) showed an elution profile including two peaks in the activity, one of which corresponds to that of P1 (data not shown). The peak eluting later, fraction P2, however, showed little activity with the template-primer, poly(dA)• oligo$(dT)_{10}$ regardless of the presence of ATP. The above results indicate that P1 represents mainly the pol III holoenzyme activity, while P2 includes one or some of pol III core enzyme, pol III' and pol III*, but not pol III holoenzyme. It has been reported (16) that the activity of DNA polymerase II (pol II) is significantly inhibited by araCTP; the finding that the activities of P1 and P2 are only slightly inhibited by this nucleotide (Table 1) indicate that pol II activity is not present in those fractions.

For the P1 fraction, it was found that the pol III holoenzyme activity was enhanced by increasing the concentration of NaCl (Fig. 1). The DNA-polymerizing activities on poly(dA)• oligo$(dT)_{10}$ measured in the absence of added ATP gradually decreased as the concentration of NaCl increased. On the other hand, the ATP-dependent activity was enhanced as the NaCl

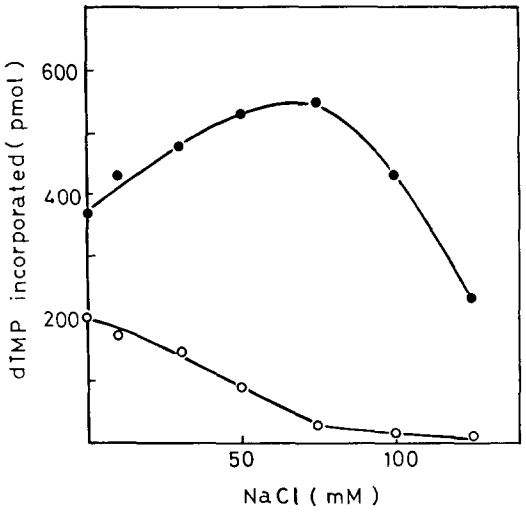

Fig. 1. Influence of NaCl concentration on DNA synthesis. DNA synthesis was measured on poly(dA)·$(dT)_{10}$ template-primer using 19 μg of P1 fraction, in the absence (○——○) and presence (200 μM) of ATP (●——●) as described in Materials and methods, except that NaCl concentration was varied. Incubation was for 30 min.

concentration increased and was maximal at about 70 mM NaCl. At this NaCl concentration, the difference between the activity of DNA-synthesizing system with ATP and that without ATP was largest. Kornberg and Gefter (9) have reported that KCl inhibits considerably the activity of pol III on the gapped template, whereas the salt slightly stimulates the activity of pol I. However, the mechanism of stimulation of the pol III holoenzyme activity by 70 mM NaCl in our experiments remains to be elucidated.

The evidence that P1 fraction catalyzes the synthesis of long-chain polymers on the template-primer, poly(dA)· oligo$(dT)_{10}$ in assay conditions different from those already reported (6,7,10) was provided by the analyses of product lengths as shown in Fig. 2. In the complete assay system, which contains 70 mM NaCl and 200 μM ATP, more than 70% of the total product was long poly$(dT)_n$ with n larger than 150, with major components up to n = 500. Only about 10% of the products were short polymers ($n \simeq 20$). In contrast to the results reported by Fay et al. (10), we found that the distribution profile of the product lengths in the absence of added spermidine (Fig. 2D) was not significantly different from that with the complete system (Fig.

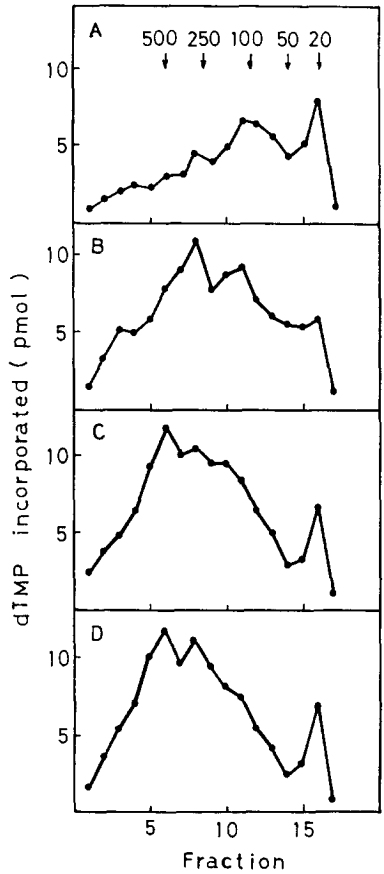

Fig. 2. Products made by P1 fraction on the poly(dA)•$(dT)_{10}$ template-primer. For product size measurement using P1 fraction (19 μg), reaction mixtures (50 μl) contained: 33 mM 4-morpholino-propane sulfonic acid-KOH (pH 7.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 80 μM (^{3}H)dTTP (110 dpm/pmol), 60 μM poly(dA)•$(dT)_{10}$, 100 μg/ml bovine serum albumin, 3.2 mM spermidine in A. Other additions were 200 μM ATP in B, and 200 μM ATP and 70 mM NaCl in C. In panel D, the reaction mixture was the same as panel C except that spermidine was omitted. Arrows indicate the sizes of polynucleotides synthesized.

2C). This result suggests that the P1 fraction contains the single-stranded DNA binding protein (SSB) (17,18). In the absence of added NaCl, the product lengths were slightly shorter (Fig. 2B), and the major products were about 250 nucleotides in length. If ATP and NaCl were omitted from the synthesizing system, long products consisting of more than 250 nucleotides markedly decreased, and the smaller amounts of relatively short poly(dT) of about 100 nucleotides and 20 nucleotides in length were mainly

synthesized (Fig. 2A). The results suggest the functioning of pol III' (5) besides the core enzyme which synthesizes the shortest poly(dT) (10).

In order to confirm the molecular size of pol III holoenzyme, gel filtration and density gradient ultracentrifugation for P1 were carried out (Fig. 3). Supprisingly, the activity of pol III holoenzyme separated into three active fractions on gel filtration, all of which were dependent on the presence of ATP on poly(dA)• oligo(dT)$_{10}$ template. The apparent molecular weights at the peaks of these fractions were estimated to be 1,000,000 or greater, 600,000 to 700,000, and 300,000 to 400,000, in the order of the elution, respectively. In density gradient ultracentrifugation fortified with ATP and Mg^{2+} (Fig. 4), the holoenzyme activities were found at 24 S and 18 S, besides a small peak of 11 S corresponding to that already reported (6,8). The pol III holoenzyme activities recovered in the gel filtration and the density gradient ultracentrifugation experiments were quite unstable.

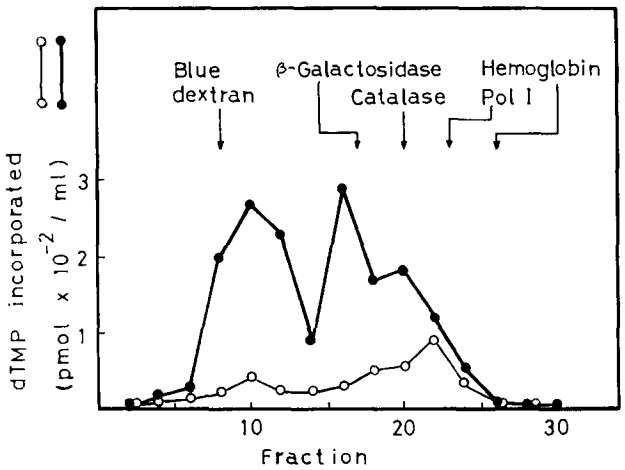

Fig. 3. Gel filtration of P1 fraction on Ultrogel AcA 22 column. P1 fraction (1 mg) was dialyzed against 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 10 mM dithiothreitol, 30% glycerol and 0.5 mg/ml bovine serum albumin and then applied on Ultrogel AcA 22 column (0.9 x 14 cm, previously equilibrated with same buffer) and eluted. Fractions of 0.4 ml each were collected and assayed for DNA-synthesizing activity on poly(dA)•(dT)$_{10}$ with ATP (●——●), without ATP (○——○) as described in Materials and methods except that 70 mM NaCl and 3.2 mM spermidine were added.

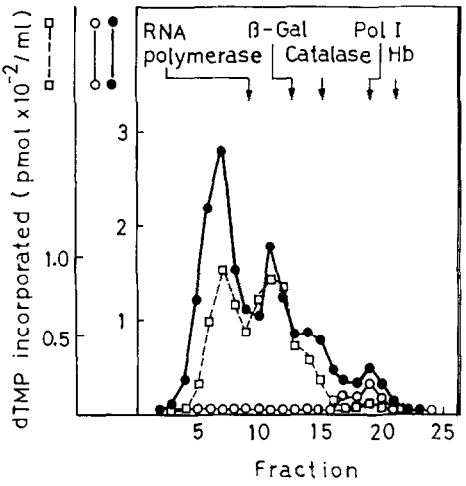

Fig. 4. Glycerol gradient sedimentation profile. P1 fraction (750 µg) was layered over 5 ml of a 20 - 40% glycerol gradient containing 20 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM EDTA and 100 µM ATP, and centrifuged for 15 h at 50,000 rev/min in a Beckman SW 65L Ti rotor at -2.5°C. Fractions of 0.2 ml were collected from the bottom of the tube. Aliquots (20 µl) of each fraction were assayed for DNA-synthesizing activity on poly(dA)•$(dT)_{10}$ with ATP (●——●), without ATP (○——○), and activated salmon sperm DNA (□- - -□) as described in Materials and methods. RNA polymerase ($s_{20,w}$ = 22 S), ß-galactosidase ($s_{20,w}$ = 15.9 S), catalase ($s_{20,w}$ = 11.3 S), pol I ($s_{20,w}$ = 5.5-5.6 S) and hemoglobin ($s_{20,w}$ = 4.5 S) were used as marker protein (14).

DISCUSSION: We reported earlier (14), that pol III* activity from wild type cells had the unusually high 17 S sedimentation value. Recently, it was suggested by McHenry (5) that pol III' contains two pol III core enzyme assemblies and two subunits of τ (tau) which might lead to a novel mechanism for DNA replication. On the basis of this presumption, the molecular weight of the pol III holoenzyme might correspond to 600,000 to 700,000 (or about 16 S to 18 S) by approximate calculation. Our experimental results suggest that the molecular weights corresponding to 24 S and 18 S fractions obtained for pol III holoenzyme activity (Fig. 4) may exceed that of E. coli RNA polymerase dimer (22 S, mol. wt. 980,000) and of ß-galactosidase (16 S, mol. wt. 540,000), respectively. As described above, the product analyses of fraction P1 also suggest the involvement of SSB in the fraction. Accordingly, it may be that the first and second fractions in the order of the elution on the gel filtration, or the 24 S

and 18 S fractions obtained in the density gradient ultracentrifugation, include some other functional proteins, such as SSB or others for DNA replication besides the known pol III holoenzyme.

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