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A Protein Cofactor That Stimulates the Activity of DNA-Dependent RNA Polymerase I on Double-Stranded DNA†

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ABSTRACT: Partially purified rat liver RNA polymerase I chromatographed on ribosomal RNA-Sepharose loses most (96%) of its activity assayed on native calf-thymus DNA templates, but loses little (8%) of its activity assayed on poly(deoxycytidylic acid) template. Polymerase I is not stimulated by polymerase II protein factor, or by bovine serum albumin. However, it is stimulated by histones, polylysine, and spermine. Addition of a protein fraction eluted by high ionic strength from the rRNA-Sepharose also restores activity on native calf-thymus DNA. Further purification yields a fraction containing two proteins of 11 000 and 12 000 molecular weight. Both proteins are distinct from histones by electrophoresis in sodium dodecyl sulfate and in acid urea. Both proteins are basic, insensitive to heat, bind to DNA, and stimulate polymerase I activity. The degree of stimulation of polymerase I is dependent upon both the enzyme/DNA and the factor/DNA ratio. The protein factors also stimulate polymerase II activity about half as effectively as polymerase I.

Eucaryotic RNA polymerases I and II are each composed of two high molecular weight and several lower molecular weight subunits (Weaver et al., 1971; Kedinger et al., 1974; Sklar et al., 1975; Hager et al., 1977). The molecular weights of the corresponding subunits of I and II are largely different; thus the molecules are distinct entities. The overall structure is analogous to that of the *Escherichia coli* core polymerase (Burgess, 1969) and implies a similarity of mechanism. This would suggest that eucaryotic RNA polymerases may require factors similar to the sigma factor (Burgess et al., 1969).

Several types of factors affecting transcription have been described in the literature. They may be divided into three groups: (a) nonspecific factors; (b) factors isolated independently that stimulate activity on double-stranded DNA but not on single-stranded DNA; and (c) factors that initially co-purify with the enzyme. Factors in the first group include DNase, which stimulates eucaryotic polymerase by the introduction of single-stranded breaks in the template and production of a greater number of initiation sites (Chambon et al., 1970; Keller and Goor, 1970), and bovine serum albumin, presumably a nonspecific protein, which stimulates both polymerases I and II to varying degrees (Rutter et al., 1973). Factors in the sec-

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ond group are capable of stimulating the activity of polymerase II on native, but not denatured, template in a highly variable fashion (Lentfer and Lezius, 1972; Seifart et al., 1973). Factors of the third group are capable of a modest stimulation of activity (Sugden and Keller, 1972; Froehner and Bonner, 1973; Higashinakagawa et al., 1972).

In this paper, we report the isolation of a low molecular weight, heat-stable factor that co-purifies with RNA polymerase I. The factor can be separated from the enzyme by chromatography on ribosomal RNA-Sepharose or by sucrose density gradient centrifugation. It is a DNA-binding protein that appears to act at the binding or initiation step, either by reducing the affinity of the enzyme for unproductive sites on the template or by facilitating enzyme entry into a productive site.

Experimental Section

Materials

Materials and their sources have been described previously (Goldberg et al., 1977). The calf-thymus histone mixture was purchased from Worthington. Individual fractions were kindly provided by Dr. R. David Cole, University of California, Berkeley.

Enzyme Assays

Enzyme assays with native calf-thymus DNA (nCT-DNA) and poly(deoxycytidylic acid) [poly(dC)] have been described previously (Goldberg et al., 1977).[1] When recombination assays were done, factor and enzyme or factor and DNA were incubated at low ionic strength for 10 min before addition of the reaction mixture.

Isolation of DNA

Calf-thymus DNA (Sigma), *Micrococcus luteus* DNA (miles), and poly(dC) (gift of Dr. Fred Bollum, University of Kentucky) were either purchased commercially or were gifts. Calf-thymus DNA was sheared in a French pressure cell at 9000 psi to obtain short double-stranded pieces and sonicated with a Branson sonicator at full power for 1–2 min to introduce nicks and gaps. Rat liver nucleolar DNA was isolated from purified nucleoli by chloroform–octanol extraction (Marmur, 1961).

Single-stranded and double-stranded molecular weights were determined by standard ultracentrifugal techniques.

Separation of Rat Liver RNA Polymerase I and Polymerase I Factor by Ribosomal RNA-Sepharose Chromatography

Rat liver RNA polymerase I was isolated through the ion-filtration step as described previously (Goldberg et al., 1977). The specific activity of the enzyme was 10–15 units/mg. The extent of purity was only 3–5%, although the two high molecular weight bands were visible on sodium dodecyl sulfate–polyacrylamide gels.

Ion-filtration enzyme was either dialyzed or diluted to an ammonium sulfate [$(NH_4)_2SO_4$] concentration of 0.1 M before application to a ribosomal RNA-Sepharose (rRNA) column. A white precipitate formed after the ionic strength had been lowered to 0.1 M $(NH_4)_2SO_4$, and was removed by centrifugation without affecting either nCT-DNA or poly(dC) activity. One milliliter of ion-filtration enzyme (0.1–0.2 mg/mL) was loaded per 0.2 mL of rRNA-Sepharose (1–2 mg of rRNA/mL bed volume) in 0.1 M $(NH_4)_2SO_4$ in TGMED buffer (0.05 M Tris (pH 7.9), 25% v/v glycerol, 0.005 M $MgCl_2$, 0.0004 M EDTA, and 0.0005 M dithiothreitol). The load was collected and the column then washed with 1–2 column volumes of equilibration buffer. Residual enzyme and factor were then eluted with 1 M $(NH_4)_2SO_4$ in TGMED. Fractions (0.2 mL) were collected and assayed for enzyme. Each fraction was heat treated and then assayed by incubation with an aliquot of flow-through enzyme prior to addition of nCT-DNA. The factor activity appeared immediately after enzyme at 0.25–0.3 M $(NH_4)_2SO_4$. Factor could be further purified by removal of a precipitate after heating at 70 °C for 30 min.

Separation of Rat Liver RNA Polymerase I and Polymerase I Factor by Sucrose Density Gradient Centrifugation

Sucrose density gradients were prepared in a manner similar to that described previously (Goldberg et al., 1977). The gradients contained, in a total volume of 5.2 mL, 5–20% sucrose–0.1 M $(NH_4)_2SO_4$ in TGMED. The ion-filtration enzyme was concentrated by $(NH_4)_2SO_4$ precipitation and stirred overnight at 4 °C. The pellets were collected by centrifugation and dissolved in TGMED at a concentration of 3–7 mg/mL. Aliquots of 250 μL were layered over each gradient and centrifuged at 400 000*g* at 4 °C for 16 h. After centrifugation, the gradients were fractionated into 0.25-mL fractions. Protein and enzyme assays were done on each fraction. A low molecular weight fraction corresponding to a peak of protein was pooled and used as a source of the factor. Recombination assays were done by incubating the factor fraction plus an aliquot of enzyme from each tube prior to addition of nCT-DNA and other assay ingredients. The factor fractions had no enzyme activity.

Chromatography of Rat Liver Factor on Denatured DNA-Cellulose

Rat liver factor, isolated by rRNA-Sepharose chromatography, was concentrated to a final volume of 0.5 mL by the Amicon ultrafiltrator. The factor was diluted with 0.1 M KCl in TGED and loaded onto a 1-mL denatured DNA-cellulose column. The column was washed with 0.1 M KCl in TGED and the factor eluted with a five column volume linear gradient of 0.1–1.0 M KCl in TGED; 0.22-mL fractions were collected.

An aliquot of each column fraction was incubated with 2 μL of sucrose gradient rat liver polymerase I for 10 min at 4 °C prior to addition of the assay mixture containing the nCT-DNA and labeled nucleoside triphosphates.

Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The procedure for sodium dodecyl sulfate gels has been described in the accompanying paper (Goldberg et al., 1977) except that the running gels contained 15% acrylamide.

Samples were prepared for electrophoresis by precipitation with 25% Cl_3CCOOH in the presence of 250 μg/mL poly(adenylic acid). Histone standards were run at the same time and included arginine-rich, lysine-rich, and slightly lysine-rich fractions.

Urea Gel Electrophoresis. Urea gel electrophoresis was carried out as described by Panyim and Chalkley (1969).

[1] Abbreviations used are: poly(dC), poly(deoxycytidylic acid); rRNA, ribosomal ribonucleic acid; $(NH_4)_2SO_4$, ammonium sulfate; nCT-DNA, native calf-thymus DNA.

TABLE I: Recombination of Polymerase I and Factor.

Source of Enzyme	Source of Factor	Activity (pmol) nCT-DNA	Activity (pmol) Poly-(dC)
A. rRNA-Sepharose[a]		0	21
	rRNA-Sepharose, high salt wash	1.1	12.2
	Heat-treated, ion-filtration enzyme	0.85	14.5
B. Ion Filtration[b]		1.8	
	rRNA-Sepharose, high salt wash	1.4	

[a] Ribosomal RNA-Sepharose enzyme was prepared by chromatography of rat liver polymerase I ion-filtration pool as described for part B. The enzyme appearing in the flow-through fractions of the rRNA-Sepharose column, and having no activity on nCT-DNA, was recombined with either the high salt wash fraction from the rRNA-Sepharose column or with a heat-treated fraction from the ion-filtration pool. The high salt wash fraction was heated to 50 °C for 30 min to destroy any residual polymerase activity and then dialyzed against TGMED to lower the ionic strength. The ion-filtration fraction was treated in the same way. Enzyme plus stimulatory fractions were incubated for 10 min at 23 °C in 0.05 M $(NH_4)_2SO_4$ prior to addition of cocktail. The final $(NH_4)_2SO_4$ concentration in the nCT-DNA cocktail was 0.05 M and in the poly(dC) cocktail it was 0.14 M. The nCT-DNA activity found after recombination is maximal and was reached by titration with increasing amounts of factor. [b] The preparation of ion-filtration enzyme has been described previously. The high salt wash fractions were prepared as noted above.

TABLE II: Polymerase I Activity after Sucrose Gradient Centrifugation.[a]

Concn of Sample (mg/mL)	% Recovery Poly(dC)	% Recovery nCT-DNA	% Recovery following Addition of Factor to Peak Fraction (nCT-DNA)
3.5	100	100	
1.75	92	56	74
0.87	84	29	120
0.44	80	13.6	

[a] Rat liver polymerase I from the ion-filtration step was precipitated with $(NH_4)_2SO_4$. The precipitate was taken up in a small volume of TGMED. Appropriate dilutions were made and 250 μL of each dilution was applied to a 5–10% sucrose gradient in TGMED. Each gradient was centrifuged at 400 000*g* for 12 h. Fractions (0.2 mL) were collected and assayed for nCT-DNA and poly(dC) activity. The initial load had a protein concentration of 3.5 mg/mL and contained 14.3 units of nCT-DNA and 28.1 units of poly(dC) activity. Percent recovery was based on the number of units of activity applied to each gradient. The peak fraction in the gradients containing 1.75 and 0.87 mg/mL was incubated with an aliquot from the concentrated pool of low molecular weight gradient fractions. The percent recovery was then determined from the ratio of the activity of that fraction (after stimulation by factor) divided by the maximum activity calculated to be in that fraction.

Factor fractions and histone standards were prepared by precipitation with 10% Cl_3CCOOH in the presence of 250 μg/mL crystalline bovine serum albumin.

Electrophoresis was conducted in the Hoefer apparatus at 200 V for 1.5 h. After the run had been completed, the slab was removed and stained in 45% methanol, 9% acetic acid, and 0.25% Coomassie blue stain overnight. The gel was destained in 45% methanol–7.5% acetic acid at room temperature.

Results

Polymerase I Activity Is Dependent on a Protein Cofactor. When rat liver polymerase is chromatographed on rRNA-Sepharose at 0.1 M $(NH_4)_2SO_4$, more than 85% of the initial poly(dC) activity is found in the flow through and the remaining 12% in the column eluate. In contrast, only 2% of the initial nCT-DNA activity is found in the flow-through fractions while another 2% is eluted from the column. A control experiment with untreated Sepharose shows that almost 80% of the initial nCT-DNA activity is recovered from such a column. In these experiments about 60% of the protein applied to the column is recovered in the fractions containing enzyme activity, and the remainder elutes at higher ionic strength (data not shown).

When this high ionic strength fraction (after removal of the salt) is added to the flow-through fractions that have lost their nCT-DNA activity, there is a substantial increase in that activity (Table I). Concomitantly, there is a loss of up to 50% in poly(dC) activity. A similar stimulation of nCT-DNA activity and an inhibition of poly(dC) activity was produced when a fraction of the initial load was heat treated to destroy catalytic activity and recombined with the flow-through fractions (Table IA). In the control experiments (Table IB), heat-treated, high salt wash fractions do not affect the activity of enzyme fractions already having nCT-DNA activity.

The activity against nCT-DNA is also lost after sucrose gradient centrifugation, and can be restored by addition of the lower molecular weight fractions. In Table II, the percent recovery of polymerase I is presented as a function of the protein concentration of the load. The recovery of poly(dC) activity is greater than 80% for all enzyme concentrations, but the recovery of nCT-DNA activity declines from 100 to less than 14%. Recombination experiments demonstrate that low molecular weight fractions from the gradient, when added to the enzyme, completely restore native activity. The association constant between polymerase I and the stimulatory factor must be low, since an excess of factor added to the enzyme before gradient centrifugation does not affect the recovery of native activity.

Characterization of Polymerase I Factor. The stimulatory activity of the fraction eluted at high ionic strength from rRNA-Sepharose is heat resistant. Heating of the fraction to 70 °C for 30 min has no effect on stimulatory activity while boiling for 1 to 2 min reduces such activity by 10%.

When the factor fraction is applied to a denatured DNA-cellulose column, it binds tightly and is not found in either the flow-through or the low ionic strength fractions. A peak of protein is eluted between 0.5 and 0.7 M KCl. The protein-containing fractions are assayed with a rat liver polymerase I preparation that is low in nCT-DNA activity. Stimulation of the nCT-DNA activity occurs only in the region where the peak of protein is found (data not shown).

The stimulatory fractions from the denatured DNA-cellulose column were analyzed both by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by acid urea–polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis disclosed two protein bands in each fraction (Figure 1, b–f) which corresponded in intensity to the degree of stimulatory activity. The proteins could barely be discerned in the gels of the enzyme (Figure 1, g,h) suggesting they were very minor components, although they were the major staining bands in the rRNA high salt wash (Figure 1a). Comparison

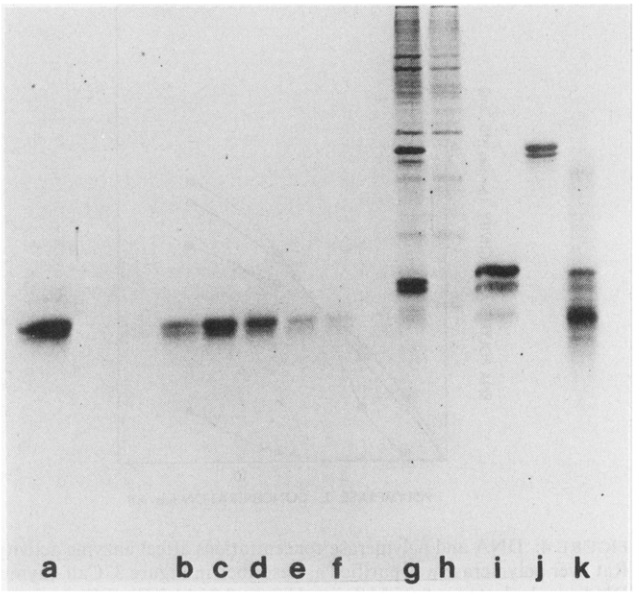

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of polymerase I factor. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out as described in the text: a is the high salt wash fraction from the rRNA-Sepharose column; b–f are the active fractions from the denatured DNA-cellulose column; g–h are representative enzyme fractions, the ion-filtration and phosphocellulose steps, respectively. The histone fractions are displayed in i–k. The major bands were identified by comparison with similar gels in the article by Panyim and Chalkley (1971); i is arginine-rich histone, j is lysine-rich histone, and k is slightly lysine-rich histone.

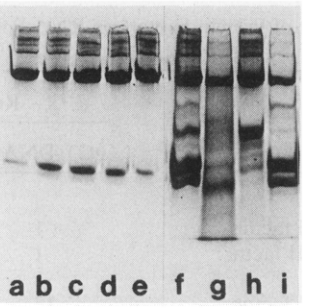

FIGURE 2: Acid–urea gel electrophoresis of polymerase I factor. Acid–urea gel electrophoresis was carried out as described in the text: a–e show the active fractions from the denatured DNA-cellulose column; f–i are the histone fractions, and include a commercial preparation (f), slightly lysine-rich histone (g), lysine-rich histone (h), and arginine-rich histone (i). The intensely staining band at the top of the gel is bovine serum albumin, which was used to facilitate precipitation of the various fractions.

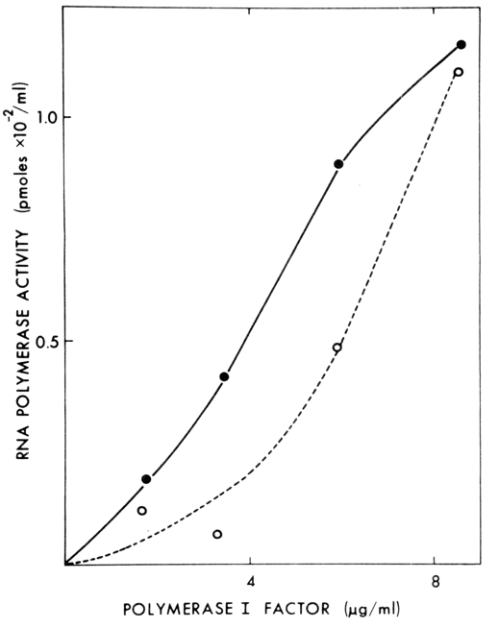

FIGURE 3: RNA polymerase I factor binds to DNA. Rat liver polymerase I was purified through the sucrose density gradient step. The enzyme was applied to the gradient at a protein concentration of 1 mg/mL (250 μL containing 7.4 nCT-DNA units) to facilitate dissociation of factor. The recovery of nCT-DNA activity was 31%. The factor was recovered from the low molecular weight fraction. Reactions were carried out by incubating enzyme (0.002 nCT-DNA unit) with the indicated concentration of factor in a total volume of 0.02 mL at 4 °C for 30 min. Reactions were started by the addition of 0.04 mL of a solution containing 40 μg of calf-thymus DNA and nucleoside triphosphates. Alternatively, incubations were done with factor plus reaction solution or factor plus enzyme plus DNA. The latter reaction was started by addition of the nucleoside triphosphate solution: (○) nCT-DNA activity after preincubation of factor plus DNA or factor plus enzyme plus DNA; (●) nCT-DNA activity after preincubation of factor plus enzyme.

with the three histone fractions (Figure 1, i–k) showed the factor(s) to have a different mobility and molecular weight than either arginine-rich histone (F3 + F2b, F2a2) or lysine-rich histone (F1). However, the mobility of the factor(s) is close to that of the major component of the slightly lysine-rich fraction (F2a1) (Panyim and Chalkley, 1971). The factor fractions (Figure 2, a–c) were also electrophoresed in acid–urea gels under the same conditions as calf-thymus histones (Figure 2, f–i). It can be seen that the factor is a single band with a mobility intermediate between those of histones F3 and F2b + F2a2 and distinct from F2a1. Since the factor has previously been shown to have a different mobility than these histones on sodium dodecyl sulfate electrophoresis, we conclude that it is not one of the major histone classes.

The binding of active enzyme and factor to rRNA-Sepharose and DNA-cellulose suggested that the factor(s) was a DNA-binding protein. This has been confirmed by an experiment in which increasing concentrations of factor were used to titrate nCT-DNA inactive polymerase I. Preincubation of factor and polymerase I at 4 °C for 10 min followed by addition of nucleoside triphosphates and DNA gives a sigmoidal titration curve (Figure 3) with less than 10% of maximal activity at the lowest factor concentrations (1.5 and 3 μg/mL). However, preincubation of factor plus DNA, or of all three (factor plus enzyme plus DNA), followed by addition of nucleoside triphosphates shifts the curve. At 3 μg/mL factor, stimulation is 30% of maximal. At a lower concentration of template (330 μg/mL nCT-DNA), stimulation by factor under identical conditions is 80% of maximum. These collective data suggest that the factor binds to DNA as well as to polymerase.

Polymerase I Factor Specificity. The stimulation of nCT-DNA activity by factor reaches a plateau at a factor concentration comparable to that of enzyme protein. Assay of poly(dC) activity disclosed a significant inhibition (up to 30% of the control) as the concentration of polymerase I factor increased. Addition of a nonspecific protein such as crystalline bovine serum albumin had no effect on nCT-DNA activity. Inhibition of poly(dC) activity in the presence of bovine serum albumin was only noticeable at concentrations exceeding 2 mg/mL.

In an experiment designed to determine the specificity of polymerase I and polymerase II factors, saturating concentrations of each were incubated with polymerase I and polymerase II and the mixture then assayed with both templates

TABLE III: Effect of Protein Factors on RNA Polymerases I and II.[a]

	Rel Act.	
	nCT-DNA	Poly(dC)
Pol I^+		
+ form I factor	1	0.3
+ form II factor	1	0.9
Pol I^-		
+ form I factor	>13	0.5
+ form II factor	1	0.2
Pol II^+		
+ form I factor	1	0.6
+ form II factor	1.6	1
Pol II^-		
+ form I factor	4.7	0.9
+ form II factor	9.2	0.8

[a] Pol I^+ was enzyme isolated through the ion-elution step. Its nCT-DNA activity was 0.3 unit/mL and its poly(dC) activity was 5.6 units/mL. Pol I^- was isolated as described in Figure 4. Pol II^+ was isolated through the DEAE-Sephadex step as described (Weaver et al., 1971). Its nCT-DNA activity was 8.5 units/mL and its poly(dC) activity was 31.6 units/mL. Pol II^- had been purified through the sucrose density gradients and had nCT-DNA activity of 0.1 unit/mL and poly(dC) activity of 9.3 units/mL. Polymerase II factor was isolated from calf thymus by Fanyela Weinberg according to the method of Stein and Hausen (1970). Enzyme and a saturating concentration of factor were incubated at 4 °C for 10 min prior to addition of cocktail. Enzyme controls were done in the absence of factor. The relative activity was the ratio of enzyme activity in the presence of factor to that in its absence. Polymerase I was assayed at 0.05 M $(NH_4)_2SO_4$ and polymerase II at 0.03 M $(NH_4)_2SO_4$ (Stein and Hausen, 1970). The maximal concentration in the assay of calf-thymus histone was approximately 1 μg.

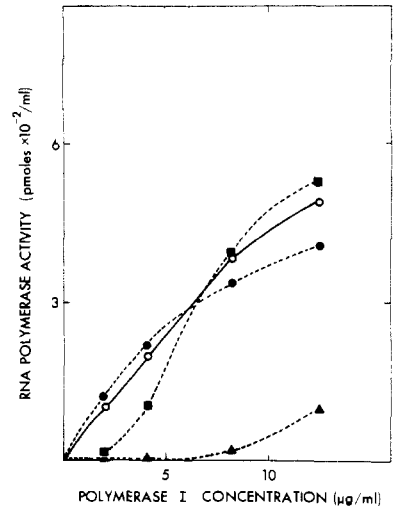

FIGURE 4: DNA and polymerase concentrations affect enzyme activity. Rat liver polymerase was purified as described in Figure 3. Calf-thymus DNA was hydrated in 0.05 M Tris (pH 7.9)–0.05 M KCl without stirring so that shearing was minimized. Reaction solutions were prepared containing the desired concentration of DNA and the reaction initiated by the addition of the desired concentration of enzyme. When assayed in the standard solution, an enzyme concentration of 2 μg/mL had an nCT-DNA activity of 0.03 unit/mL: (○) polymerase I^+, 640 μg/mL nCT-DNA; (●) polymerase I^-, 80 μg/mL nCT-DNA; (■) polymerase I^-, 640 μg/mL nCT-DNA; (▲) polymerase I^-, 1280 μg/mL nCT-DNA.

(Table III). Two different preparations of each enzyme were employed: one that had not been chromatographed on rRNA-Sepharose and was not activated by factor (i.e., Pol I^+), and one that had been chromatographed on rRNA-Sepharose such that it was activated by the factor (i.e., Pol I^-) (Table III, column I). The activity of Pol I^+ on nCT-DNA does not change upon addition of either polymerase I or polymerase II factor. However, when Pol I^- is recombined with the protein cofactors, only polymerase I factor is capable of stimulating activity.

Since polymerase I factor is a basic protein, other known basic proteins were assayed for stimulatory activity. Commercially available calf-thymus histone stimulated polymerase I^- 2.5-fold, while having no effect on polymerase I^+, II^+, or II^-. Preincubation of histone plus enzyme and histone plus DNA shifted the stimulation curve in the same manner as for the factor, lending additional support to the hypothesis that the factor is a DNA-binding protein.

At protein concentrations greater than 10 μg/mL, stimulation by calf-thymus histone is no longer optimal and at still higher concentrations inhibition is observed. There is no synergism between factor and histone. Addition of either after the reaction has started in their absence increased the amount of activity less (11–33%) than occurs at initiation (57–60% activation) and suggests both may act at an early step in the reaction sequence.

Other experiments have shown that the lysine-rich fraction of calf-thymus histone and polylysine stimulate polymerase I^- equally. Spermine at very low concentrations (0.3 mM) stimulates polymerase I^- approximately 30% as effectively as histone or factor, although at 5 mM it inhibits both polymerase I^- and polymerase I^+ at the low ionic strengths used for assay. A similar concentration-dependent effect of spermine on pig kidney polymerase has been demonstrated by Janne et al. (1975).

Polymerase I and DNA Concentration Affect Enzyme Activity. In Figure 4 enzyme activity at several nCT-DNA concentrations is plotted as a function of enzyme concentration. When Pol I^+ is assayed, the activity in the presence of 600 μg/mL nCT-DNA is linear until a concentration of 8 μg/mL protein is reached and then falls off when template becomes limiting. The same phenomenon occurs with Pol I^- at low DNA concentrations (80 μg/mL). However, at comparable concentrations of DNA (600 μg/mL), there is a marked decrease of the initial rate. The inhibition for Pol I becomes more severe as the template concentration is increased to 1200 μg/mL.

Inhibition by template also appears to be a function of the source of DNA and may be related to its method of preparation. In Table IV enzyme activity is presented as a function of the concentration and source of DNA. When the activity of Pol I^+ at a single protein concentration is measured on unsheared calf-thymus DNA, it increases 75% when the DNA concentration is raised eightfold, as if template saturation were occurring. The same effect is seen with sheared calf-hymus DNA and nucleolar DNA. On the other hand, the activity of Pol I^- decreases as the concentration of DNA is increased (with the exception of *M. luteus* DNA). The inhibition is most pronounced when sheared or sonicated calf-thymus DNA is present (640 μg/mL).

The lack of activity by Pol I^- on calf-thymus DNA is apparently not due to a nonspecific inhibitor in the DNA preparation. When the DNA is denatured, the relation of enzyme activity to DNA concentration observes normal saturation kinetics (not shown). Furthermore, Pol I^+ is not inhibited by increasing concentrations of nCT-DNA.

TABLE IV: Template Concentration Affects Polymerase Activity.[a]

DNA	Concn (μg/mL)	Act. (pmol/10 min) Polymerase I⁻	Polymerase I⁺
Unsheared nCT-DNA	80	2.2	1.2
	640	1.1	2.0
Sheared nCT-DNA	80	2.5	1.8
	640	0.6	3.9
Sonicated nCT-DNA	80	1.7	
	640	0.2	
Rat nucleolar DNA	50	3.4	1.0
	400	2.2	4.5
M. luteus DNA	40	1.2	
	330	4	

[a] Polymerase I⁺ was isolated through the ion-filtration step and had an activity of 0.3 nCT-DNA unit/mL. Polymerase I⁻ was purified as described in Figure 3 and had an activity of 0.9 nCT-DNA unit/mL. The isolation and production of the various DNAs have been described in the Experimental Section.

Discussion

A Protein Cofactor Affects Polymerase I Activity. Since the discovery and identification of multiple DNA-dependent RNA polymerases in the cells of eucaryotic organisms (Roeder and Rutter, 1969), considerable effort has been expended in the search for a protein cofactor(s) that could modulate their activity. Most of the experimental protocols have been patterned after those for the isolation and recombination of *E. coli* sigma protein and core RNA polymerase (Burgess et al., 1969). No protein has as yet been found in eucaryotes that exactly fits these criteria, although a number of stimulatory fractions are known (Seifart, 1970; Goldberg and Moon, 1970; Sugden and Keller, 1972; Lee and Dahmus, 1973).

In the course of purifying polymerase I from both ascites and rat liver, we have found that the ratio of enzyme activities, when assayed using native calf-thymus DNA and poly(deoxycytidylic acid), often decreased during the isolation procedures. These changes were particularly pronounced when the enzyme was either chromatographed on rRNA or poly(U)-Sepharose (Goldberg et al., 1977) or diluted and then centrifuged through a sucrose gradient (Table II). The data suggested that a (protein) cofactor was dissociated from the enzyme.

We have devised isolation procedures that can reproducibly separate polymerase I from a stimulatory fraction. They include chromatography on rRNA-Sepharose and sucrose density gradient centrifugation. In each case, addition of fractions not containing any enzyme activity to the reaction mixture restores enzyme activity on nCT-DNA.

Characterization and Specificity of Polymerase I Factor. The polymerase I factor appears to be a basic, low molecular weight compound(s). We infer that the factor is basic because it binds tightly to cation exchangers, such as phosphocellulose, and weakly, or not at all, to DEAE-Sephadex. Heat stability usually indicates the presence of low molecular weight components (<40 000). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the high salt eluate of the DNA-cellulose column reveals the presence of two bands of molecular weight from 11 000 to 12 000. The activity is presumed to reside in one (or both) of these components and is distinct from the putative enzyme subunit that appears to affect RNA synthesis from native calf-thymus DNA.

A rough estimate can be made of the amount of factor required for saturation of the enzyme. Approximately 50 molecules of factor may be required for maximal stimulation of (assuming 20% purity of 15 000 molecular weight factor and 25% purity of enzyme) 1 mol of polymerase under our assay conditions. This value may not be surprising in view of the fact that sucrose gradient centrifugation of factor and enzyme shows them to have a low association constant. A similar calculation for the histone fraction indicates that 100–200 mol of histone per mol of enzyme is required for optimal effects.

Relationship of Polymerase I Factor to Other Stimulatory Proteins. Stimulatory proteins for RNA polymerases may bind to the enzyme, or to the template or both. Sigma, the subunit of *E. coli* DNA dependent RNA polymerase, is obviously a polymerase-specific factor. It binds to the enzyme apparently mediating its ability to discriminate between nonspecific DNA binding sites and promoters (Burgess et al., 1969; Hinkle and Chamberlin, 1972a,b) and is subsequently released during transcription. Several accessory proteins are also known which are thought to bind to holoenzyme, and to stimulate initiation on a variety of templates (Leavitt et al., 1972; Ramakrishnan and Echols, 1973).

Although the polymerase I factor(s) that we have isolated co-purifies with the enzyme, it does not have the properties of sigma. In fact, polymerase I factor bears a remarkable resemblance to an unusual class of either procaryotic or eucaryotic proteins capable of affecting RNA polymerase activity. For example, the procaryotic factors D (Ghosh and Echols, 1972) and H. (Jacquet et al., 1971) are a group of heat-stable, basic, low molecular weight (~20 000) DNA binding proteins whose stimulatory activity is a function of the enzyme/DNA ratio. *E. coli* polymerase holoenzyme is inhibited by high concentrations of DNA (limiting enzyme) and addition of D or H protein relieves this inhibition. Conversely D or H protein is capable of inhibiting enzyme activity at very low concentrations of DNA (limiting DNA). The degree of stimulation is also related to the factor/DNA ratio. Both factors appear to operate by causing changes in the DNA temperature transition profile, stabilizing certain regions of the DNA while inducing local melting in others (Cukier-Kahn et al., 1972; Crepin et al., 1975). In this sense, D and H factors are analogous to the DNA unwinding proteins (Sigal et al., 1972; Molineux and Gefter, 1974), which also reduce the denaturation temperature of DNA, presumably by binding to what would otherwise be transient single strands and preventing them from renaturing.

In eucaryotes, histones at low concentrations are capable of performing many of the functions of these basic factors. At limiting enzyme, both yeast and *E. coli* polymerases are stimulated by calf-thymus histone (Hall et al., 1973; Konishi and Koide, 1970). The degree of stimulation is dependent on both the enzyme/DNA and histone/DNA ratio.

Other similar chromatin binding proteins are also capable of affecting RNA synthesis (Teng et al., 1971; Shea and Kleinsmith, 1973; Kostraba et al., 1975). A low molecular weight (12 000) stimulatory activity has been isolated from yeast (DiMauro et al., 1972). More recently, a "histone-like" nonhistone chromosomal protein has been purified from rat liver nucleoli. Its levels were markedly reduced during nuclear hypertrophy induced by regeneration or by administration of thioacetamide (Goldknopf et al., 1975). Several workers have shown there are a number of similar tissue-specific, low molecular weight proteins (10 000–30 000) that have unusual electrophoretic mobility (Wakabayashi et al., 1973, 1974; Goodwin et al., 1973; Goodwin and Johns, 1973) and whose function is not yet known.

Possible Mechanism of Action of Polymerase I Factor. Our evidence suggests that polymerase I factor acts at the initiation step. If so, there are several possible ways in which it may modulate enzyme activity. Polymerase I, bound to unproductive sites, may not transcribe or may terminate abortively at a very early stage of RNA synthesis. Polymerase I factor could then act negatively, like sigma, by preventing the enzyme from binding to unproductive sites. It might do so by functioning as a DNA-unwinding protein which would stabilize the site so that polymerase would no longer bind there.

Alternatively, polymerase I factor could act in a positive manner by facilitating the interaction of polymerase with potential initiation sites. By acting at the initiation step after polymerase has bound to DNA, it would increase the likelihood that polymerase would start and continue RNA synthesis. As an unwinding protein it could alter the transient equilibrium between open and closed promoters and freeze the promoter in an open configuration. Alterations in promoter availability depend upon whether that promoter is likely to be used under a given set of physiological conditions (Travers, 1974).

In addition, the existence of factor could further explain the initiation requirement of polymerase I for single-stranded gaps of 2–50 nucleotides. As shown by Dezelee et al. (1974) and by Hossenlopp et al. (1974), only supercoiled DNA or DNAs with single-stranded gaps are effective templates for polymerase I. In linear duplex, the unpaired structure required for chain initiation easily might be induced by a protein factor acting as a DNA-unwinding protein. A study of the action of these factors may reveal one of the significant aspects of regulation of transcriptive activity.

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