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I Protein: Bacteriophage T7-Coded Inhibitor of *Escherichia coli* RNA Polymerase

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Bacteriophage T7-coded inhibitor of *Escherichia coli* RNA polymerase, termed I protein, was purified from an inactive *E. coli* RNA polymerase-I protein complex isolated from phage T7-infected cells. A molecular weight of about 7,000 to 9,000 was assigned to the purified I protein by acrylamide-sodium dodecyl sulfate gel electrophoresis, Sephadex G-50 gel filtration, and glycerol gradient centrifugation analysis. I protein inhibits initiation of RNA synthesis by directly binding to the RNA polymerase holoenzyme and prevents the binding of the enzyme to the promoter sites on the template T7 DNA. However, once a highly stable transcriptional preinitiation complex between RNA polymerase holoenzyme and T7 DNA is formed at the promoter site on T7 DNA in the absence of nucleoside triphosphates, I protein does not inhibit the initiation of RNA synthesis by this preformed complex upon addition of nucleoside triphosphates. RNA synthesis by the core RNA polymerase and the binding of core RNA polymerase with template DNA are not inhibited by I protein, although a partial association between the core enzyme and I protein can be observed. I protein does not bind to sigma factor or T7 DNA. Therefore, binding of I protein with the RNA polymerase, which results in the inhibition of initiation of RNA synthesis, requires the presence of sigma factor in the RNA polymerase holoenzyme form.

Bacteriophage T7 development in *Escherichia coli* involves a unique transcription control mechanism in the "early to late" switch of T7 gene expression. *E. coli* RNA polymerase which transcribes T7 early mRNA is inactivated at about the same time that T7-specific RNA polymerase (5), which is one of the T7 early gene products, begins to transcribe T7 late mRNA. This switch from the host to phage-coded enzyme had been known for some time, but the exact mechanism which "shuts off" the activity of *E. coli* RNA polymerase was not understood even though involvement of a phage T7-coded protein in the host shutoff was speculated (2, 3, 19).

We have previously shown that the shutoff of *E. coli* RNA polymerase in the T7-infected cell is due to an association of an inhibitor protein, very likely a T7-coded protein, with the enzyme, thus forming an inactive enzyme complex (7, 9). The inhibitor protein, termed I protein, was isolated from the inactive RNA polymerase complex prepared from T7-infected cells, and we were able to show that purified I protein inhibits in vitro RNA synthesis by *E. coli* RNA polymerase holoenzyme at initiation but does not inhibit RNA synthesis by *E. coli* RNA polymerase core enzyme or T7-specific RNA polymerase (7). After our report of these findings, a similar inhibitor protein of RNA polymerase was isolated from phage T7-infected *E. coli* cells (17).

In the preceding paper, we described the results of our genetic analysis which indicated that gene 2 of phage T7 codes for the I protein (8). In this paper, we describe further purification and characterization of the I protein and the mode of inhibition of *E. coli* RNA polymerase by purified I protein. By refining the analytical procedures, we estimated a molecular weight of about 7,000 to 9,000 for the I protein, a value slightly smaller than our previous estimation (7). We found that the inhibition of initiation of RNA synthesis by I protein is due to the prevention of binding of *E. coli* RNA polymerase holoenzyme to the promoter sites on T7 DNA through direct association of I protein with the enzyme.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]UTP (22.7 Ci/mmol), [*G*-^{3}H]leucine (40 Ci/mmol), and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear Corp.; ATP, CTP, GTP, and UTP were from Sigma Chemical Co.; rifampin was from Schwarz/Mann; poly[d(A-T)] was from P-L Biochemical Co.

Standard buffer solutions. The following buffer solutions were frequently used in this work: (i) buffer

A, consisting of 20 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol; (ii) buffer B, consisting of 10 mM Tris-hydrochloride (pH 7.9), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 2% glycerol; (iii) buffer C, consisting of 50 mM Tris-hydrochloride (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol; and (iv) buffer G, consisting of 50 mM Tris-hydrochloride (pH 7.6), 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM β-mercaptoethanol, and 5% glycerol.

Purification of *E. coli* RNA polymerase holoenzyme from T7-infected and uninfected cells. Usually 100 g (wet weight) of *E. coli* D10 F^- cells was harvested from large-scale M9-glucose medium cultures without infection or 9 min after T7 infection at 30°C. The cells were disrupted by grinding with alumina, extracted with buffer G, and then treated with DNase (0.5 μg/ml) for 30 min at 4°C. Disruption by sonic extraction of cells suspended in the same buffer resulted in a similar yield of the enzyme. Cell debris and alumina were removed from the crude extract by centrifugation at 13,000 × *g* for 10 min. The remaining debris and ribosomes were removed by centrifugation at 100,000 × *g* for 165 min. The resulting supernatant fluid was fractionated with ammonium sulfate. The 33 to 50% fraction was dissolved in buffer A and diluted to approximately 250 ml with a conductivity of 5.5 mmho (equal to buffer A with 0.09 M KCl). This solution was applied to a DEAE-cellulose (DE 52) column (2.5 by 20 cm) followed first by 50 ml of buffer A and then 100 ml of buffer A containing 0.09 M KCl. The column was then eluted with a 600-ml linear gradient containing 0.09 M to 0.4 M KCl in buffer A. The eluted fractions with the enzyme activity were pooled and precipitated with 60% ammonium sulfate, resuspended in 9 ml of buffer A, and dialyzed for 6 h against three changes of buffer A. This material was centrifuged in a 50-ml, 10 to 30% glycerol gradient in buffer A (no KCl) by using an SW25.2 rotor at 24,000 rpm for 24 h at 4°C. Less than 100 mg of protein was applied to each gradient. The gradients were fractionated, and the fractions containing the enzyme activity were pooled and precipitated with 60% ammonium sulfate. This partially purified enzyme preparation from T7-infected cells was used for the subsequent purification of the inhibitor I protein. However, to obtain highly purified *E. coli* RNA polymerase from both T7-infected and uninfected cells, this enzyme preparation was subjected to a second centrifugation in a 12-ml, 10 to 30% glycerol gradient in buffer A containing 1.0 M KCl using an SW41 rotor at 40,000 rpm for 24 h at 4°C. The gradient fractions containing the enzyme activity were pooled and precipitated with 2 volumes of saturated ammonium sulfate and then resuspended in buffer A containing 50% glycerol and stored at −70°C.

This procedure was similar to that of Burgess (4) with some modifications as described above. The *E. coli* RNA polymerase activity in preparations from T7-infected cells was monitored by the enzyme activity on poly[d(A-T)] up to the first glycerol gradient step due to the low enzyme activity on T4 DNA (see Table 1).

Purification of *E. coli* RNA polymerase core enzyme and sigma factor from T7-infected and uninfected cells. *E. coli* RNA polymerase core enzyme and sigma factor were separated by subjecting purified RNA polymerase holoenzyme to phosphocellulose column chromatography as described by Berg et al. (1). Sigma factor was recovered from the flow-through fractions at 0.08 M KCl, and the core polymerase was eluted at 0.35 to 0.40 M KCl. Sigma factor was further purified by DEAE-cellulose column chromatography and one cycle of glycerol gradient centrifugation.

Purification of I protein. I protein was purified from a partially purified *E. coli* RNA polymerase preparation from T7-infected cells harvested 10 min after infection. For radioactive I protein purification, 2 g of T7-infected cells labeled only after infection with 5 mCi of [^{3}H]leucine per 2 liters of culture was mixed with 50 g of nonradioactive T7-infected cells, and the same procedure was followed. Partially purified RNA polymerase was obtained by using our standard RNA polymerase purification procedure up to the first glycerol gradient centrifugation in the absence of KCl as described above (Table 1, step 5). Glycerol gradient fractions containing RNA polymerase activity measured on a poly[d(A-T)] template (almost no activity on T4 or T7 DNA) were pooled and precipitated with 60% ammonium sulfate, suspended in 2 ml of buffer C, and dialyzed for 8 h with three changes of buffer C, 4 liters each. This solution was applied to a phosphocellulose column (1.2 by 20 cm), allowed to equilibrate for 30 min, and then washed with 100 ml of buffer C containing 0.08 M KCl. The flow-through and wash fractions containing the inhibitor activity were directly applied to a DEAE-cellulose column (1.2 by 10 cm). The column was eluted with a 70-ml gradient of 0.08 M to 0.4 M KCl in buffer C. The inhibitor activity was eluted at about 0.3 M KCl. Sigma factor activity, which was present together with the inhibitor activity in the phosphocellulose column flow-through and wash fractions, was eluted at about 0.25 M KCl. The fractions showing the inhibitor activity were pooled and dialyzed against buffer A with five 1-hourly changes, and then applied to a small DEAE-cellulose column (total volume 1 ml). The inhibitor activity, concentrated by retention on the column, was eluted with buffer A containing 0.5 M KCl in a total volume of 1 ml. After dialysis against buffer B with three changes every 2 h, this material was applied to a Sephadex G-100 column (1.2 by 45 cm) and eluted with buffer B at a flow rate of 9 to 10 ml/h. The inhibitor activity was eluted as a single peak. The fractions containing the inhibitor activity were pooled and concentrated on a small DEAE-cellulose column (total volume 1 ml) and eluted with buffer A containing 0.5 M KCl. This material contained only a single protein and was termed I protein. The inhibitor activity of I protein decreased by 50% after a 2-week period of storage at 4°C. Therefore, after dialysis against buffer A, equal volumes of glycerol and then bovine serum albumin (at a final concentration of 1 mg/ml) were added, and the I protein preparation was stored at −20°C to minimize the loss of inhibitor activity.

Assay for *E. coli* RNA polymerase. The standard assay mixture contained, in a total volume of 0.25 ml: 10 mM Tris-hydrochloride (pH 7.9), 150 mM KCl, 10

mM $MgCl_2$, 12 mM β-mercaptoethanol, 50 μg of bovine serum albumin, 50 nmol each of ATP, CTP, and GTP, and 20 nmol of [^{3}H]UTP (1 μCi/20 nmol). Varying amounts of purified RNA polymerase holoenzyme or core RNA polymerase and template DNA were added as indicated. Reactions were always carried out at 37°C for 10 min unless specified. The reaction was terminated by the addition of 0.30 ml of 10% trichloroacetic acid. Radioactivity in acid-insoluble material retained on glass fiber filters (Reeve Angel, 934AH) was measured by liquid scintillation counting. The activity of enzyme preparations was given as nanomoles of UMP incorporated into RNA in standard reaction at 37°C in 10 min. One activity unit equaled 1 nmol of UMP incorporated into RNA at 37°C in 10 min. Specific activity was given as activity units per milligram of protein.

Assay for the inhibition of *E. coli* RNA polymerase by I protein. The standard RNA-synthesizing system described above was used with the addition of 3 μg of RNA polymerase holoenzyme and template DNA as indicated. Varying amounts of purified I protein or inhibitory material were added as specified. The standard reaction was carried out at 37°C for 10 min and usually resulted in an incorporation of 60,000 cpm of [^{3}H]UMP from [^{3}H]UTP into RNA in the presence of 10 μg of T7 DNA. The inhibitor activity was presented as the reduction in counts per minute from this control value, or as the reduction in nanomoles of UMP incorporation from the control value.

Assay of binding of RNA polymerase with T7 DNA by nitrocellulose filter retention. The binding of RNA polymerase to T7 DNA was measured by the retention of the enzyme-DNA complexes on nitrocellulose filters as described by Hinkle and Chamberlin (10). The binding reaction mixture contained indicated amounts of RNA polymerase holoenzyme or core enzyme, [^{3}H]thymidine-labeled T7 DNA, and 50 μg of bovine serum albumin in a total volume of 0.10 ml of binding buffer (10 mM Tris-hydrochloride [pH 8.0], 50 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM EDTA). The reaction mixture was incubated at 37°C for 5 min, diluted into 1 ml of the binding buffer containing 1 mg of bovine serum albumin per ml, and immediately filtered through a nitrocellulose filter with gentle suction (1 ml/20 s). The filter was dried and the radioactivity was measured by scintillation counting. Reaction mixtures containing no RNA polymerase retained approximately 5% of the radioactivity of input ^{3}H-labeled T7 DNA. Nitrocellulose filters used in this work were from Schleicher & Schuell Co. (type B-6, 24-mm filters).

RESULTS

Purification of *E. coli* RNA polymerase holoenzyme from T7-infected and uninfected cells. Purification of RNA polymerase holoenzyme from *E. coli* D10 F$^-$ cells harvested 9 min after T7 infection and from uninfected cells was carried out as described in Materials and Methods and the results are summarized in Table 1. The RNA polymerase preparation from T7-infected cells was almost inactive when assayed on T4 DNA through the first glycerol gradient centrifugation in the absence of KCl (Table 1, step 5). Therefore, residual activity of the enzyme on poly[d(A-T)] was monitored during the purification process. When the inactive RNA polymerase preparation after the first glycerol gradient step was subjected to the second glycerol gradient centrifugation in the presence of 1 M KCl (Table 1, step 6), RNA polymerase activity on T4 DNA was restored to the enzyme preparation due to the release of the inhibitor I protein bound to the enzyme.

Purification of I protein from T7-infected cells. Purification of I protein from inactive *E. coli* RNA polymerase complexes prepared from T7-infected cells is summarized in Table 2. The inactive RNA polymerase complexes, purified through the first glycerol gradient centrifugation step (Table 1, step 5), were subjected to phos-

TABLE 1. *Purification of E. coli RNA polymerase from uninfected and T7-infected cells*

Purification step	Uninfected cells					T7-infected cells (9 min after infection)				
	Total enzyme U on T4 DNA	Total enzyme U on poly-[d(A-T)]	Ratio[a] T4: poly-[d(A-T)]	Yield[b] (%)	Total protein (mg)	Total enzyme U on T4 DNA	Total enzyme U on poly-[d(A-T)]	Ratio T4: poly-[d(A-T)]	Yield[c] (%)	Total protein (mg)
1. Extract (low-speed supernatant)	12,000	17,100	1.4	100	7,600		2,800			7,500
2. Extract (high-speed supernatant)	11,800	15,700	1.5	95	5,500		1,900			5,160
3. Ammonium sulfate (33-50%)	13,100	15,400	1.7	109	1,900		2,700			2,200
4. DEAE-cellulose column	12,900	17,200	1.5	107	330	450	3,200	0.29	4	370
5. Glycerol gradient (no KCl)	10,600	14,100	1.5	89	66	550	3,600	0.34	6	75
6. Glycerol gradient (1 M KCl)	8,800	10,400	1.7	73	10	6,400	9,170	1.4	54	8

[a] (Nanomoles of UMP incorporation on T4 DNA)/{nanomoles of UMP incorporation on poly[d(A-T)]} × 2.
[b] Based on enzyme units on T4 DNA.
[c] Normalized to value of uninfected cells.

TABLE 2. *Purification of I protein*[a]

Purification step	Inhibitor activity (U[b])	Yield (%)	Total protein (mg)
Phosphocellulose column	4,300	100	70
DEAE-cellulose column	3,200	74	2.4
Sephadex G-100 column	2,400	56	0.070

[a] Starting material was partially purified, almost inactive *E. coli* RNA polymerase complexes prepared from 50 g of T7-infected *E. coli* D10 F^- cells harvested 10 min after infection. This material had been processed by using our standard RNA polymerase purification procedure through the first glycerol gradient centrifugation step in the absence of KCl as described in the text.

[b] Reduction in nanomoles of UMP incorporation in the standard RNA-synthesizing system due to the inhibition by I protein.

phocellulose column chromatography. The RNA polymerase core enzyme was bound to the column, whereas most of the other proteins including I protein and sigma factor passed through the column in flow-through fractions at a low concentration of salt (0.08 M KCl). The flow-through fractions were pooled and directly placed on a DEAE-cellulose column and chromatographed. As shown in Fig. 1, I protein, assayed by its inhibitor activity on RNA polymerase holoenzyme, was eluted from the column at approximately 0.3 M KCl, whereas sigma factor was recovered at about 0.25 M KCl. The fractions containing the inhibitor activity were pooled, concentrated, and subjected to Sephadex G-100 gel filtration. Figure 2 shows that the inhibitor activity was eluted in a single peak. The protein in this peak material was considered as "pure" I protein and, after concentration, was used in the following experiments.

Purity of I protein. Estimation of the purity of I protein preparation by staining the protein bands after gel electrophoresis did not yield accurate results because only a limited amount of I protein in a diluted form was available. Under the conditions used for gel electrophoresis and staining of proteins, it was difficult to identify proteins of small molecular weights and small quantities.

However, when I protein was purified from T7-infected cells labeled with radioactive amino acids before and after infection, or only after the infection, a single radioactive protein band, labeled with the radioisotope added only after the infection, appeared in acrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis (Fig. 3). The data shown in Fig. 3, which was included in our preliminary report (7), indicated that our

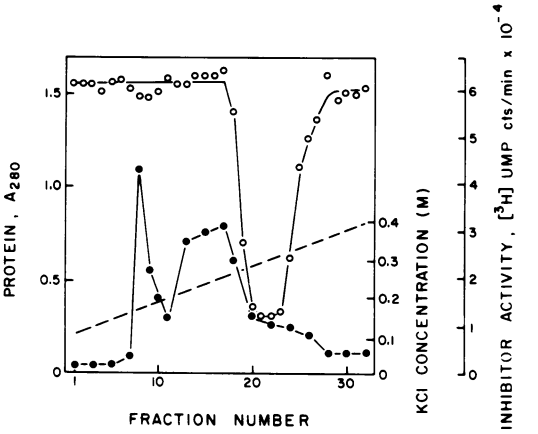

FIG. 1. *DEAE-cellulose column chromatography of I protein. E. coli RNA polymerase was partially purified from 50 g of T7-infected cells up to the first glycerol gradient centrifugation step without KCl as described in the text. The gradient fractions containing almost inactive enzyme, due to the association of I protein, were pooled, concentrated, and applied to a phosphocellulose column. On elution of the column with buffer C containing 0.08 M KCl, the inhibitor activity was separated from the core RNA polymerase and eluted in the flow-through fractions with the sigma factor, while the core polymerase was eluted from the column at about 0.35 M KCl. The flow-through fractions from the phosphocellulose column were directly applied to a DEAE-cellulose column (1.2 by 10 cm) and eluted with 70 ml of 0.08 M to 0.4 M KCl gradient in buffer C. A volume of 5 μl from each fraction was assayed for the inhibitor activity as described in the text. Sigma factor was usually recovered from the fractions eluted at about 0.25 M KCl. Symbols: ●, relative protein content, A_{280}; ○, inhibitor activity, reduction in [^{3}H]UMP incorporated into RNA; -----, KCl concentration.*

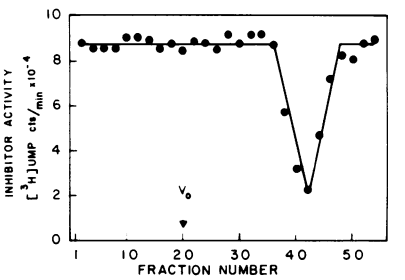

FIG. 2. *Sephadex G-100 gel filtration of I protein. The fractions from the DEAE-cellulose column which contained the inhibitor activity of I protein (Fig. 1) were pooled and concentrated and applied to a Sephadex G-100 column (1.2 by 45 cm). The column was eluted with buffer B at a flow rate of 9 to 10 ml/h and fractions of 1 ml each were collected. A volume of 5 μl from each fraction was assayed for the inhibitor activity as described in the text. The fractions which contained the inhibitor activity were pooled, concentrated, and used as "pure" I protein.*

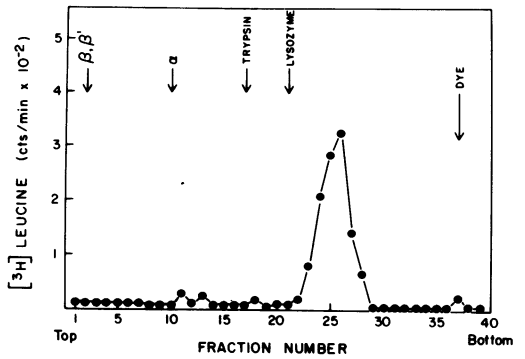

FIG. 3. *Acrylamide-SDS gel electrophoretic profile of purified I protein. I protein was purified as described in the text from T7-infected cells labeled with [^{3}H]leucine (5 mCi/2.5 μg per ml of culture) for 9 min after infection. The fraction from the Sephadex G-100 column containing inhibitor activity was subjected to acrylamide (15%)-SDS slab gel electrophoresis as described previously (7), and the radioactivity of [^{3}H]leucine in each gel slice was counted. Marker proteins, E. coli RNA polymerase β', β and α subunits, trypsin, and lysozyme were subjected to electrophoresis in the same slab gel.*

I protein preparation was almost pure and supported the notion that I protein is a phage T7-coded product. As described in the accompanying paper (8), we have evidence that I protein is the product of T7 gene 2.

Molecular weight of I protein. When I protein was purified from T7-infected cells labeled with [^{3}H]leucine only after the infection and subjected to acrylamide-SDS gel electrophoresis, a single peak of radioactive material migrating in the gel slightly faster than a lysozyme marker was detected (Fig. 3). The molecular weight of this radioactive I protein was estimated to be about 9,400 ± 700 (Fig. 4).

In our earlier report (7), we tentatively assigned a molecular weight of less than 14,000 for I protein according to our preliminary molecular weight estimation by Sephadex G-100 gel filtration and glycerol gradient centrifugation. By refining these methods, a more precise estimation of the molecular weight of I protein was accomplished by employing Sephadex G-50 gel filtration for determining the Stokes Radius value of I protein together with a determination of the sedimentation coefficient for I protein by glycerol gradient centrifugation.

The result from Sephadex G-50 gel filtration is presented in terms of K_{av} as defined by Laurent and Killander (14). The parameter K_{av} is defined as $(Vol_{elution} - Vol_{void})/(Vol_{total} - Vol_{void})$. The square root of $-\log K_{av}$ is then plotted versus the Stokes Radius, a, of the standards as shown in Fig. 5B.

Figure 5A shows the elution profiles of I protein and the standard protein myoglobin from a Sephadex G-50 column. I protein was eluted at fraction 50 ± 1, giving a value of $(-\log K_{av})^{1/2} = 0.547 \pm 0.023$. This value gave a corresponding Stokes Radius $a = 12.2 \pm 0.8$ Å, as shown in Fig. 5B.

Figure 6A shows sedimentation profiles of I protein and a standard myoglobin in a 5 to 15% glycerol gradient. I protein sedimented in fraction 14 ± 1, giving an estimated sedimentation coefficient value S of 1.36 ± 0.1 as determined from the standard curve presented in Fig. 6B.

From these experimental data, the molecular weight of I protein can be calculated by the following equation (14, 20):

$$\text{Molecular weight} = \frac{6\pi\eta N a S}{1 - \bar{v}\rho}$$

where a = Stokes radius, S = sedimentation coefficient, $\bar{v}$ = partial specific volume, η = viscosity of medium, ρ = density of medium, and N = Avogadro's number.

A molecular weight of 7,100 ± 1,000 was calculated for I protein. This value is comparable to that estimated by acrylamide-SDS gel electrophoresis of radioactive I protein as described above.

Characterization of the inhibition of *E. coli* RNA polymerase by I protein. We have shown previously that purified I protein inhibits

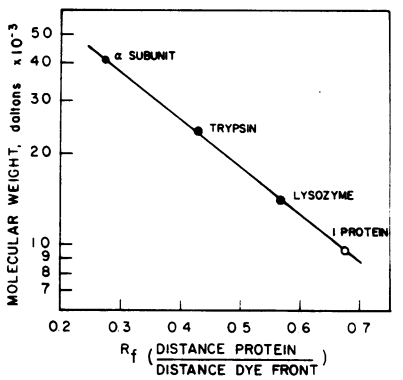

FIG. 4. *Acrylamide-SDS gel electrophoresis of purified I protein. [^{3}H]leucine-labeled I protein was purified as described in the text and subjected to electrophoresis in 15% acrylamide-SDS slab gel as described previously (7). E. coli RNA polymerase α subunit, trypsin, and lysozyme were subjected to electrophoresis in the same slab gel and stained with Coomassie brilliant blue. The relative migration distance (R_f) of each protein was plotted against the log of the molecular weight. From the R_f value of I protein, an estimated molecular weight of 9,400 ± 700 was assigned to I protein.*

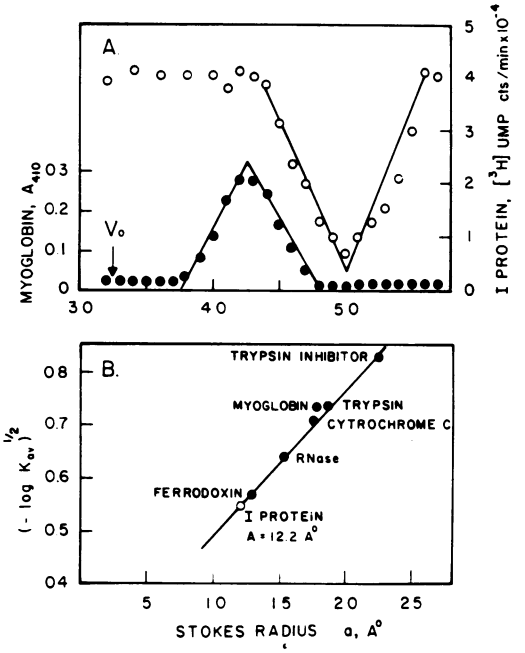

FIG. 5. *Sephadex G-50 gel filtration analysis of purified I protein. A Sephadex G-50 column (1.2 by 45 cm) was calibrated using the following standard protein markers: soyabean trypsin inhibitor (Stokes Radius 22.48 Å); trypsin (18.45 Å); horse heart myoglobin (17.96 Å); cytochrome c (17.7 Å); RNase (15.42 Å); and ferrodoxin (13.03 Å). All standards and purified I protein were applied in a 0.5-ml volume and eluted with buffer B at a flow rate of 9 to 10 ml/h. Each fraction was assayed for the inhibitor activity of I protein as described in the text. (A) Elution profiles of I protein and one of the standards, myoglobin; (B) Stokes Radius value determination for I protein (12.2 Å).*

RNA synthesis by *E. coli* RNA polymerase holoenzyme at the initiation step but does not inhibit RNA synthesis by the core RNA polymerase on poly[d(A-T)] or by T7-specific RNA polymerase on T7 DNA (7). Further characterization of the mode of inhibition of *E. coli* RNA polymerase by I protein is described in the following sections.

I protein inhibits RNA polymerase holoenzyme purified from T7-infected cells. As described in Table 1, *E. coli* RNA polymerase holoenzyme was purified from T7-infected cells. The last step removed I protein from inactive RNA polymerase-I protein complexes and recovered the activity of the enzyme (Table 1, step 6).

Figure 7 shows that I protein inhibits purified RNA polymerase holoenzyme from T7-infected cells and uninfected cells equally. Although the purified enzyme from T7-infected cells had usually about 20 to 30% less activity than the enzyme from uninfected cells, probably due to phosphorylation of the enzyme protein by a T7 phage-coded protein kinase as described previously (8), the enzyme from which I protein had been removed showed a sensitivity to be again inhibited by I protein to a similar degree as that of the enzyme from uninfected cells. The result suggests that the binding of I protein with the RNA polymerase holoenzyme is a salt concentration-dependent reversible process and that the inhibition of the enzyme by I protein does not involve any denaturation of the enzyme.

I protein binds to RNA polymerase holoenzyme. We have shown that purified I protein directly binds to RNA polymerase holoenzyme and forms an inactive complex (7). Almost all of purified radioactive I protein molecules cosedimented with RNA polymerase holoenzyme molecules in a glycerol gradient when an esti-

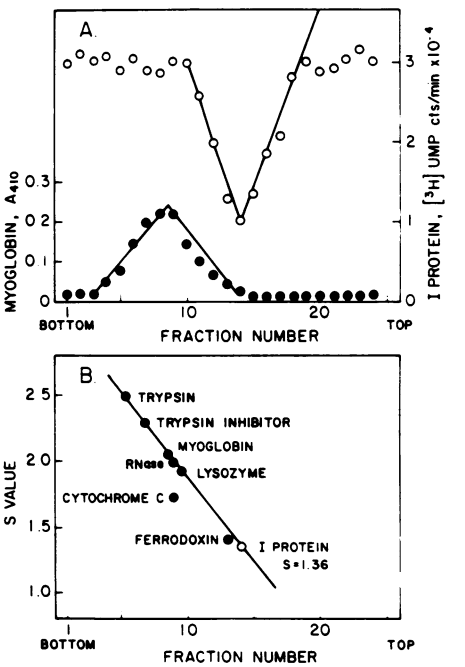

FIG. 6. *Glycerol gradient sedimentation analysis of purified I protein. Purified I protein was subjected to glycerol gradient centrifugation by using 5 ml of 5 to 15% glycerol in buffer A containing 0.15 M KCl in an SW50.1 rotor at 49,000 rpm for 30 h. The following standard proteins were analyzed in parallel with the I protein: trypsin (S = 2.5); trypsin inhibitor (S = 2.30); horse heart myoglobin (S = 2.04); RNase (S = 2.00); lysozyme (S = 1.91); cytochrome c (S = 1.71); and ferrodoxin (S = 1.4). Each gradient fraction was assayed for the inhibitor activity of I protein as described in the text. (A) Glycerol gradient sedimentation profiles of I protein and one of the standards, myoglobin; (B) S value determination for I protein (S = 1.36).*

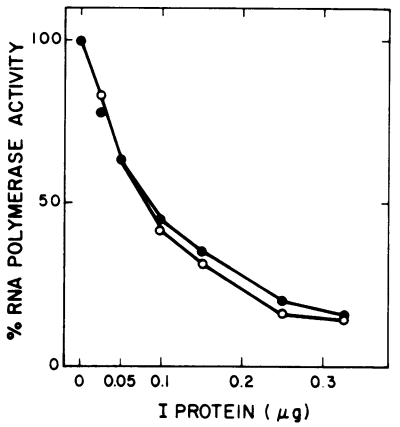

FIG. 7. *Inhibition of purified E. coli RNA polymerase holoenzyme from T7-infected and uninfected cells by I protein. The inhibitor activity of I protein on E. coli RNA polymerase holoenzyme was measured in the standard RNA-synthesizing system with 10 μg of T7 DNA. The RNA polymerase holoenzymes were purified from T7-infected cells and uninfected cells as described in the text and Table 1, and had specific activities on T7 DNA of 660 U/mg and 830 U/mg, respectively. The RNA-synthesizing reaction was carried out at 37°C for 10 min. Symbols: ○, E. coli RNA polymerase holoenzyme from T7-infected cells (2.5 μg) (the 100% value was 48,000 cpm per reaction); ●, E. coli RNA polymerase holoenzyme from uninfected cells (2.5 μg) (the 100% value was 68,000 cpm per reaction).*

mated equal molar ratio of I protein and the holoenzyme were mixed and subjected to centrifugation. The formation of the I protein-holoenzyme complexes resulted in a loss of both RNA polymerase activity and the inhibitor activity of the I protein simultaneously. Data supporting these findings have been reported in a previous paper (7).

I protein does not interact with sigma factor. The core RNA polymerase can synthesize RNA on synthetic template DNA such as poly[d(A-T)], but not on natural DNA template. It has been shown that core polymerase contains the enzymatic machinery for elongation of the RNA chain but lacks the necessary specificity for the initiation of RNA synthesis on a natural DNA template that is provided by the sigma factor (15). Because the inhibition by I protein of RNA polymerase depends on the presence of sigma factor with the enzyme, i. e., I protein inhibits the holoenzyme but not the core enzyme, physical interaction of I protein with the sigma factor was investigated.

The RNA polymerase holoenzyme was separated into the core polymerase (containing α_2, β, β' subunits) and the sigma factor by phosphocellulose column chromatography (1). [^{3}H]leucine-labeled purified I protein was mixed with an approximately equal amount of sigma factor and sedimented in a glycerol gradient with parallel gradients containing the same amount of the respective protein. The result (Fig. 8) indicates that I protein has no affinity for the sigma factor. There was no reduction in sigma factor activity when the two proteins were mixed and sedimented together (Fig. 8A and B) and no loss in the inhibitor activity of I protein (Fig. 8B and C). A loss of about 60% of the initial sigma factor activity in the gradient shown in Fig. 8A and B was due to the instability of the sigma factor sedimented in the absence of the stabilizer, bovine serum albumin (1). It is also clear by a comparison of the distribution of [^{3}H]leucine-labeled I protein in Fig. 8B and C that there was no evidence of physical association between I protein and sigma factor.

Table 3 shows an experiment in which competition between I protein and sigma factor was tested. The result provided additional evidence that I protein does not interact with sigma factor. In the standard RNA-synthesizing reaction mixture, known amounts of I protein, which should inhibit RNA synthesis by RNA polymerase holoenzyme by 68 and 83%, were mixed with different amounts of sigma factor prior to addition of the holoenzyme, and the synthesis of RNA was carried out after addition of the enzyme. As shown in Table 3, prior mixing of increasing amounts of sigma factor with I protein had no effect on the inhibition of subsequent RNA synthesis by I protein; i.e., inhibition of RNA synthesis by I protein was not overcome by sigma factor which was added at a concentration up to three times excess over the holoenzyme. The result agrees with the result obtained by the glycerol gradient centrifugation analysis (Fig. 8).

Therefore, it is concluded that I protein does not directly interact with free sigma factor, although inhibition of RNA polymerase by I protein is exerted only when the sigma factor is associated with the enzyme and forming the holoenzyme.

Partial binding of I protein with core RNA polymerase. Physical association of I protein with the core polymerase was examined by glycerol gradient centrifugation analysis as described above for sigma factor. The result (Fig. 9) indicates that the activity of core polymerase, assayed on poly[d(A-T)], was not reduced at all after centrifugation in a glycerol gradient together with I protein (Fig. 9A and B), although some radioactivity of the [^{3}H]leucine-labeled I protein cosedimented with the core polymerase

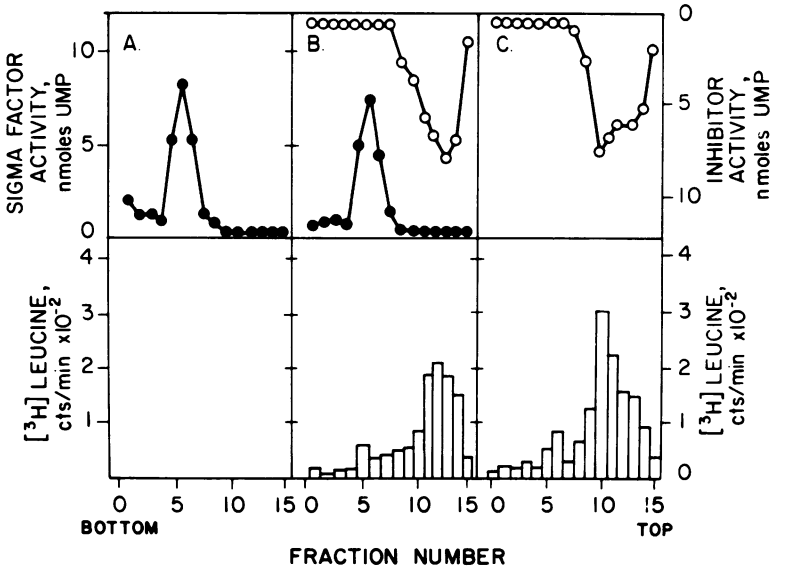

FIG. 8. *Glycerol gradient sedimentation analysis of interaction between I protein and sigma factor. The interaction of I protein with sigma factor was examined by glycerol gradient centrifugation. Three samples contained: (A) sigma factor (19 μg of protein and 56 sigma factor U); (B) sigma factor as in A and [³H]-leucine-labeled I protein (968 cpm and 45 inhibitor U); (C) [³H]leucine-labeled I protein as in B alone. These samples were layered on 5-ml, 5 to 20% glycerol gradients in buffer A containing 0.15 M KCl and centrifuged in an SW50.1 rotor at 50,000 rpm for 17 h. A volume of 40 μl from each gradient fraction from A and B was assayed for the sigma factor activity in the standard RNA-synthesizing system with 5.6 μg of T7 DNA and 4 μg of purified RNA polymerase core enzyme. Sigma factor activity was plotted as the increase in nanomoles of UMP incorporated per reaction. A volume of 40 μl from each fraction from gradients B and C was assayed for the inhibitor activity of I protein as described in the text and expressed as the reduction in nanomoles of UMP incorporated per reaction. Fifty micrograms of bovine serum albumin was added to the remaining part of each fraction and the trichloroacetic acid-insoluble radioactivity of [³H]leucine was determined. Symbols: ●, sigma factor activity; ○, inhibitor activity of I protein; □, radioactivity of [³H]-leucine-labeled I protein.*

TABLE 3. *Effect of sigma factor on the inhibition of RNA polymerase holoenzyme by I protein*[a]

I protein (μg)	RNA synthesis (nmol of UMP incorporated into RNA)			
	0 μg[b]	0.2 μg[b]	1.0 μg[b]	2.0 μg[b,c]
0	2.36	2.36	2.39	2.33
0.106	0.75 (68%)[d]	0.75	0.83	0.87
0.212	0.42 (82%)	0.54	0.53	0.58

[a] The standard RNA-synthesizing system contained 2.8 μg of RNA polymerase holoenzyme and 8.4 μg of T7 DNA in a total volume of 0.25 ml. Indicated amounts of I protein and sigma factor were mixed prior to the addition of the RNA polymerase holoenzyme and kept at 4°C for 5 min. After addition of the enzyme, the reaction mixture was incubated at 37°C for 10 min and the trichloroacetic acid-insoluble radioactivity of [³H]UMP was counted.

[b] Sigma factor.

[c] The molar ratio of 2.0 μg of sigma factor to 2.8 μg of RNA polymerase holoenzyme was about 3:1 considering the purity of the sigma factor.

[d] Percent inhibition by I protein.

and some loss in the inhibitor activity of I protein was noticed (Fig. 9B and C).

Several repeated experiments showed cosedimentation of 10 to 50% of the radioactive input I protein with the core polymerase without subsequent inhibition of the core polymerase activity assayed on poly[d(A-T)]. The unbound, remaining I protein was detected across the top portion of the gradient either by the inhibitor activity or by the radioactivity.

If the observed partial binding of I protein with the core polymerase was attributable to a small amount of contaminating holoenzyme, then a reduction in the enzyme activity after the sedimentation with I protein should have been observed because I protein inhibits holoenzyme activity on poly[d(A-T)]. However, the RNA polymerase activity on poly[d(A-T)] shown in Fig. 9A and B was the same.

The effect of core polymerase on the inhibition of holoenzyme by I protein was examined to determine whether excess core polymerase

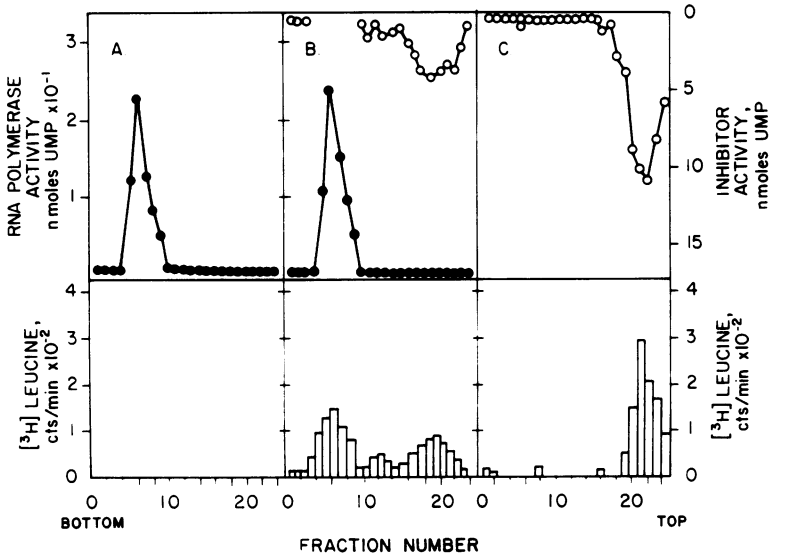

FIG. 9. *Glycerol gradient sedimentation analysis of interaction between I protein and the core RNA polymerase. Three samples contained: (A) core RNA polymerase {81 μg with a total activity of 57 U on poly[d(A-T)]}; (B) core RNA polymerase as in A and [³H]leucine-labeled I protein (968 cpm and 45 inhibitor U); (C) [³H]leucine-labeled I protein as in B alone. These samples were centrifuged in 12-ml, 10 to 30% glycerol gradients in buffer A with 0.15 M KCl using an SW41 rotor at 40,000 rpm for 20 h. Each gradient fraction from A and B was assayed for the activity of core RNA polymerase in the standard RNA-synthesizing system with 5 μg of poly[d(A-T)], and the activity was presented in nanomoles of UMP incorporated into RNA per reaction. Each gradient fraction from B and C was assayed for the inhibitor activity of I protein as described in the legend for Fig. 8. The rest of each fraction from B and C was used for the measurement of trichloroacetic acid-insoluble radioactivity of [³H]leucine-labeled I protein after addition of 50 μg of bovine serum albumin. Symbols: ●, core RNA polymerase activity on poly[d(A-T)]; ○, inhibitor activity of I protein; □, radioactivity of [³H]leucine-labeled I protein.*

might reduce the inhibition of holoenzyme by I protein by virtue of its partial binding to I protein. An amount of I protein sufficient to inhibit a given amount of holoenzyme was mixed with core polymerase prior to the addition of holoenzyme to the RNA-synthesizing mixture. If the partial binding of I protein with the core polymerase is irreversible, increasing amounts of core polymerase should take up increasing amounts of I protein and result in less inhibition of the activity of holoenzyme by I protein on T7 DNA. This would be observed as an overall increase in the RNA polymerase activity on T7 DNA. However, addition of core polymerase at three times the molar concentration of I protein did not reverse the inhibition of the activity of holoenzyme by I protein.

Therefore, we conclude that the observed partial binding of I protein with the core polymerase has no significant functional effect.

I protein does not bind to T7 DNA. A possible binding of I protein to T7 DNA was examined by glycerol gradient centrifugation. When radioactive I protein was mixed with T7 DNA and sedimented in a glycerol gradient, there was no association of I protein with the T7 DNA. As reported previously (7), our purified I protein preparation had no DNase or RNase activity.

I protein prevents binding of RNA polymerase holoenzyme with T7 DNA. RNA polymerase holoenzyme first binds randomly to all regions of T7 DNA, leading to a strong association at the promoter site of the DNA, and then a highly stable preinitiation complex is formed. These events are attributed to the presence of the sigma factor (10). The highly stable preinitiation complexes formed between holoenzymes and T7 DNA at the promoter sites are retainable on nitrocellulose filters, whereas free T7 DNA molecules pass through the filters. Chamberlin's group has refined the method for DNA retention on nitrocellulose filters by RNA polymerase and presented detailed information on the binding of RNA polymerase to T7 DNA (6, 10–13). It appears that one RNA polymerase molecule bound to T7 DNA is sufficient to retain the DNA on filters. However, up to nine molecules of the enzyme may bind with a high affinity at the promoter region of the T7 DNA.

When I protein was mixed with the RNA polymerase holoenzyme prior to the addition of T7 DNA, the DNA retention on nitrocellulose filters was greatly reduced to about 30% of the control value without I protein (Fig. 10). Therefore, the prevention of RNA polymerase holoenzyme binding to the template T7 DNA by I protein, through its direct association with the enzyme molecule, is considered the major mechanism by which I protein inhibits the initiation of RNA synthesis by RNA polymerase holoenzyme.

Since I protein inhibits the activity of RNA polymerase holoenzyme by greater than 90%, it might be expected that I protein would almost completely prevent the retention of T7 DNA by the enzyme. In our experiment, however, we found that increasing the ratio of I protein to the enzyme did not reduce the amount of T7 DNA retained on the filter. However, we would like to eliminate the possibility that core RNA polymerase might have been present in our RNA polymerase holoenzyme preparation. Although, as will be described in the following section, I protein does not prevent the binding of core polymerase with T7 DNA, our holoenzyme preparation used in this experiment was saturated with sigma factor; the enzyme preparation was mixed with an excess amount of sigma factor and repurified by glycerol gradient sedimentation. Furthermore, the addition of excess sigma factor did not alter the value of filter-retainable enzyme-DNA complex formation in this experimental system.

The formation of stable complexes between the holoenzyme and T7 DNA that are retainable on filters required less than 1 min at 37°C (Fig. 11). If I protein was present in the complex formation mixture, the filter-retainable complex

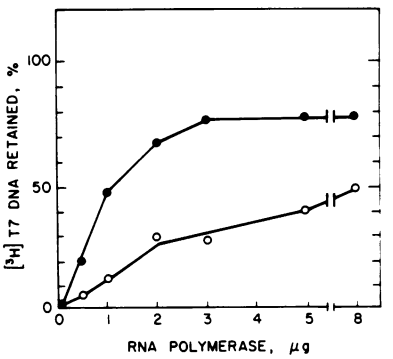

FIG. 10. *Inhibition of the binding of RNA polymerase holoenzyme to T7 DNA by I protein measured by the retention of [³H]thymidine-labeled T7 DNA on nitrocellulose filters. The binding reaction mixture contained 6.1 µg of [³H]thymidine-labeled T7 DNA (41,600 cpm) and increasing amounts of RNA polymerase holoenzyme as indicated and also 50 µg of bovine serum albumin in 0.10 ml of binding buffer as described in the text. The RNA polymerase holoenzyme was saturated with sigma factor and repurified by glycerol gradient centrifugation (1) before the experiment. The amount of I protein was calculated to give twice the amount required to give maximum inhibition for the amount of enzyme present in the reaction according to our previous data (7). In the reactions containing I protein, the enzyme and I protein were mixed prior to the addition of T7 DNA. The binding reaction mixture was incubated at 37°C for 5 min and filtered through a nitrocellulose filter, and the filter-retainable radioactivity of [³H]-thymidine-labeled T7 DNA was measured. Reactions without RNA polymerase holoenzyme retained approximately 5% of the input radioactivity and this value was subtracted from the experimental value. Symbols: ●, retention of [³H]-labeled T7 DNA by RNA polymerase holoenzyme; ○, retention of [³H]-labeled T7 DNA by RNA polymerase holoenzyme in the presence of I protein.*

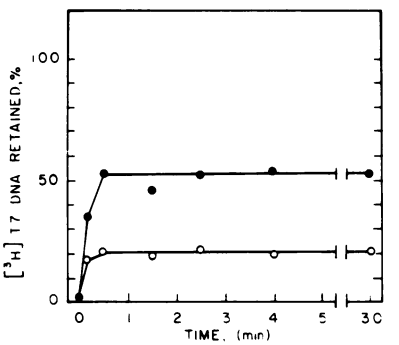

FIG. 11. *Kinetics of formation of nitrocellulose filter-retainable complexes between RNA polymerase holoenzyme and T7 DNA in the presence and absence of I protein. The binding reaction mixture contained 6.1 µg of [³H]thymidine-labeled T7 DNA (41,600 cpm), 1 µg of RNA polymerase holoenzyme, and 50 µg of bovine serum albumin in 0.10 ml of binding buffer as described in the text. Where indicated, I protein was mixed with the enzyme prior to the addition of T7 DNA to the reaction mixture. The enzyme-to-DNA ratio was chosen from the result shown in Fig. 10 so that the enzyme was not saturating the DNA. The reaction was initiated by incubation at 37°C and terminated at indicated times by dilution into 1 ml of binding buffer containing 1 mg of bovine serum albumin per ml and immediately filtered through a nitrocellulose filter as described in the text. A reaction mixture which contained ³H-labeled T7 DNA and the enzyme but filtered before incubation retained approximately 5% of the radioactivity of input DNA. This value was subtracted from the experimental value. Symbols: ●, filter-retainable complex formation with RNA polymerase holoenzyme; ○, filter-retainable complex formation with RNA polymerase holoenzyme in the presence of I protein.*

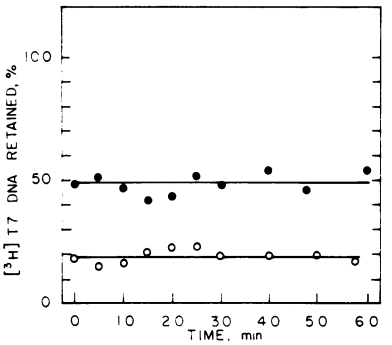

FIG. 12. *Kinetics of dissociation of RNA polymerase holoenzyme-T7 DNA complexes formed in the presence and absence of I protein. The RNA polymerase holoenzyme-T7 DNA complex formation was carried out at 37°C for 5 min in a 2.0-ml reaction mixture which contained 20 μg of RNA polymerase holoenzyme and 122 μg of [3H]thymidine-labeled T7 DNA (832,000 cpm) in the presence or absence of I protein as described in the legend for Fig. 10. At this time, aliquots of 0.10 ml were removed and diluted with an equal volume of binding buffer containing 50 μg of bovine serum albumin, incubated at 37°C for an additional 5 min, and then diluted in 2 ml of binding buffer containing 1 mg of bovine serum albumin per ml and filtered. The filter-retainable radioactivity of 3H-labeled T7 DNA served as the "zero time" control. After the removal of the zero time control samples, 642 μg of nonradioactive T7 DNA in 1.7 ml of binding buffer was added to the remaining 1.7 ml of reaction mixture and the incubation was continued at 37°C. At indicated times, 0.20-ml volumes (containing 1 μg of RNA polymerase holoenzyme and 41,600 cpm of 3H-labeled T7 DNA) were removed and diluted in 2 ml of binding buffer with 1 mg of bovine serum albumin per ml and filtered. The radioactivity of filter-retainable 3H-labeled T7 DNA was counted. Symbols: ●, filter-retainable radioactivity of 3H-labeled T7 DNA-RNA polymerase holoenzyme complexes formed in the absence of I protein; Zero time control contained 20,562 cpm of 3H radioactivity (equivalent to 49% retention of input T7 DNA); ○, filter-retainable radioactivity of 3H-labeled T7 DNA-RNA polymerase holoenzyme complexes formed in the presence of I protein. Zero time control contained 7,987 cpm of 3H radioactivity (equivalent to 19% retention of input T7 DNA).*

formation was inhibited to about 30% of that obtained without I protein, in agreement with the result presented in Fig. 10.

The dissociation rate of the enzyme-DNA complex formed in the presence or absence of I protein was measured and is shown in Fig. 12. [3H]thymidine-labeled T7 DNA was incubated with RNA polymerase holoenzyme to form filter-retainable complexes in the presence or absence of I protein, and then an excess amount of nonradioactive T7 DNA was added. The result (Fig. 12) shows that the filter-retainable complexes once formed with radioactive T7 DNA, either in the presence or absence of I protein, were stable, and nonradioactive T7 DNA did not replace the radioactive DNA. Therefore, the filter-retainable enzyme-DNA complexes formed in the presence of I protein (30% of the amount of that formed in the absence of I protein) represented an experimentally stable complex, but the significance of this complex is not known. There is, however, a possibility that a fraction of holoenzyme molecules used in this experiment were enzymatically inactive and unable to bind with I protein, but still able to bind and retain T7 DNA on a filter.

I protein does not prevent binding of core RNA polymerase with T7 DNA. Core RNA polymerase has been shown to bind all regions of T7 DNA equally but with a low affinity compared to the binding by the holoenzyme (10). Increasing amounts of [3H]thymidine-labeled T7 DNA were retained on nitrocellulose filters with increasing amounts of core RNA polymerase until 100% of input DNA was retained (Fig. 13). The efficiency of the retention of DNA by the core polymerase was lower than that by the holoenzyme, as previously reported (10). Addition of excess I protein to the core polymerase did not alter the retention of T7 DNA (Fig. 13). This result is consistent with the previous ob-

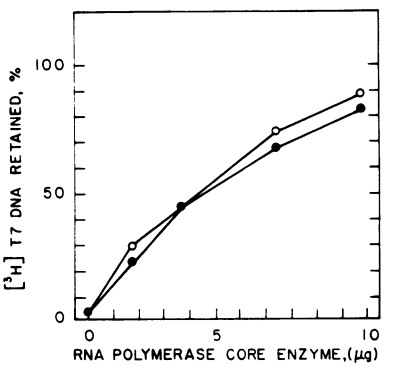

FIG. 13. *Binding of core RNA polymerase to T7 DNA in the presence and absence of I protein measured by the retention of [3H]thymidine-labeled T7 DNA on nitrocellulose filters. The binding reaction and nitrocellulose filtering were the same as described in the legend for Fig. 10, except that core RNA polymerase was used instead of the holoenzyme. The amount of I protein was in 100% excess over the amount for maximum inhibition of an equivalent amount of RNA polymerase holoenzyme on poly[d(A-T)]. The percentage of input [3H]thymidine-labeled T7 DNA (41,600 cpm) retained on nitrocellulose filters was plotted. Symbols: ●, retention of 3H-labeled T7 DNA by core RNA polymerase; ○, retention of 3H-labeled T7 DNA by core RNA polymerase in the presence of I protein.*

servation that no inhibition of core polymerase activity was exerted by I protein, although a partial association of I protein with core polymerase was observed. Therefore, partial association of I protein with the core polymerase appears to have no effect on the binding of core polymerase with T7 DNA.

I protein does not inhibit initiation of RNA by highly stable preinitiation complexes formed between RNA polymerase holoenzyme and T7 DNA. RNA polymerase holoenzyme forms a highly stable complex with T7 DNA at a low concentration of salt (0.05 M NaCl) (10). The enzyme is known to bind with a high affinity to one unique promoter region on T7 DNA, now known to have three independent promoter sites (16), with a high affinity, and this region can accommodate binding of a maximum of nine enzyme molecules (10).

Rifampin, an inhibitor of the initiation of RNA synthesis, was shown to prevent the binding or utilization of the first nucleoside triphosphate by *E. coli* RNA polymerase (12). It was also shown that rifampin inactivates the RNA polymerase-T7 DNA complex at a slower rate than the free enzyme but is still able to inactivate the enzyme-DNA complex completely in less than 1 min (12). We previously showed that I protein inhibits the initiation of RNA synthesis by RNA polymerase holoenzyme similar to the inhibition by rifampin (7).

In the experiment shown in Fig. 14, the effect of I protein on the activity of preformed RNA polymerase holoenzyme-T7 DNA complex was investigated in comparison to the effect of rifampin. I protein or rifampin was added to preformed enzyme-T7 DNA complexes formed in the absence of nucleoside triphosphates. After incubation of the complexes with the inhibitor for the indicated times, aliquots were removed and further incubated for 2 min with nucleoside triphosphates and [^{3}H]UTP to measure the activity of the enzyme complexes to initiate and synthesize RNA. The RNA-synthesizing reaction was terminated after 2 min, prior to the completion of one round of RNA synthesis on T7 DNA, to measure the activity of only the enzyme molecules that had already bound to the DNA and were therefore capable of initiating RNA synthesis (6).

The results (Fig. 14A and B) revealed a difference between the effects of I protein and rifampin on the enzyme activity of the highly stable holoenzyme-T7 DNA complexes formed in the presence of 0.05 M NaCl. Figure 14A shows that I protein, in an amount 100% in excess of that required to completely inhibit free RNA polymerase holoenzyme, did not inhibit the activity of the highly stable complexes

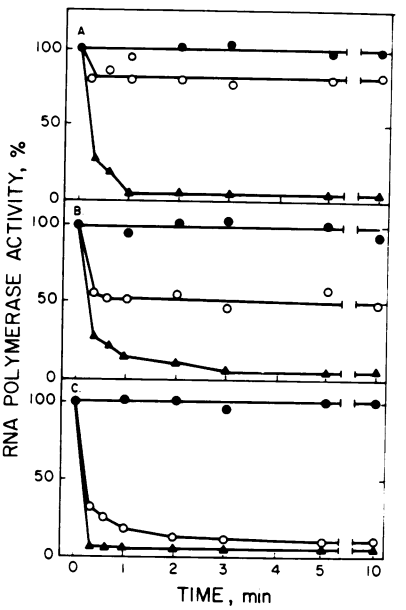

FIG. 14. *Effects of I protein and rifampin on the activity of preformed RNA polymerase holoenzyme-T7 DNA complexes. The kinetics of the inactivation of preformed RNA polymerase holoenzyme-T7 DNA complexes by I protein and rifampin were measured at varying concentrations of NaCl and different molar ratios of the enzyme:DNA. The enzyme-DNA complexes were formed by incubation of RNA polymerase holoenzyme and T7 DNA in the standard RNA-synthesizing system with the NaCl concentrations as indicated and without nucleoside triphosphates. At time zero, I protein or rifampin was added and the incubation was continued. At indicated times, 0.20-ml volumes were removed and added to an 0.05-ml solution containing all four nucleoside triphosphates and [^{3}H]UTP to initiate RNA synthesis at 37°C. RNA synthesis was terminated after 2 min prior to the completion of one round of RNA synthesis on T7 DNA, and trichloroacetic acid-insoluble radioactivity was counted. The amount of I protein was in 100% excess over the amount required to cause maximum inhibition. Rifampin was added to make a final concentration of 2.5 μg/ml. (A) 0.05 M NaCl and an enzyme-to-DNA ratio of 5:1 (0.88 μg of RNA polymerase holoenzyme and 10 μg of T7 DNA); (B) 0.05 M NaCl and an enzyme-to-DNA ratio of 13:1 (0.88 μg of RNA polymerase holoenzyme and 4 μg of T7 DNA); (C) 0.15 M NaCl and an enzyme-to-DNA ratio of 5:1 (0.88 μg of RNA polymerase holoenzyme and 10 μg of T7 DNA). Symbols: ●, RNA synthesis in 2 min without inhibitor; the 100% values were (A) 7,480 cpm; (B) 6,740 cpm; (C) 6,700 cpm; ○, RNA synthesis in 2 min in the presence of I protein; the 100% values were (A) 7,689 cpm; (B) 5,432 cpm; (C) 7,734 cpm; ▲, RNA synthesis in 2 min in the presence of rifampin; the 100% values were (A) 7,134 cpm; (B) 5,738 cpm; (C) 7,300 cpm.*

formed between the holoenzyme and T7 DNA without the first nucleoside triphosphate, ATP or GTP. On the other hand, rifampin rapidly inactivated the activity of the complexes almost completely within 1 min. This set of experiments was performed at an enzyme-DNA ratio of 5:1 and all enzyme molecules were expected to bind the promoter region of T7 DNA (10).

When the enzyme-DNA ratio was changed to 13:1 under the identical conditions, I protein inhibited about 50% of the original activity of the enzyme-DNA complexes (Fig. 14B). Since at this enzyme-DNA ratio only about one-half of the added enzyme molecules were expected to form stable complexes with T7 DNA, assuming that a maximum of nine enzyme molecules can bind to the promoter region of T7 DNA (10), the result shown in Fig. 14B indicates that I protein inhibits enzyme molecules that have not bound to DNA.

This notion was further supported by the experiment shown in Fig. 14C. When the effects of I protein and rifampin were tested in the presence of 0.15 M NaCl, an NaCl concentration which allows the formation of functionally reversible enzyme-DNA complexes (10), I protein rapidly inhibited almost all of the enzyme activity at a rate similar to that of the inhibition by rifampin.

DISCUSSION

I protein, the inhibitor of *E. coli* RNA polymerase. An inhibitor of *E. coli* RNA polymerase associated with partially purified inactive RNA polymerase complex from T7 phage-infected cells was purified. The molecular weight of this purified protein (I protein), determined by acrylamide-SDS gel electrophoresis, Sephadex G-50 gel filtration, and glycerol gradient sedimentation, was about 7,000 to 9,000. This value is slightly different from that previously reported by Sephadex G-100 gel filtration analysis (7). The molecular weight of T7 gene 2 product has not been determined (21), mainly due to its small size. However, we have presented evidence that I protein is the direct product of T7 gene 2 (8), and if so, this work represents the first purification and molecular weight determination of gene 2 protein.

One interesting aspect of the procedure used for the purification of I protein is that we followed a standard RNA purification step (4) until partially purified RNA polymerase was obtained. At this stage, the enzyme preparation contained several proteins other than the subunits of the enzyme, α, β, β', and sigma. Although the enzyme was almost inactive when tested on natural phage DNA templates, some residual activity of the enzyme on poly[d(A-T)] enabled us to monitor the fractions which contained the enzyme. I protein was separated from the inactive enzyme complex by glycerol gradient sedimentation with 1 M KCl, resulting in a recovery of active RNA polymerase holoenzyme, or by phosphocellulose column chromatography which recovered active core RNA polymerase. This procedure seems to be applicable for the isolation and purification of other regulatory protein factors associated with the RNA polymerase molecules and controlling the template specificity or the activity of the enzyme.

Ponta et al. (17) reported that they had also isolated an inhibitor protein of *E. coli* RNA polymerase from crude ribosomes of T7-infected cells. Their protein does not inhibit T7-specific RNA polymerase or core RNA polymerase and has a molecular weight of 14,000. It appears that their protein is identical or similar to the I protein.

Mode of inhibition of *E. coli* RNA polymerase by I protein. The results presented in this paper showed that I protein directly binds with the RNA polymerase holoenzyme and prevents the binding of the enzyme to the promoter sites of the template T7 DNA. However, once highly stable preinitiation complexes are formed between the holoenzyme and T7 DNA at the promoter sites, I protein can not inactivate the preformed enzyme-DNA complexes to prevent initiation of RNA synthesis. With these data, we can now explain why I protein inhibits in vitro RNA synthesis at the initiation step but cannot inhibit already initiated RNA synthesis until one round of RNA synthesis is completed. The mode of inhibition by I protein on overall RNA synthesis appears to be very similar to that by rifampin as shown previously (7).

Our detailed analysis, however, revealed that the mode of inhibition of RNA polymerase by I protein is significantly different from that by rifampin. Rifampin is known to bind directly to the β subunit of the RNA polymerase (15), but the drug does not inhibit the binding of the enzyme to the template DNA (12). On the other hand, I protein blocks the binding of the enzyme to the template DNA by directly binding to the holoenzyme.

Rifampin is also known to inhibit the binding or utilization of the initial purine nucleoside triphosphate which forms the 5'-terminus of the RNA chain by the RNA polymerase (12). Therefore, it has been shown that rifampin can inactivate the highly stable preinitiation enzyme-DNA complex formed without nucleoside triphosphates within 1 min, although this rate is somewhat slower than that of inhibition of free RNA polymerase by rifampin (12). I protein, on

the other hand, cannot inhibit the highly stable preinitiation complex from initiating RNA synthesis when nucleoside triphosphates are supplied to the enzyme complex.

Another interesting finding derived from this study is that the inhibition of RNA polymerase by I protein depends on the presence of sigma factor. I protein inhibits RNA polymerase holoenzyme but does not inhibit core RNA polymerase activity on poly[d(A-T)]. This difference results from the fact that I protein blocks the binding of holoenzyme to the promoter site of T7 DNA, whereas random binding of core RNA polymerase to all regions of T7 DNA is not inhibited by I protein. The prevention of the binding of holoenzyme to T7 DNA is caused by a direct physical binding of I protein to the holoenzyme. Since I protein does not show any direct interaction with the sigma factor, and since a partial association of I protein with the core polymerase does not affect the binding of the core polymerase to the DNA template nor the activity of the core polymerase, the presence of sigma factor appears to be essential for the binding of I protein to the RNA polymerase and the subsequent inhibition of enzyme activity.

It seems likely that the association of sigma factor with the core polymerase, and thus the formation of the holoenzyme, results in conformational changes to the polymerase subunits. Because I protein does not bind to sigma factor directly, it is also likely that I protein binds to one of the polymerase subunits only when that subunit is in the holoenzyme form but not in the core enzyme form. Since the β' subunit is presumably responsible for the binding of the enzyme to DNA (15), this subunit is a possible candidate for the binding site of I protein. Since it is known that the rifampin binding site is the β subunit of RNA polymerase (15), the difference between the effects of I protein and rifampin on the inhibition of RNA polymerase might be explained by the different enzyme subunits to which the two different inhibitors bind.

Early to late switch in phage T7 development and the host shutoff function. In addition to the well known switch from the host RNA polymerase to the T7-coded RNA polymerase transcribing early mRNA and late mRNA, respectively, the host shutoff function of the phage has an important role to achieve a complete early to late switch to halt any further synthesis of early mRNA and host RNA by inactivating the host RNA polymerase.

I protein was isolated and purified from an inactive *E. coli* RNA polymerase complex prepared from T7-infected cells. Purified I protein inhibited *E. coli* RNA polymerase effectively with a nearly stoichiometric ratio but did not inhibit T7-specific RNA polymerase coded by the phage. These facts provided direct evidence at the enzyme level that I protein is involved in the host shutoff function. Our recent genetic analysis of T7 genes using many amber and deletion mutants of T7 indicated that gene 2 codes for I protein (8). However, as described in the accompanying paper (8), it appears that to manifest a complete shutoff of *E. coli* RNA polymerase activity in vivo, the function of T7 gene 0.7 which codes for a protein kinase (18, 22) is also required, although I protein plays a major role in the activation of the enzyme.

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