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Stimulation of RNA Synthesis by Two Protein Factors in Extracts of *Escherichia coli*

(RNA polymerase/ribosomal RNA/template specificity)

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ABSTRACT **Two separate protein factors that markedly stimulate DNA-dependent RNA polymerase have been partially purified from ribosome-free extracts of *E. coli* Q13 cells. These proteins appear to differ from sigma, M, H, psi, and the factor described by Mahadik and Srinivasan. The stimulation by either factor requires the presence of the sigma protein. Using RNA–DNA hybridization competition analyses, we also demonstrate that neither of the new factors preferentially stimulates the synthesis of ribosomal RNA. The purification, properties, and mechanism of action of these new factors are examined.**

The activity of *Escherichia coli* RNA polymerase is strongly influenced by various proteins and effector molecules. The sigma protein, which is normally purified as part of the RNA polymerase holoenzyme, and the rho protein are required for the correct initiation and termination of RNA chains on several DNA templates (1, 2). In the presence of these two proteins, the RNA synthesized *in vitro* on several phage DNA templates resembles, both qualitatively and quantitatively, the RNA synthesized *in vivo* during the early phases of phage infection (3–5). In addition to these comprehensive factors, there are many genetically defined cytoplasmic elements that play a decisive role in the regulation of specific genes and operons. It seems likely that these positive and negative control elements exert their influence at the level of mRNA synthesis. This expectation has been realized in the case of the lambda and phage 434 repressors (6, 7), *lac* repressor (8), CAP (or CRP) (8, 9), and the C-gene product of the arabinose operon (9, 10). Conversely, several protein factors have been identified by their ability to stimulate RNA synthesis *in vitro*, but their relationship to the regulation of DNA transcription and gene expression has not been defined. In this communication, we report partial purification and properties of two separable factors from *E. coli* extracts that belong to the latter category. These factors stimulate DNA transcription by the holoenzyme ($\beta\beta'\alpha_2\sigma$), but not by the "core" RNA polymerase ($\beta\beta'\alpha_2$). The stimulatory activity of these factors is additive when the RNA polymerase concentration limits the reaction. Several lines of evidence suggest that the stimulation results from an increase in RNA chain initiation, rather than from an increase in the rate of RNA chain elongation. Hybridization-competition analyses of the synthesized RNA indicate that these factors do not preferentially stimulate ribosomal RNA (rRNA) synthesis.

Abbreviations: $NadodSO_4$, sodium dodecyl sulfate; SSC buffer, 0.15 M NaCl–0.015 M Na_3 citrate.

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MATERIALS AND METHODS

Materials. Frozen midlog-phase *E. coli* Q13 cells were obtained from General Biochemicals. Whatman brand microgranular diethylaminoethyl-cellulose DE 52 and phosphocellulose P-11 were obtained from H. Reeve Angel & Co. Agarose (Bio-Gel A-0.5 M, 100–200 mesh) was obtained from Bio-Rad Laboratories. Nitrocellulose membranes (type HA, 0.45 μm), used to collect trichloroacetic acid precipitates, were purchased from Millipore Corp., while those used to immobilize DNA for RNA–DNA hybridizations (type B-6) were purchased from Carl Schleicher and Schuell & Co. [^{3}H]-UTP (13 Ci/mmol) and Liquiflor were purchased from New England Nuclear. [^{14}C]ATP (29.2 Ci/mol) was purchased from Schwarz/Mann.

DNA Preparations. E. coli DNA was isolated from freshly grown cells of *E. coli* strain B or Q13 by the method of Miura (11). Bacteriophage T4 DNA was isolated from bacteriophage that were purified from cell lysates by polyethylene glycol precipitation, followed by two successive isopycnic bandings in cesium chloride gradients. Phage DNA was prepared by sodium dodecyl sulfate ($NadodSO_4$)–phenol extraction of the purified phage.

Preparation of RNA Polymerase. E. coli RNA polymerase was prepared from frozen *E. coli* B cells by the method of Berg *et al.* (12). The specific activity with T4 DNA as a template was 1200 units/mg, where one unit is defined as the incorporation of 1 nmol of [^{14}C]AMP in 10 min at 37°. This preparation was at least 90% pure, as judged by polyacrylamide gel electrophoresis in $NadodSO_4$. Core RNA polymerase was prepared (12) from portions of this RNA preparation by phosphocellulose chromatography.

Assay of Y Factors. The Y factors were estimated by their ability to stimulate RNA synthesis in a standard reaction mixture. The mixture contained in 0.25 ml: 0.04 M Tris·HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.04 M KCl, 0.5 mg/ml of bovine serum albumin, 0.2 mM UTP, CTP, 0.04 mM [^{14}C]ATP (5 Ci/mol), 1.25 μg of *E. coli* RNA polymerase, 2.3 μg of *E. coli* Q13 DNA, and various amounts of the Y factor preparations. Incubation mixtures in which the Y factors were omitted were run in parallel in order to determine the extent of stimulation. After incubation for 10 min at 37°, the mixtures were precipitated with 3 ml of 5% trichloroacetic acid (0°). After 10 min at ice temperature, the precipitates were collected on Millipore filters, washed with 5% trichloroacetic acid, dried, and counted by liquid scintillation counting in Liquiflor–toluene scintillation fluid.

Preparation of RNA for Hybridization. Isotopically-labeled RNA was synthesized in 500-μl reaction mixtures containing

0.04 M Tris·HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.04 M KCl, 0.5 mg/ml of bovine serum albumin, 0.2 mM ATP, GTP, CTP, and [^{3}H]UTP (4 Ci/mol), 4.6 μg of *E. coli* DNA, 6 μg of RNA polymerase, and Y_I or Y_{II}, as indicated. The reactions were terminated after 10 min at 37° by the addition of NadodSO$_4$ (0.1% final concentration) and 5 μg of rat-liver tRNA. The mixture was extracted with water-saturated phenol, and the RNA in the aqueous phase was precipitated by the addition of 0.05 ml of 2 M ammonium acetate and 2.4 ml of ethanol. After 2 hr at −70°, the precipitate was collected by centrifugation, drained of the supernatant fluid, and dried under reduced pressure. The dry powder was dissolved in 200 μl of 50% formamide -3 × SSC buffer.

RNA–DNA Hybridization. *E. coli* DNA was denatured and trapped on nitrocellulose filters (13); in these experiments the salt concentration was lowered to 3 × SSC (14). A sequential hybridization-competition procedure was used in which the products of the *in vitro* RNA polymerase reaction served as a competitor for highly purified [^{14}C]rRNA isolated from labeled ribosomes. Nitrocellulose filters containing 6 μg of denatured DNA were incubated for 16 hr at 37° in 200 μl of 3 × SSC–50% formamide that contained graded amounts of RNA transcripts. The filters were removed and washed sequentially in 3 × SSC and 3 × SSC–50% formamide. Filters were then incubated for an additional 16 hr at 37° in 200 μl of 3 × SSC–50% formamide that contained a saturating amount of [^{14}C]rRNA (0.2 μg, 12,300 cpm). The filters were then washed in 2 × SSC, treated with pancreatic ribonuclease (50 μg/ml, 30 min, room temperature), and washed again with 2 × SSC. The filters were dried and counted in Liquiflor–toluene scintillation fluid. The rRNA content of the RNA transcripts was estimated by comparison of their effectiveness as a competitor for the [^{14}C]rRNA with that observed for pure unlabeled rRNA.

Preparation of Y Factors. 50 g of frozen *E. coli* Q13 cells were thawed and suspended in 50 ml of Buffer A[10 mM Tris·HCl (pH 7.9)–0.1 mM EDTA–0.1 mM dithiothreitol–5% glycerol]. Egg-white lysozyme (300 μg/ml final concentration) was added, and the suspension was frozen and thawed twice. After gentle mixture of the resulting lysate with 100 ml of Buffer A containing 1 M KCl, ribosomes, together with the bulk of the DNA, were removed by centrifugation for 2 hr at 105,000 × *g*. This and later steps were performed at 0–4°. The clear supernatant was brought to 50% saturation with $(NH_4)_2SO_4$, and the resulting precipitate was collected by centrifugation and was dissolved in 20 ml of Buffer B [20 mM Tris·HCl (pH 7.9)–10 mM $MgCl_2$–0.1 mM EDTA–0.1 mM dithiothreitol–5% glycerol]. The solution was applied to a Bio-Gel A 0.5 M Agarose column (60 × 700 mm) equilibrated with Buffer B containing 0.1 M KCl. The column was eluted at a flow rate of 2 ml/min with Buffer B containing 0.1 M KCl. The first protein fractions to emerge from the column contained all of the RNA polymerase activity; they were discarded. The balance of the eluate was combined and brought to 50% saturation with solid ammonium sulfate. The precipitate was collected, dialyzed against Buffer B, and passed over a phosphocellulose column (17 × 200 mm) that had been equilibrated with Buffer B containing 0.05 M KCl. The fraction that eluted with the same buffer contained most of the stimulating activity. The phosphocellulose eluate was applied to a DEAE-cellulose column (17 × 200 mm) equilibrated with Buffer B, and eluted with a 300-ml linear gradient from 0.05 to 0.4 M KCl in Buffer B. Two separable peaks of the stimulating activity that were eluted at 0.13 and 0.2 M KCl concentrations were designated the Y_I and Y_{II} factors, respectively (Fig. 1). Each peak fraction was precipitated with one volume of saturated neutral ammonium sulfate. The precipitates were collected, dissolved in Buffer B, and dialyzed. Each fraction was applied to a DEAE-cellulose column (12 × 140 mm) and eluted with a 100-ml linear gradient of 0.1–0.4 M KCl in Buffer B. The peak fractions were concentrated by precipitation with saturated ammonium sulfate solution, dissolved, and adjusted to 50% glycerol in Buffer B and stored at −70°. The Y_I and Y_{II} fractions were stable for at least 1 month under these conditions.

RESULTS

Purification of Y_I and Y_{II}

The rationale that guided the initial phase of this work was that proteins that stimulate the RNA polymerase could most easily be identified in crude extracts of *E. coli* after the endogenous RNA polymerase, DNA, and rho protein had been removed. To this end, we used a gentle lysis of *E. coli* cells that allowed the removal of virtually all of the DNA by centrifugation of the lysate, followed by molecular sizing on an Agarose column to separate the RNA polymerase from smaller proteins, and finally passage of the extract over columns of phosphocellulose to remove the rho protein. The protein fraction thus obtained, when fractionated on DEAE-cellulose columns, reveals two peaks of stimulatory activity that are not easily discerned if any of the previous steps had been omitted (Fig. 1). The inhibition of RNA polymerase activity that immediately follows Y_{II} in the DEAE-cellulose elution profile is most likely due to the very substantial amount of ATPase that elutes in that position. Furthermore, the relative amounts of Y_I and Y_{II} shown in Fig. 1 are probably also affected by failure to completely resolve Y_{II} from the ATPase.

Stimulation of RNA synthesis by Y_I and Y_{II}

Several reagents and experimental conditions stimulate *E. coli* RNA polymerase. Most notable among these are ionic

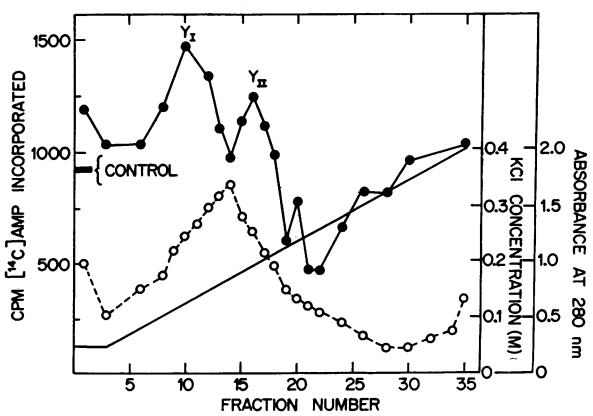

FIG. 1. DEAE-cellulose chromatography. Phosphocellulose eluate was applied to a DEAE-cellulose column and eluted. 10-μl portions of each 9-ml fraction were tested in the standard assay for stimulation (●——●). The control (unstimulated) incorporation is indicated on the *ordinate*. *Open circles* represent absorbance at 280 nm.

TABLE 1. *Y_I and Y_{II} are proteins*

Reaction	[^{14}C]AMP incorporation (pmol)	(%)
I. Control	125	100
+ Y_I	414	331
+ Y_{II}	236	189
+ heated Y_I	129	103
+ heated Y_{II}	120	96
II. Control	133	100
+ trypsin + soybean inhibitor	109	82
+ Y_I	528	397
+ trypsinized Y_I + soybean inhibitor	135	101
+ Y_{II}	254	191
+ trypsinized Y_{II} + soybean inhibitor	105	79

Y_I and Y_{II} were either heated to 90° for 2 min or treated with 20 μg of trypsin for 10 min at 37°. Trypsin treatment was terminated by the addition of 20 μg of soybean trypsin inhibitor. Where indicated, 18 μg of native or treated Y_I and 32 μg of native or treated Y_{II} were included in the reaction mixtures.

strength (15), manganese ion (16), and polyamines such as spermine (17). The stimulation observed with either the Y_I or Y_{II} fractions cannot be ascribed to small ions contained in these fractions. Both Y_I and Y_{II} are thermolabile, and are completely inactivated by heating to 90° for 2 min (Table 1). The protein nature of these stimulating factors is indicated both by the purification schemes and their sensitivity to trypsin (Table 1).

Both Y_I and Y_{II} stimulate the incorporation of ATP, UTP, and GTP (CTP was not tested) into acid-precipitable material when all four triphosphates are present, showing that the RNA synthesized in their presence is a heteropolymer (Table 2). The fact that the incorporation of each nucleotide is stimulated to about the same degree suggests that the RNA synthesized in the presence or absence of Y_I and Y_{II} has about the same base composition. This indication was further substantiated by RNA–DNA hybridization analysis. In these experiments, the products from equivalent amounts of the RNA polymerase reactions were either precipitated with acid to measure UTP incorporation, or were hybridized with filter-bound DNA from *E. coli*. The results shown in Table 3 demonstrate that the two factors stimulated the synthesis of RNA complementary to the DNA template.

TABLE 2. *Stimulation of nucleotide incorporation by Y_I and Y_{II}*

	Nucleotide incorporated (pmol)		
	[^{14}C]AMP	[^{3}H]UMP	[^{14}C]GMP
Control	93.4	90.5	117
+ Y_I	291 (312)	279 (308)	325 (278)
+ Y_{II}	214 (229)	188 (208)	217 (185)

Standard assay reactions were used except that the labeled nucleotide was either [^{14}C]ATP (5 Ci/mol), [^{3}H]UTP (20 Ci/mole), or [^{14}C]GTP (10 Ci/mol). Where indicated 18 μg of Y_I and 32 μg of Y_{II} were included in the assay. The figures in parentheses give % stimulation by Y_I or Y_{II} over the appropriate control value.

TABLE 3. *Hybridization analysis of RNA synthesized in the presence or absence of Y_I and Y_{II}*

	[^{3}H]UMP incorporation		Hybridization	
	(cpm)	(%)	(cpm)	(%)
Control	5,612	110	2329	100
+ Y_I	17,858	318	7097	305
+ Y_{II}	13,977	249	5159	222

Filters containing 75 μg of immobilized, denatured *E. coli* B DNA (RNA/DNA > 1000) were incubated (40 hr, 37°) with the indicated amounts of [^{3}H]RNA synthesized in the presence of 18 μg of Y_I, 32 μg of Y_{II}, or in their absence.

The effects of different concentrations of Y_I and Y_{II} on the RNA polymerase reaction are shown in Fig. 2. At low protein concentrations, the stimulation of RNA synthesis is about proportional to the amount of Y_I or Y_{II} added, while at higher concentrations the factors become saturating. At saturation, the stimulation activity by Y_I and Y_{II} is about 4- and 2-fold, respectively. Furthermore, the stimulations of RNA synthesis are additive when both factors are present together. From this result, we conclude that Y_I and Y_{II} are different from each other, and that neither is grossly contaminated with the other. When either RNA polymerase or DNA is omitted from the reaction mixture, the amount of RNA made is negligible, even in the presence of large amounts of the stimulating factors (Fig. 2).

The stimulation of RNA synthesis can not only be saturated with Y_I and Y_{II}, but also with the RNA polymerase itself. At relatively low concentrations of the RNA polymerase, there is

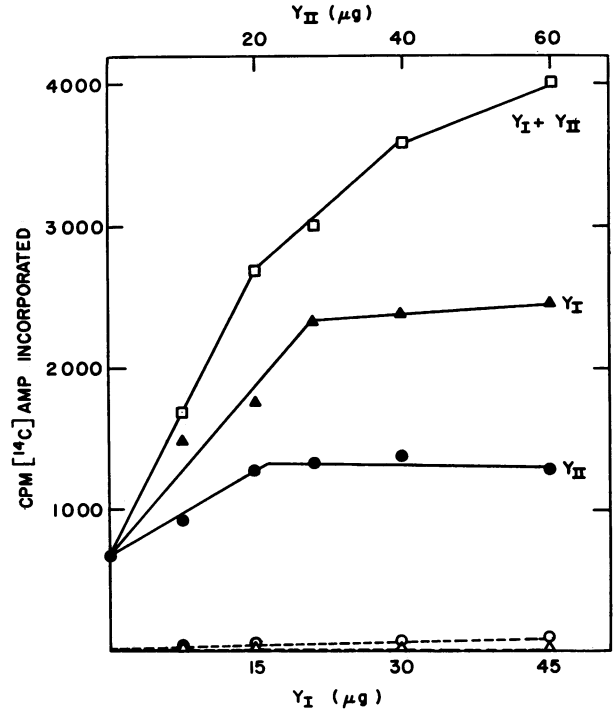

FIG. 2. Effect of Y_I and Y_{II} concentration of RNA polymerase transcription of *E. coli* DNA. The standard assays containing the indicated amounts of Y_I (▲——▲), Y_{II} (●——●), or Y_I plus Y_{II} (□—□). The incorporation observed in the absence of RNA polymerase, but with Y_I (△——△) or Y_{II} (○——○) is also shown.

TABLE 4. *Specificity of Y_I and Y_{II}*

Enzyme*	DNA template	[^{14}C]AMP incorporated (pmol) Control	+ Y_I	+ Y_{II}
Holo	*E. coli*†	162	493	403
Core	*E. coli*	66.5	71.4	74.9
Holo	poly(dA–dT)‡	147	172	163
Core	poly(dA–dT)	335	320	292
Holo	T_4‡	313	580	592
Core	T_4	14.7	20.4	26.1

* 1.8 μg of holoenzyme or 2.3 μg of core enzyme was used throughout.
† 2.3 μg.
‡ 2.0 μg.

a marked stimulation by either Y_I or Y_{II} (Fig. 3). However, at higher concentrations of the RNA polymerase, the percentage stimulation by the factors diminished, while the total stimulation increment remained constant. These results suggest that the Y_I and Y_{II} factors included in the reaction mixtures have become saturated with 1.8 μg of RNA polymerase, although gross incorporation was not saturated by 8 μg of RNA polymerase.

The DNA dependence of the reaction is changed by the presence of either Y_I or Y_{II} (Fig. 4). In the presence of either factor the reaction shows a complete dependence upon the presence of DNA, but reactions including Y_I or Y_{II} require 1–2 μg of DNA to saturate them, whereas the control is saturated by 0.5 μg. As in the control, high concentrations of DNA lead to a suppression of activity.

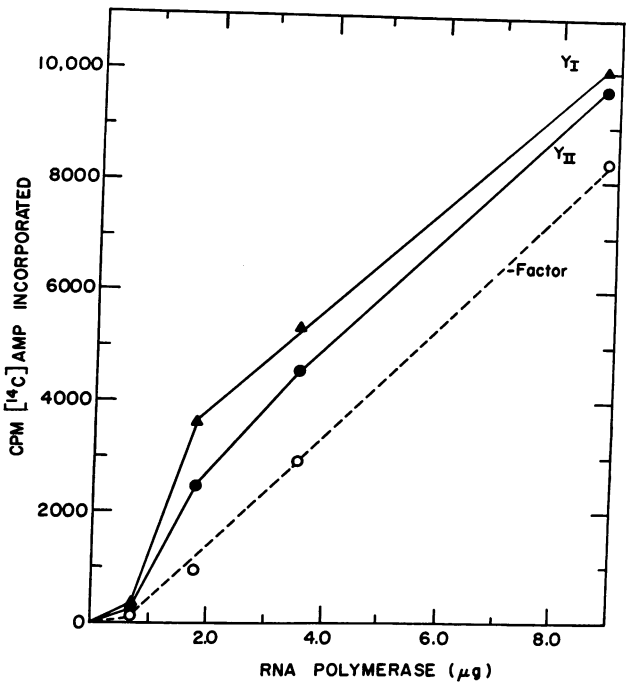

FIG. 3. Effect of RNA polymerase concentration on the Y_I and Y_{II} mediated stimulation. Incorporation in the absence of Y_I or Y_{II} (O——O), with 30 μg of Y_I (▲——▲), and with 52 μg of Y_{II} (●——●) are shown.

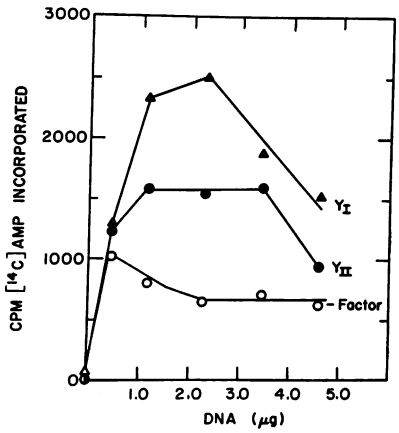

FIG. 4. Effect of DNA concentration on Y_I and Y_{II} activity. Incorporation of [^{14}C]ATP in the presence of 30 μg of Y_I (▲——▲), 52 μg of Y_{II} (●——●), or in the absence of factors (O——O) was measured as a function of DNA concentration.

Specificity of stimulation

The stimulation of DNA transcription by Y_I and Y_{II} is not restricted to an *E. coli* DNA template, but is also demonstrable on T4 DNA (Table 4). In contrast, reactions in which poly(dA–dT) serves as the template are not stimulated appreciably by either factor. It is also apparent that both factors stimulate only the holoenzyme. This dependence on the presence of sigma, and the failure of poly(dA–dT)-directed reactions to show a stimulation, suggests that the factors function at the level of initiation rather than chain elongation (see *Discussion*).

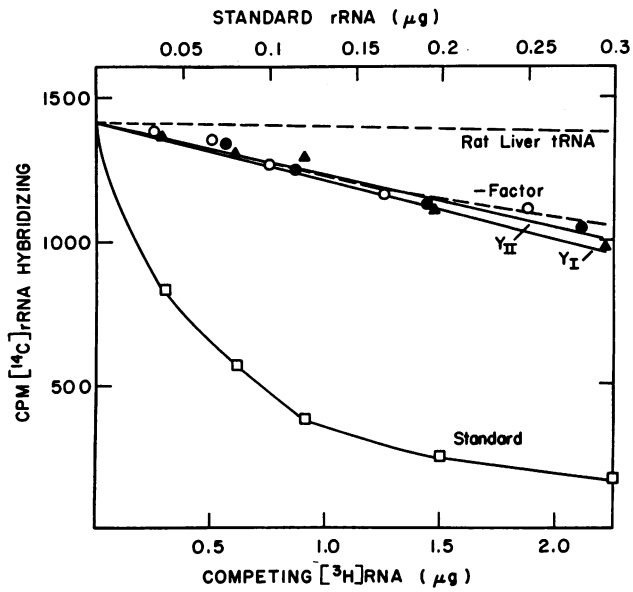

FIG. 5. Hybridization-competition analysis of [^{3}H]RNA synthesized in the presence or absence of Y_I and Y_{II}. Filters containing 6 μg of immobilized denatured DNA were incubated (16 hr, 37°) with the indicated amounts of [^{3}H]RNA synthesized in the presence of Y_I (▲——▲), Y_{II} (●——●), or in their absence (O——O). Filters were washed free of unbound RNA and incubated with saturating amounts of [^{14}C]rRNA (0.2 μg, 12,300 cpm) for 16 hr at 37°. The standard curve was prepared in an identical way, but purified rRNA was used as indicated on the *upper abscissa* in the initial incubation.

The product of the stimulated transcription of *E. coli* DNA is not appreciably enriched in rRNA. Under the conditions we used, about 2% of the unstimulated transcription products behave as rRNA in hybridization-competition reactions. The percentage of rRNA in RNA transcripts from reactions stimulated with either Y_I or Y_{II} are indistinguishable from the controls (Fig. 5). Equivalent results were obtained with DNA from *E. coli* Q13 or *E. coli* B as a template, as well as with freshly prepared or aged DNA.

DISCUSSION

These results demonstrate that extracts of *E. coli* freed of RNA polymerase, DNA, and the rho factor can be resolved by DEAE-cellulose chromatography into two fractions that stimulate *E. coli* RNA polymerase. The stimulatory activity in these two fractions are distinct from one another, since they can be rechromatographed on DEAE-cellulose without further resolution, and since their stimulation of the RNA polymerase is additive.

The increased RNA synthesis observed in the presence of either of these factors cannot be attributed to the presence of polynucleotide synthesizing enzymes (e.g., polynucleotide phosphorylase) in the fractions, since the reaction is completely dependent upon the presence of the RNA polymerase and DNA. The possibility that the stimulated synthesis is due to an enzyme that requires an RNA primer for activity is equally unlikely, since neither Y_I nor Y_{II} stimulates transcription when poly(dA–dT) or when core RNA polymerase are used, although RNA synthesis occurs in both cases.

Vogt (18) has cautioned that low levels of deoxynuclease stimulate RNA polymerase reactions by introducing single-strand scissions in the DNA template. Such trivial explanations of the stimulation by present factors are difficult to rule out, since nucleases are ubiquitous and neither Y_I nor Y_{II} is homogeneous. Nonetheless, the specificity of the stimulation observed with the factors makes such explanations unlikely. Mild nuclease treatments of DNA stimulate the transcription by both the *E. coli* core RNA polymerase (18) and the sigma-deficient enzymes obtained from T4-infected cells (19). However, neither Y_I nor Y_{II} stimulates core RNA polymerase transcription of any template tested. The failure of the factors to stimulate when poly(dA–dT) serves as a template, and the reaction characteristics shown in Figs. 2, 3, and 4, also are not easily accommodated by trivial explanations.

The rate of RNA synthesis can be increased either by an increase of the number of RNA chains under construction, i.e., increasing the rate of chain initiation, or by an increase of the average rate at which nucleotide residues are added to growing RNA chains. The data shown in Table 4 suggest that stimulation of RNA synthesis is only observed with the Y factors when both the enzyme and template are competent to mediate natural chain initiation. Although the core RNA polymerase initiates RNA chains, the site of initiation does not correspond to that observed *in vivo* (4). Similarly, the template poly(dA–dT) is likely not to contain natural promoter sites. This specificity suggests that the Y factors affect the rate of chain initiation, rather than the rate of chain propagation.

In some of their properties Y_I and Y_{II} resemble stimulatory proteins described by others. However, the present factors can be distinguished from these in several ways. Both the M protein of Davison *et al.* (20, 21) and the factor described by Mahadik and Srinivasan (22) stimulate core RNA polymerase about 30-fold when phage DNA templates are transcribed. In contrast, Y_I and Y_{II} do not stimulate the core RNA polymerase on any template tested. Furthermore, Y_I and Y_{II} are purified from ribosome-free lysates, whereas both other factors are purified from high-salt extracts of the ribosomal fraction. The present factors also differ from the activity of psi_r, as originally described by Travers (23), in that they do not stimulate the transcription of ribosomal cistrons.

The relationship of either Y_I or Y_{II} to the heat-stable (H) protein described by Jaquet *et al.* (24) is difficult to discuss, because of insufficient data. The stimulation of RNA polymerase by either the present factors or the H protein is similar in degree, and responds similarly to variation of DNA and RNA polymerase concentrations. However, the position of the Y factors in the agarose chromatography eluate suggests that they are considerably larger than 10,000 molecular weight, the value ascribed to the H protein. Furthermore, the Y factors are both thermolabile. However, both apparent molecular weight and heat stability might be altered by secondary, nonspecific associations with other proteins. A meaningful comparison must await further purification of both H and Y factors.

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