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Stimulation of DNA-Dependent RNA Synthesis by a Protein Associated with Ribosomes

(*E. coli*/RC^{rel} strains/RNA polymerase core/sigma factor)

S. P. MAHADIK AND P. R. SRINIVASAN

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, N. Y. 10032

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ABSTRACT **A protein factor partially purified from ribosomes of *Escherichia coli* RC^{rel} cells starved for an amino acid markedly stimulates DNA-dependent RNA synthesis by core and whole RNA polymerase. This protein factor appears to differ from the sigma factor and the M factor. The properties of this new factor and its mechanism of action have been explored.**

In *Escherichia coli*, the synthesis of RNA is linked to protein synthesis under the control of the genetic locus RC (1). Bacterial strains carrying the RC^{str} (*rel*$^+$) allele exercise stringent control over RNA synthesis, and starvation for a required amino acid prevents the synthesis of bulk RNA and protein. In RC^{rel} (*rel*$^-$) strains, the control of RNA synthesis is relaxed; amino acid auxotrophs deprived of an exogenous supply of the required amino acid continue to synthesize RNA in the absence of protein synthesis (2). All three major kinds of RNA—ribosomal, transfer, and messenger—are made in relaxed strains during starvation (3, 4). The unabated synthesis of RNA during starvation of *rel*$^-$ strains suggested the occurrence of protein factors that stimulated transcription under these conditions. In this communication, we report the isolation of a protein associated with the ribosomes of starved *rel*$^-$ cells. The partially purified protein stimulated the *in vitro* transcription of DNA by core polymerase and whole polymerase.

MATERIALS AND METHODS

E. coli K12 *met*$^-$ *ura*$^-$ *rel*$^-$ was grown in a modified M9 minimal medium (5) supplemented with 10 μg/ml of L-methionine, 10 μg/ml of uracil, and 0.5% glucose in a 50-liter fermentor. The cells were harvested in mid-logarithmic phase by centrifugation at 15°C and resuspended in the original volume of methionine-free medium. After starvation for 120 min, the cells were collected and stored at −70°C for the preparation of the ribosome-associated protein. Under these conditions of starvation the amount of RNA increased by 80%, as measured by the orcinol reaction.

50 grams of frozen cells were ground with 100 grams of alumina and the resultant paste was suspended in 150 ml of Tris·HCl buffer (pH 7.9), containing 50 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, and 50 mM KCl. The clear extract obtained by centrifugation at 14,000 × *g* for 15 min was centrifuged at 75,000 × *g* for 120 min. The ribosomal pellet was extracted with 25 ml of the above buffer (containing all the additions, except that the concentration of KCl was raised to 0.5 M) by gentle homogenization and then centrifuged at 105,000 × *g* for 90 min. 30 ml of the ribosomal wash was brought to 40% saturation with $(NH_4)_2SO_4$ and centrifuged. The supernatant was brought to 70% saturation and then centrifuged. The pellet was dissolved in 5 ml of buffer A [0.01 M Tris·HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 M $MgCl_2$, 0.05 M KCl, and 5% glycerol]. This fraction, which contained all the stimulating activity, was dialyzed against buffer A and applied to a DEAE-cellulose column (1.2 × 20 cm) previously equilibrated with buffer A. The column was then washed with buffer A and all the stimulating activity was eluted with the unadsorbed material. This eluate was brought to 70% saturation with neutral, saturated, $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation and was dissolved in 50 mM Tris·HCl buffer (pH 7.9) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM KCl, and 5% glycerol. This fraction was dialyzed extensively against the same buffer and then passed through a phosphocellulose column (1.2 × 10 cm) previously equilibrated with the same buffer. The fraction that eluted with the same buffer contained most of the stimulating activity. This fraction was either concentrated by Diaflo ultrafiltration or by precipitation with neutral, saturated, $(NH_4)_2SO_4$ solution. The fraction was brought to 50% glycerol and stored at −70°C.

E. coli RNA polymerase holoenzyme was prepared from exponentially grown cells of *E. coli* K12 U49 by the method of Burgess (6). The specific activity of the enzyme preparation was 540 units per mg of protein (1 unit = 1 nmol of [^{14}C]ATP incorporated into RNA in 10 min at 37°C with native calf-thymus DNA as template). The RNA polymerase was further fractionated into a core enzyme and sigma factor on a phosphocellulose column (7).

E. coli DNA was isolated by the method of Marmur (8). T2 and T4 DNA were prepared by the procedure of Mandel and Hershey (9). Ribosomal RNA was extracted from washed ribosomes of *E. coli* Q13. Catalase, alcohol dehydrogenase, and lysozyme were obtained from Worthington Biochemical Corp., Freehold, N.J. SV40[^{14}C]DNA was a generous gift of Dr. Dorothy Srinivasan.

The assay mixture for RNA synthesis contained in 0.25 ml: 40 mM Tris·HCl buffer (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.2 M KCl, 125 μg of bovine serum albumin, 25 μg of DNA, 0.2 mM radioactive nucleoside triphosphate (ATP or GTP or CTP; 1 Ci/mol) and 0.2 mM each of the other three unlabeled triphosphates, and either *E. coli* RNA polymerase or core polymerase. Incubation was at 37°C for 10 min. The reaction mixture was cooled and

3 ml of cold 5% trichloroacetic acid containing 0.01 M pyrophosphate was added to stop the reaction. The precipitate was collected on Whatman GF/C filters or on Millipore filters and washed three times with 3 ml of 5% TCA containing 0.01 M pyrophosphate. The filters were fixed to planchets, dried at 60°C, and counted in a low background, gas-flow counter.

For estimation of the size of RNA product formed, reaction mixtures of 0.5 ml were incubated for 30 min with [^{14}C]GTP and [^{14}C]CTP with either 60 μg of RNA polymerase or 60 μg of core polymerase and 400 μg of stimulatory factor. At the end of the incubation, carrier ribosomal RNA was added and the mixture was extracted three times with phenol saturated with 0.5% sodium dodecylsulfate in 0.02 M potassium phosphate buffer, pH 7.0. Nucleic acid in the aqueous layer was precipitated by the addition of 0.25 volumes of 1 M potassium acetate buffer, pH 5.0, and 2 volumes of ethanol. After standing overnight at 4°C, the precipitated RNA was collected by centrifugation and dissolved in acetate buffer. The ethanol precipitation was repeated twice. The [^{14}C]RNA was finally dissolved in 0.02 M Tris·HCl buffer, pH 7.5, containing 0.15 M NaCl. The RNA was either used immediately or stored at −20°C.

RESULTS

The ribosome-associated protein purified by phosphocellulose column chromatography had an $A_{280}:A_{260}$ ratio of 1.4–1.6 and contained no detectable RNA or DNA or ribonuclease activity. The preparations from two independent isolations were tested for deoxyribonuclease activity with SV40[^{14}C]DNA, which contained 60% Form I and 40% Form II. A single nick is sufficient to convert Form I to Form II (10, 11). After incubation of 40 μg and 80 μg of the factor preparation with 1 μg of SV40[^{14}C]DNA for 10 min at 37°C, analysis on alkaline sucrose gradients (11) indicated no significant change in the ratio of Form I to Form II. The ratio of factor to DNA in the above incubation mixture is five to eight times greater than that normally employed in the assay mixtures. The molecular weight of the factor was estimated by velocity sedimentation centrifugation in sucrose gradients with the following markers:

TABLE 1. *Stimulation of RNA synthesis with native and denatured DNA templates by factor*

	[^{14}C]AMP incorporated (nmol)			[^{14}C]GMP incorporated (nmol)		
	E. coli	T2	T4	*E. coli*	T2	T4
Native DNA templates						
Core polymerase	2.43	0.88	0.80	2.05	0.92	0.35
Core polymerase with factor	5.42	9.76	10.8	6.72	8.92	8.48
Whole polymerase	1.34	—	—	1.46	3.99	—
Whole polymerase with factor	1.67	—	—	2.29	5.78	—
Denatured DNA templates						
Core polymerase	—	0.57	0.14	—	0.33	0.09
Core polymerase with factor	—	13.25	8.69	—	12.0	8.24

30 μg of core polymerase or 10 μg of whole polymerase and 300 μg of factor were used as indicated.

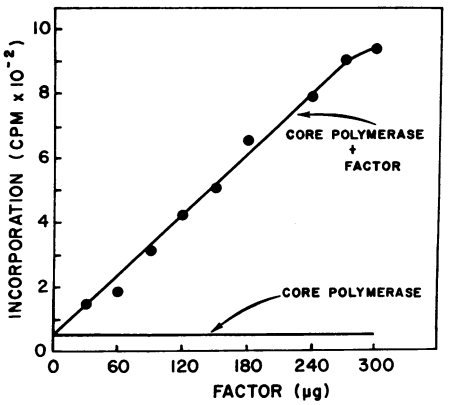

FIG. 1. Effect of variation of the new factor concentration on RNA synthesis by core polymerase. Each assay contained 30 μg of core polymerase; T2 DNA was used as template.

sigma factor, catalase, alcohol dehydrogenase and lysozyme. The position of the new factor and sigma factor in individual, as well as in mixed, gradients was determined by the capacity of the various fractions to stimulate the transcription of native T2 DNA by core polymerase. The new factor has a sedimentation value of about 7.7 S and is distinct from the sigma factor. The new factor was also isolated from other amino-acid auxotrophs of *E. coli* that carry the relaxed gene. It could only be isolated from the ribosomes of cells grown under conditions of amino-acid starvation; it could not be isolated from ribosomes of cells grown exponentially.

The stimulation of RNA synthesis by this factor with core polymerase and T2 DNA is shown in Fig. 1. Core polymerase alone catalyzes a low level of incorporation that proceeds at a linear rate. Addition of the factor markedly enhances incorporation; at very high concentrations a plateau is reached. At this high concentration, a 20-fold stimulation of RNA synthesis can be observed. The ribosome-associated protein, alone or with T2 DNA, did not cause any incorporation of radioactivity.

The effect of the factor on RNA synthesis with core polymerase, whole polymerase, and various native and denatured

TABLE 2. *Effect of rifampicin on the stimulation of core polymerase with either sigma factor or the new factor*

	[^{14}C]GMP incorporated (nmol)		
	Control without rifampicin	Rifampicin at 0 min	Rifampicin after 1 min
Core polymerase*	0.20	0.09	0.18
Core polymerase with sigma factor	6.13	0.09	4.04
Core polymerase with ribosome-associated factor	1.81	0.07	1.28

The concentration of core polymerase, sigma, and the new factor used was 30, 10, and 300 μg, respectively. 1 μg of rifampicin was included at the beginning of incubation or after 1 min of incubation as indicated. The total incubation period was 10 min at 37°C. T2 DNA was used as a template.

* The activity of the core polymerase depends on the T2 DNA preparation, but the extent of stimulation by the new factor is always the same.

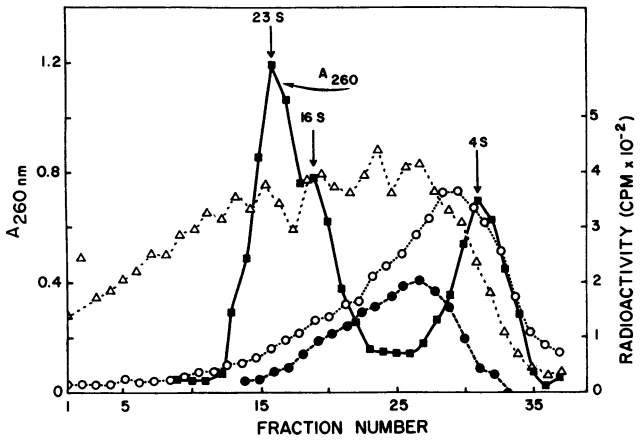

FIG. 2. Size of the RNA product formed by core polymerase with native and denatured T2 DNA as template in the presence of the new factor, and by whole polymerase with native T2 DNA as template. 1-ml samples were layered on 28 ml of 5–20% sucrose gradients in 0.02 M Tris·HCl buffer (pH 7.5)–0.15 M NaCl–10% formamide and centrifuged at 25,000 rpm in a SW 25.1 rotor for 24 hr at 4°C. 10-drop fractions were collected; 100 μg of bovine-serum albumin was added to each fraction as a carrier before precipitation with 1.5 ml of 5% trichloroacetic acid contining 0.01 M pyrophosphate buffer. The precipitates were collected on Whatman GF/C filters, washed with 5% TCA, dried at 60°C, and counted in a Nuclear-Chicago Scintillation Counter with omnifluor scintillator. Δ- - -Δ, whole polymerase and native T2 DNA, O- - -O, core polymerase, factor, and denatured T2 DNA; ●- - -●, core polymerase, factor, and native T2 DNA.

templates is presented in Table 1. The synthesis of RNA was measured with both [^{14}C]ATP and [^{14}C]GTP in separate assays. With core polymerase, there is a 3-fold increase in RNA synthesis with *E. coli* DNA as template, whereas with T2 and T4 native DNAs as templates, the stimulation is about 12-fold with [^{14}C]ATP and 10- and 24-fold, respectively, with [^{14}C]-GTP. In contrast, the enhancement in RNA synthesis with whole polymerase by the factor is only between 1.5- and 2-fold. The core polymerase activity is decreased with denatured T2 and T4 DNAs as templates. The inclusion of the factor in the assay mixtures markedly enhances the synthesis of RNA, and the degree of stimulation is considerably greater than that observed with native DNA as template.

Enhancement of RNA synthesis by the factor could be due to either a direct effect on RNA chain elongation, or on chain initiation, or on both. Rifampicin, a potent inhibitor of chain initiation (12), was examined for its effects on the stimulation of RNA synthesis by both the new factor and Sigma factor; the results are summarized in Table 2. If the drug is added before initiation of RNA synthesis, the stimulation of RNA synthesis by the ribosome-associated factor is completely abolished. However, if the drug is introduced after 1 min of RNA synthesis, the stimulation is inhibited by only 30%. Similar findings are obtained with sigma factor, and are in agreement with the earlier observations of Travers and Burgess (13). The similarity in the behavior of these two factors raised the question as to whether the addition of the new factor to core polymerase containing a large excess of sigma factor will further enhance the synthesis of RNA. Core polymerase was first titrated with sigma factor to determine the saturation value, i.e., addition of more sigma factor did not further increase the

TABLE 3. *The effect of the new factor on RNA synthesis with either core polymerase or core polymerase and excess sigma factor*

Concentration of factor per 0.35 ml	[^{14}C]GMP incorporated (nmol)	
	Core polymerase	Core polymerase with excess sigma
0	0.17	3.48
60	0.66	4.14
120	1.38	4.75
300	3.26	5.35

The final volume of the incubation mixture was raised to 0.35 ml. 30 μg of core polymerase was used with T2 DNA as template. Sigma factor was used at a concentration of 10 μg/0.35 ml; this concentration is at least three times the amount required to saturate 30 μg of the core polymerase used here.

incorporation. The effect of various concentrations of the new factor on core polymerase and a large excess of sigma factor is illustrated in Table 3. The results obtained indicate that even in the presence of a large excess of sigma factor, addition of the ribosome-associated factor further enhances RNA synthesis. With 60 and 120 μg of the new factor, the effect is additive; with 300 μg of factor (the amount that yields maximum stimulation with core polymerase), the increase in activity over the value seen with core polymerase plus excess sigma factor is 60%. These results are in accord with the observation that the new factor also enhances RNA synthesis with whole polymerase. However, these results do not exclude the possibility that both the new factor and sigma factor may function through a common mechanism.

The size of the RNA product was investigated by sucrose gradient centrifugation, in the presence of 10% formamide to prevent aggregation. The amount of RNA synthesized with core polymerase alone was insignificant and was not tested. The whole RNA polymerase, with T2 DNA as template, yielded a heterogeneous product with a size range of 4–46 S. The size of the RNA made in the presence of native T2 DNA, core polymerase, and the new factor was slightly bigger than with denatured T2 DNA as template, but both of these RNA products showed a broad size distribution ranging from 4 to 16 S.

DISCUSSION

The results presented here indicate the existence of a protein factor associated with the ribosomes of starved cells that enhances the synthesis of RNA by purified core polymerase. In the presence of whole polymerase the stimulation was 2-fold. In some of its functional properties, the new factor behaves like the sigma factor, but it is clearly distinct from sigma as shown in velocity sedimentation gradients. Whereas the sigma factor appears to play a catalytic role (13), the amount of ribosome-associated factor required for maximum stimulation is large. Moreover, addition of this factor to core polymerase and saturating amounts of sigma factor further enhances RNA synthesis. The new factor is also distinct from the M factor described by Davidson *et al.* (14) in its sedimentation value (the latter has a sedimentation value of about 4 S) and in its elution properties from DEAE-cellulose and phosphocellulose columns. The stability of the ribosome-associated factor also differs from that of M factor. The former is inacti-

vated by dialysis against 1 M KCl for 5 hr, whereas the M factor was isolated by extraction of ribosomes and DNA with 2 M NH_4Cl for 18 hr.

E. coli ribosomes are bound to nascent RNA both *in vivo* (15, 16) and *in vitro* (17, 19) and stimulate DNA transcription *in vitro* (20–23) by about 2-fold at 0.05 M KCl. At 0.25 M KCl, a 10-fold stimulation of RNA synthesis by ribosomes in a chromatin system has recently been reported (24). The 10- to 24-fold stimulation observed in our system was in 0.2 M KCl, and the new factor may, therefore, function by associating with nascent RNA and facilitating its removal from the template. Other mechanisms can also be envisaged. The factor may function by promoting a stable ternary complex of DNA, RNA polymerase, and nucleotide. The inhibitory effect of rifampicin lends support to such an idea. However, the size of the RNA product formed under the influence of the factor with core polymerase and T2 DNA suggests a participatory role for the factor in elongation of RNA chains.

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