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DNA-directed *in vitro* synthesis of proteins involved in bacterial transcription and translation

(transducing phages/λ*rif*^{d}*18*/λ*fus3*)

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ABSTRACT **The *in vitro* synthesis of elongation factor (EF)-Tu (*tufB*), the ββ′ subunits of RNA polymerase, ribosomal proteins L10 and L12 directed by DNA from the transducing phage λ*rif*^{d}*18*, EF-Tu (*tufA*), EF-G, and the α subunit of RNA polymerase directed by DNA from the transducing phage λ*fus3* has been investigated in a crude and a partially defined protein-synthesizing system. Proteins L10 and L12 are synthesized in the partially defined system almost as well as in the crude system. However, the synthesis of EF-Tu, EF-G, and the α and ββ′ subunits of RNA polymerase is far less efficient in the partially defined system. An active fraction that stimulates the synthesis of these latter proteins has been obtained by fractionation of a high-speed supernatant on DEAE-cellulose. Because previous studies showed that this fraction (1 M DEAE salt eluate) contains a protein, called L factor, that stimulates β-galactosidase synthesis *in vitro*, L factor was tested for activity. Although L factor stimulates the synthesis of the ββ′ subunits, it has little or no effect on the *in vitro* synthesis of the other products studied. In the present experiments, the ratio of L12/L10 and of EF-Tu (*tufA*)/EF-G formed is 4–6. These values are consistent with *in vivo* results.**

DNA-directed *in vitro* protein-synthesizing systems afford a valuable tool with which to study the regulation of gene expression (for review, see ref. 1). Because of the complexity of the system, these *in vitro* studies have used relatively crude extracts to obtain protein synthesis. However, a great deal of new information might be obtained if gene expression could be achieved in a more defined system. Our laboratory has initiated studies on the DNA-directed *in vitro* synthesis of β-galactosidase with the hope of obtaining synthesis of this protein in a completely defined system (2–7). It has been possible to obtain significant synthesis of β-galactosidase in a partially defined system that contained many highly purified factors in addition to several partially purified fractions (7).

In conjunction with the studies on the synthesis of β-galactosidase, experiments were initiated on the expression of the two elongation factor (EF)-Tu genes, *tufA* and *tufB* (Fig. 1), carried by the transducing phages λ*fus3* and λ*rif*^{d}*18*, respectively (8, 9). In addition, quantitative assays have been developed to measure the expression of other genes on these phages, such as ribosomal proteins L10 and L12, EF-G, and the α and ββ′ subunits‡ of RNA polymerase (see Fig. 1). The results of these studies using a crude and partially defined system are described here.

MATERIALS AND METHODS

Uniformly labeled L-[^{14}C]leucine (≈300 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) and L-[^{35}S]methionine (≈1000 Ci/mmol) were purchased from Amersham/Searle. [^{3}H]Formaldehyde (≈90 Ci/mol) and sodium boro[^{3}H]hydride (≈10 Ci/mmol) were purchased from New England Nuclear. 2-Methoxy-2,4-diphenyl-3-(2*H*)-furanone (MDPF I) was obtained from Hoffmann–La Roche.

Bacteriophages λ*rif*^{d}*18* and λ*fus3* were isolated from *Escherichia coli* H105 (J. B. Kirschbaum, Harvard University) and *E. coli* NO1380 (M. Nomura, University of Wisconsin), respectively. The phages were purified and DNA was extracted as described (10, 11). Preparation, from *E. coli* Z19i^q (provided by G. Zubay, Columbia University), of the ribosomal wash, washed ribosomes, and a supernatant extract (S-200) has been reported (2, 12). The S-200 extract was further fractionated by chromatography on DEAE-cellulose into 0.25 M and 1.0 M DEAE salt eluates, as described (4, 13). The preparation or source and purity of the other protein factors used in the partially defined *in vitro* system has been described in a recent paper (7).§

Preparation of Antisera and Tritiated Protein Standards. Specific antisera to purified preparations of EF-Tu, EF-G, ribosomal protein L12, and RNA polymerase were raised in goats or rabbits by injecting 100–300 μg of protein in a suspension containing 500 μl of phosphate-buffered saline (1.5 mM KH_2PO_4/6.5 mM Na_2HPO_4/140 mM NaCl) and 500 μl of Freund's complete adjuvant.

The purified proteins were labeled by reductive methylation with [^{3}H]formaldehyde (14) or sodium boro[^{3}H]hydride (15). The specific activities (cpm/pmol) routinely obtained were: EF-Tu, 30–80; EF-G, 40–80; L12, 30–60; and RNA polymerase, 100–150 (distribution of the label was 55% in the ββ′ subunits and 20% in the α subunit).

DNA-Directed *In Vitro* Protein Synthesis. The complete system (70 μl) contained 15 mM Tris acetate (pH 8.0); 10 mM

Abbreviations: EF, elongation factor; IF, initiation factor; RF, release factor; NaDodSO$_4$, sodium dodecyl sulfate; MDPF I, 2-methoxy-2,4-diphenyl-3-(2*H*)-furanone.

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‡ The β and β′ subunits were not resolved in these experiments and are referred to as ββ′ in the text. The values given in the tables and figures represent the total amount of synthesis of both subunits.

§ The purified factors used in the present study were routinely prepared in our laboratory with the following exceptions. Tyrosyl- and methionyl-tRNA synthetases from *E. coli* were kindly supplied by C. Bruton (Imperial College of Science and Technology, London), as was cysteinyl-tRNA synthetase from *Bacillus stearothermophilus*. Tryptophanyl-tRNA synthetase was kindly supplied by K. Muench (University of Miami), and RF-1 and RF-2 by T. Caskey and J. Campbell (Baylor College of Medicine). For these studies only prolyl-tRNA synthetase was not >80% pure, but was purified 150- to 200-fold. Typical gel patterns of the other factors used have been presented (7).

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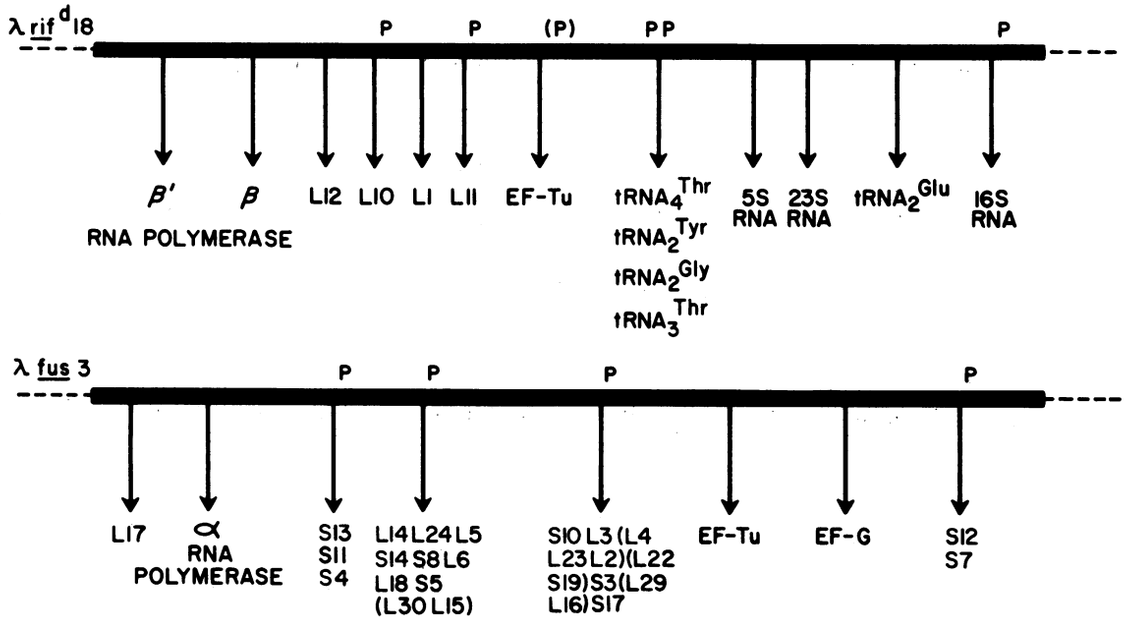

FIG. 1. Schematic genetic map of the λ*rif*^{d}*18* and λ*fus3* transducing phages. This has been taken from the results of Nomura and coworkers (8, 9).

Na dimethylglutarate (pH 6.0); 35 mM NH_4OAc; 65 mM KOAc; 13 mM $Mg(OAc)_2$; 2 mM dithiothreitol; 0.7 mM UTP, CTP, and GTP; 2.7 mM ATP; 30 mM phospho*enol*pyruvate; 0.8 μg of pyruvate kinase; 2.5 mg of polyethylene glycol 6000; 0.8 mM spermidine; 0.125 mM each of 19 amino acids (minus either leucine or methionine); 1.6 nmol of L-[*U*-^{14}C]leucine or 40 pmol of L-[^{35}S]methionine plus 2 nmol of methionine; 1.5 nmol of $N^{5,10}$-methenyl-H_4folate; 50 μg of *E. coli* B tRNA; 1.5–3.0 A_{260} units of NH_4Cl-washed ribosomes; and saturating amounts of λ*rif*^{d}*18* DNA or λ*fus3* DNA as template (between 2 and 5 μg). The following protein components were then added. The crude system (4) contained a 0.25 M DEAE salt eluate (240 μg of protein), a 1 M DEAE salt eluate (16 μg of protein), and a ribosomal high salt wash (100 μg of protein). For the partially defined system (7), the crude extracts were replaced with the following: RNA polymerase (3–4 μg), transformylase (0.03 μg), 50–150 units each of 20 aminoacyl-tRNA synthetases (7), initiation factor (IF)-1 (1.8 μg), IF-2 (0.8 μg), IF-3 (0.8 μg), EF-Tu (12 μg), EF-Ts (0.5 μg), EF-G (8 μg), release factor (RF)-2 (or RF-1 or both) (0.2 μg), and ribosome release factor (1.5 μg). In separate experiments it was shown that the amounts used of all the factors was saturating. An Ehrlich's ascites cell extract (20 μg of protein) was also added because it was shown to stimulate the reaction (7).¶

The reaction mixture was incubated at 37°C for 60 min, treated with 1 μg of DNase I (Worthington) for an additional 5 min at 37°C, and then assayed for the incorporation of amino acids into total protein as described (17).

Slab-Gel Electrophoresis of Products Synthesized *In Vitro*. An aliquot (2–5 μl) of the DNase-treated reaction mixture was analyzed by electrophoresis on 7–15% gradient polyacrylamide slab gels in the presence of sodium dodecyl sulfate ($NaDodSO_4$) (0.1%) (50–60 V, 16 hr) (18). The gels were treated with 2,5-diphenyloxazole in dimethyl sulfoxide (22.5% wt/vol) for fluorographic detection (19) and then exposed to Kodak X-Omat R film at −190°C for 3–5 hr.

Immunoprecipitation and Quantitation of Products Synthesized *In Vitro*. EF-G, L12 (and L10), and the RNA polymerase subunits were immunoprecipitated as described (20). Aliquots of the DNase-treated reaction mixture were mixed with 4–6 μg of [^{3}H]EF-G, [^{3}H]L12, or [^{3}H]RNA polymerase plus ≈50–70 μl of the corresponding antiserum in a final volume of 200 μl. Previous studies have shown that L10 interacts with L12 and is coprecipitated with L12 by L12 antiserum (21, 22). For EF-Tu, an aliquot of the DNase-treated reaction mixture was mixed with 3.5 μg of [^{3}H]EF-Tu plus 12 μg of unlabeled EF-Tu (including that amount contributed by the incubation aliquot) and 200 μl of EF-Tu antiserum in a final volume of 500 μl. The immunoprecipitation reaction mixtures were incubated at 37°C for 2–4 hr, and then chilled at 4°C for an additional 15 min. The immunoprecipitates were washed three times (20), suspended in 50 μl of 2% $NaDodSO_4$/0.1 M 2-mercaptoethanol, and dissolved by heating for 2 min at 90°C. A 5-μl aliquot was removed and assayed for radioactivity. Forty-five microliters of the dissolved immunoprecipitates were mixed with 4–8 μg of the appropriate fluorescent (MDPF I)-conjugated standard polypeptide (23, 24) and the samples were subjected to electrophoresis on polyacrylamide gels in the presence of $NaDodSO_4$ (0.1%) at 3 mA/tube for ≈3 hr (18). The reaction of the standard proteins with MDPF I allowed direct visualization of the protein bands both during and after disc-gel electrophoresis by using long-wavelength (352 nm) UV illumination.

The gels were cut manually to determine the radioactivity in each protein product, and the gel sections were extracted with 0.5–1.0 ml of 0.1% $NaDodSO_4$ for 1 hr at 55°C. Radioactivity was determined by addition of 7 ml of Instabray (National Diagnostics, Parsippany, NJ) and use of a Beckman liquid scintillation counter. The tritiated proteins served as internal standards for the estimation of the recovery of the synthesized products after immunoprecipitation and gel electrophoresis. The amount of the *in vitro* products formed was determined from the specific activity of the amino acid used and the amino acid composition of the individual proteins (25–29).

¶ It has recently been shown that the ascites extract contains a factor that simulates the *in vitro* synthesis of β-galactosidase, very likely by protecting mRNA against degradation (16). Because the ascites extract also stimulated protein synthesis in the present study, it was routinely added.

RESULTS

Gel Analysis of Products by Using λ*rif*^{d}*18* DNA and λ*fus3* DNA. The present experiments attempted to see whether the expression of several gene products directed by λ*rif*^{d}*18* and λ*fus3* DNA could be achieved in a partially defined *in vitro* system with 30 highly purified factors. The formation of several of the products that were of interest (EF-Tu, EF-G, and the α and ββ′ subunits of RNA polymerase) could be easily detected by slab-gel analysis. Fig. 2 (gel A) shows the radioactive products formed with λ*rif*^{d}*18* DNA as template and a crude protein-synthesizing system. The radioactive bands corresponding to the ββ′ subunits and EF-Tu (*tufB*) are clearly visible; however, ribosomal proteins L10 and L12 cannot be clearly distinguished because they comigrate with lower molecular weight products. Gel B shows the *in vitro* products when this phage DNA was used as template in the partially defined system. Much lower amounts of EF-Tu and the ββ′ subunits were synthesized compared to the crude system although total protein synthesis (hot Cl_3CCOOH-insoluble material) was depressed only 50% or less. As mentioned above, the amounts of L10 and L12 cannot be accurately estimated by slab-gel analysis; however, it appears that the synthesis of products in the 10,000–15,000 molecular weight range were not markedly depressed in the partially defined system. Quantitative studies on L10 and L12 formation confirmed this, as will be shown below.

It is apparent from a comparison of gels A and B in Fig. 2 that there are additional stimulatory components in the crude extracts that are required for the synthesis of EF-Tu and the ββ′ subunits and are lacking in the partially defined system. Preliminary experiments showed that of the three extracts used in the crude system (ribosomal wash and 0.25 M and 1 M salt eluates from DEAE), the 1 M DEAE salt eluate was effective in stimulating the synthesis of all of the gene products analyzed in the present study. The results of a typical experiment in which the 1 M DEAE salt eluate was added to the partially defined system are shown in Fig. 2, gel C. The increased synthesis of both EF-Tu and the ββ′ subunits (as well as other products not identified in this study) is evident in the partially defined system supplemented with the 1 M DEAE salt eluate.

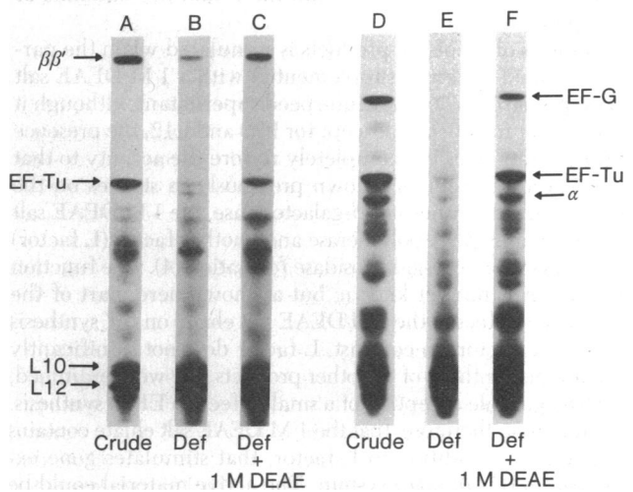

FIG. 2. Slab-gel electrophoresis of radioactive reaction products from λ*rif*^{d}*18* and λ*fus3* DNA-directed incubations. The incubations used 2 μg of λ*rif*^{d}*18* DNA (gels A–C) and 4 μg of λ*fus3* DNA (gels D–F) as templates. Two microliters of the incubation mixtures was applied to the gels. Def, partially defined system. Where indicated, 30 μg of a 1 M DEAE salt eluate was added. Gels A, C, D, and F, amounts of total protein synthesized (hot Cl_3CCOOH-insoluble material) were ≈300 pmol; gels B and E, ≈175 pmol.

Table 1. Quantitation of λ*rif*^{d}*18* and λ*fus3* DNA-directed products synthesized *in vitro*

	λ*rif*^{d}*18**			λ*fus3**		
Product	Crude	Defined	Defined + 1 M DEAE	Crude	Defined	Defined + 1 M DEAE
EF-Tu	3.5	0.2	1.5	7.6	0.8	4.0
L12	30	24	32	—	—	—
L10	7.0	5.0	8.0	—	—	—
ββ′	0.2	0.02	0.1	—	—	—
EF-G	—	—	—	1.5	0.1	0.6
α	—	—	—	2.0	0.2	1.0

* Values are in pmol.

A comparable series of experiments were done with λ*fus3* DNA as template. The results are seen in Fig. 2 (gels D, E, and F). Similar to the results with λ*rif*^{d}*18* DNA, the synthesis of EF-Tu (*tufA*), EF-G, and the α subunit of RNA polymerase directed by λ*fus3* DNA was much less in the partially defined system (Fig. 2, gel E) relative to the crude system (Fig. 2, gel D). As seen in Fig. 2, gel F, the addition of the 1 M DEAE salt eluate to the partially defined system also stimulated the synthesis of EF-Tu, EF-G, and the α subunit.

Quantitation and Ratio of Synthesized Products. Because the slab-gel analysis gave only semiquantitative results for the products that could be identified and no information on proteins L10 and L12, the amount of each of the products synthesized was quantitated by immunoprecipitation and disc-gel analysis. The results are shown in Table 1. The synthesis of EF-Tu and the ββ′ subunits directed by λ*rif*^{d}*18* DNA was generally decreased 80–90% in the partially defined system compared to the crude system. However, L10 and L12 synthesis showed only a slight decrease in the partially defined system, usually less than 30%. The 1 M DEAE salt eluate stimulated the synthesis of both EF-Tu (*tufA* and *tufB*) and ββ′ to about ⅓–½ of the value of the crude system. The synthesis of EF-Tu, EF-G, and the α subunit from λ*fus3* DNA was also decreased 80–90% in the partially defined system, but could be stimulated by the 1 M DEAE salt eluate.

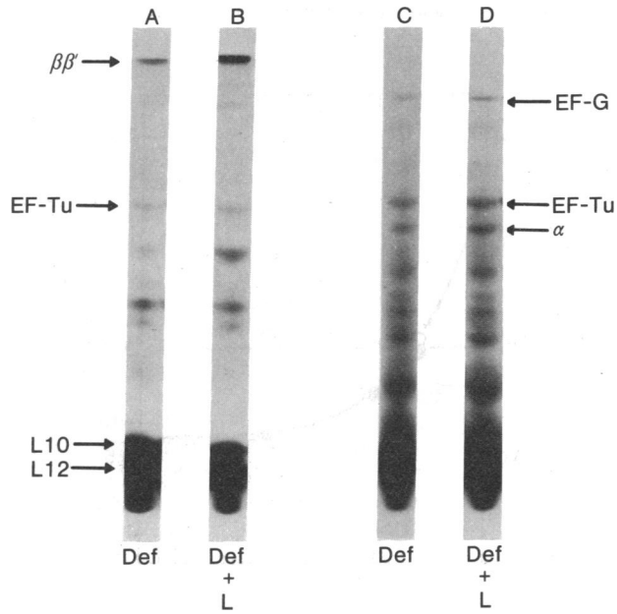

FIG. 3. Gel electrophoresis showing the effect of L factor in the partially defined system. Gels A and B, incubations using λ*rif*^{d}*18* DNA (2 μg); gels C and D, incubations using λ*fus3* DNA (4 μg). Where indicated, 5 μg of purified L factor was added.

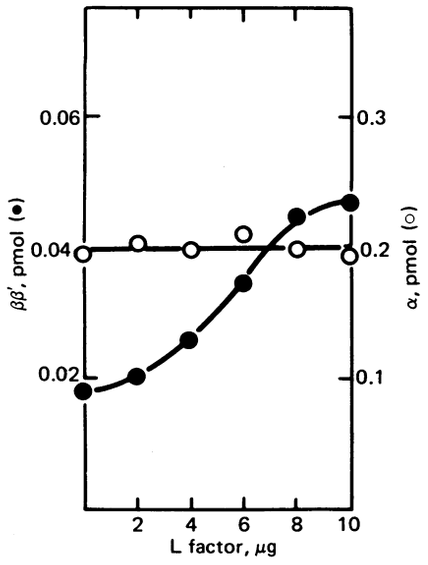

FIG. 4. Effect of L factor concentration on α and $\beta\beta'$ subunit synthesis. The indicated amounts of L factor were added to the partially defined *in vitro* system with λrif^d18 DNA or $\lambda fus3$ DNA as template. The incubation mixtures contained 4 μg of RNA polymerase. The α and $\beta\beta'$ subunits were quantitated by immunoprecipitation and disc-gel electrophoresis.

The results in Table 1 also demonstrate that in these *in vitro* studies, the ratios of L12/L10 and EF-Tu/EF-G agree with *in vivo* data. There are four copies of L12 per ribosome (30), whereas L10, like the other ribosomal proteins, is present in one copy per ribosome (31). In both the crude and partially defined systems, the L12/L10 ratio averaged between 3 and 6 in a large number of experiments. The ratio of L12/L10 obtained with the crude system in this study agrees with the *in vitro* results recently obtained in studies on the regulation of the synthesis of L12 (32). The bulk of the EF-Tu synthesized in the cell comes from the *tufA* gene and the EF-Tu/EF-G ratio *in vivo* is about 5 (33–35). As shown in Table 1, in both the crude and the partially defined systems the ratio of EF-Tu/EF-G formed, with $\lambda fus3$ DNA as template, was about 5 and ranged between 4 and 6.

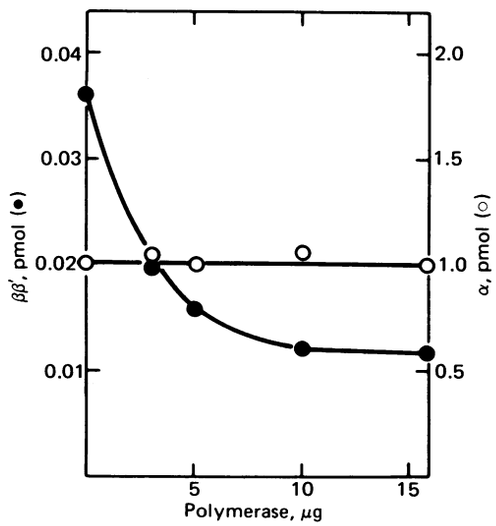

FIG. 5. Effect of RNA polymerase on α and $\beta\beta'$ subunit synthesis. Varying amounts of RNA polymerase holoenzyme were added to the partially defined system containing 30 μg of the 1 M DEAE salt eluate. α and $\beta\beta'$ subunit formation were then quantitated.

Studies on 1-M DEAE Eluate. Preliminary studies have been done on the stimulatory effect of the 1 M DEAE salt eluate in the partially defined system. Previous experiments on the *in vitro* synthesis of β-galactosidase showed that the 1 M DEAE salt eluate contains a factor, called L factor, that is necessary for β-galactosidase synthesis (4), and L factor was therefore examined in the present system. Fig. 3 shows that L factor stimulated the synthesis of the $\beta\beta'$ subunits, but did not appear to significantly affect the synthesis of EF-Tu, EF-G, or the α subunit. Quantitative studies were done to examine the effect of L factor more carefully. L factor did not stimulate EF-Tu, α subunit, L12, or L10 synthesis although a slight stimulatory effect ($\approx$30%) was occassionally observed on EF-G synthesis (data not shown). The effect of L factor concentration on the synthesis of the α and $\beta\beta'$ subunits is shown in Fig. 4. L factor stimulated $\beta\beta'$ synthesis about 2- to 3-fold and only partially replaced the 1 M DEAE salt eluate, which stimulated the amount of $\beta\beta'$ formed about 5-fold (Table 1).

Additionally, it was observed that higher levels of the 1 M DEAE salt eluate caused a specific inhibition of $\beta\beta'$ synthesis. The 1 M DEAE salt eluate contains RNA polymerase (4), and there is evidence from *in vivo* studies that $\beta\beta'$ synthesis is under autogenous regulation (36, 37). Recent *in vitro* studies have also shown that β subunit synthesis is inhibited by RNA polymerase holoenzyme (38). This observation was confirmed in our studies (Fig. 5). Further addition of RNA polymerase to a partially defined system containing the 1 M DEAE salt eluate caused a 70–80% inhibition of the synthesis of the $\beta\beta'$ subunits, although there was no effect on the synthesis of the α subunit. These levels of RNA polymerase also did not inhibit the synthesis of EF-Tu (*tufA* and *tufB*), EF-G, or ribosomal proteins L10 and L12 in this *in vitro* system (data not shown).

DISCUSSION

One of the goals in using a defined system for studying gene expression is to identify factors that are required for the regulation of the synthesis of specific proteins. The present experiments indicate that in a partially defined system the efficiency of synthesis of several gene products is quite different. For example, although L10 and L12 are synthesized quite well in the partially defined system compared to the crude system, EF-Tu (both *tufA* and *tufB*), EF-G, and the α and $\beta\beta'$ subunits of RNA polymerase are not.

Synthesis of all of the products is stimulated when the partially defined system is supplemented with a 1 M DEAE salt eluate prepared from the high-speed supernatant, although it is important to note that, except for L10 and L12, the presence of this extract does not completely restore the activity to that of the crude system. As shown previously in studies on the DNA-directed synthesis of β-galactosidase, the 1 M DEAE salt eluate contains RNA polymerase and another factor (L factor) that is essential for β-galactosidase formation (4). The function of L factor is not yet known, but as shown here, part of the stimulatory effect of the 1 M DEAE salt eluate on $\beta\beta'$ synthesis is due to L factor. In contrast, L factor does not significantly increase the synthesis of the other products that were examined, with the possible exception of a small effect on EF-G synthesis. It is apparent, therefore, that the 1 M DEAE salt eluate contains a factor(s), in addition to L factor, that stimulates gene expression in this *in vitro* system. The active material could be a transcription or translation factor or might function by protecting nucleic acid templates against degradation.

We have also confirmed in the partially defined system the results of Fukuda *et al.* (38), who showed that high levels of RNA polymerase specifically inhibited the synthesis of the $\beta\beta'$ subunits. The α subunit is not affected by either L factor or RNA polymerase in our experiments.

The partially defined system used in the present experiments contains 30 purified factors (RNA polymerase, transformylase, 20 aminoacyl-tRNA synthetases, IF-1, IF-2, IF-3, EF-Tu, EF-Ts, EF-G, RF-2, and ribosome release factor) as well as a small amount of an ascites extract. The ascites extract generally gave about a 2- to 3-fold stimulation of all the products, including L10 and L12. It does not appear to be supplying any of the known factors required for prokaryote protein synthesis, but may contain a factor that protects mRNA against degradation, as has been shown for β-galactosidase (16). It should be stressed that the partially defined *in vitro* system supplemented with the 1 M DEAE salt eluate does not support the synthesis of β-galactosidase (<2% of the crude system) directed by DNA from a transducing phage carrying the lactose operon. As reported previously (7), additional fractions (L_γ and L_Δ) were required in a partially defined system to obtain reasonable synthesis of β-galactosidase as compared to a crude system. In preliminary studies on EF-Tu synthesis with λ*rif*^{d}*18* and λ*fus*3 DNA as templates, L_γ and L_Δ gave little or no stimulation and could not replace the 1 M DEAE salt eluate. These results indicate that L_γ and L_Δ do not contain factors that are essential for the expression of all genes.

It is apparent that the optimum synthesis of EF-Tu, EF-G, and the α and ββ′ subunits of RNA polymerase requires other factors in addition to the basic 30 used in the partially defined system. However, the partially defined system does maintain the observed *in vivo* ratios of ribosomal proteins L12/L10 and EF-Tu (*tufA*)/EF-G, as well as the autogenous regulation of ββ′ synthesis by RNA polymerase. Therefore, the present system may be useful for elucidating new factors required for gene expression, as well as for studying the regulation of the synthesis of several gene products.

These studies were submitted as part of a dissertation by T.Z.-S. to the Department of Human Genetics and Development, Columbia University, in partial fulfillment of the requirements for the Doctor of Philosophy degree. We thank Dr. Stanley Stein for many helpful discussions and expert advice. We also thank Carlos Spears for supplying many of the purified factors used in this study.

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