DNA-DIRECTED IN VITRO SYNTHESIS OF ELONGATION FACTOR Tu

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SUMMARY: The in vitro synthesis of EF-Tu was studied using the transducing phage λrif^{d}18 DNA as template. The EF-Tu synthesized was identified by its immunological properties, gel analysis, and its ability to interact with GDP and EF-Ts. Two other unidentified polypeptides were also precipitated from the incubation mixture by EF-Tu antiserum. The synthesis of total EF-Tu immunoprecipitable material was depressed about one-half by guanosine-5'-diphosphate-3'-diphosphate which was shown to inhibit the transcription process.

The transducing phage λrif^{d}18 (1) has been found to contain the structural genes for ribosomal RNA (16S, 23S, 5S), several ribosomal proteins (L1, L10, L11, L12), $tRNA_2^{Glu}$, RNA polymerase subunits β and β' and EF-Tu (2-4). The synthesis of EF-Tu is of special significance. It is now clear that there are at least two EF-Tu genes on the Escherichia coli chromosome (2) and that the synthesis of EF-Tu is controlled in a unique way since this protein may represent > 5% of the soluble protein of E. coli (5). The availability of DNA-directed in vitro systems makes it possible to investigate the synthesis of specific proteins and their regulation in cell free systems. Jaskunas et al. (2) recently demonstrated the synthesis of EF-Tu in vitro using the transducing phage λrif^{d}18 as template. The present study using a reconstituted protein synthesizing system described previously (6,7) shows that the EF-Tu synthesized in vitro is biologically active and under the regulation of guanosine-5'-diphosphate-3'-diphosphate (ppGpp).

MATERIALS AND METHODS: Uniformly [^{14}C]labeled L-amino acid mixture (54 mci/mAtom carbon) was purchased from Amersham/Searle. ppGpp was provided by Dr. Alan Cook of Hoffmann-La Roche. [^{3}H]EF-Tu was prepared by growing E. coli B in the presence of [^{3}H]valine and [^{3}H]alanine and purifying EF-Tu to homogeneity as previously described (8). EF-Ts was purified from E. coli as described previously (9). E. coli H105, obtained from Dr. J.B. Kirschbaum, was used as the source of λrif^{d}18 DNA (7). The

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λdlac DNA was prepared from E. coli RV cells (10). The preparation of the ribosomal wash, washed ribosomes, and a supernatant extract which was further fractionated by DEAE chromatography into 0.25 M and 1.0 M DEAE salt eluates has been described elsewhere (6,10).

Preparation of EF-Tu antiserum. An EF-Tu-thyroglobulin conjugate was prepared by incubating 15 mg of purified EF-Tu and 55 mg of thyroglobulin at 37° in 0.01 M NaCl at pH 5.9 with 0.25 mg of water soluble carbodiimide (final concentration of protein: 15 mg/ml). At the end of 30 min incubation, the reaction mixture was mixed with one-tenth its volume of a concentrated phosphate buffered saline solution (KH_2PO_4, 0.015 M; Na_2HPO_4, 0.065 M; NaCl, 1.4 M). Previous experiments had verified that under these conditions virtually all of the EF-Tu was conjugated to the thyroglobulin. Three hundred µl of this solution containing about 750 µg of EF-Tu was mixed with 1.5 ml of complete Freund's adjuvant and injected weekly into a goat for 8 weeks. EF-Tu antibodies were observed in the goat's serum after four weeks of injections and remained at about the same level for the next four weeks. Five hundred ml of blood were obtained from the goat after the 9th week and the serum used as the source of EF-Tu antibodies.

System for protein synthesis. The complete system (70 µl) for EF-Tu synthesis was similar to that described previously (7). The reaction mixture was incubated with shaking at 37° for 90 min. The reaction was stopped by the addition of 0.7 µg of pancreatic ribonuclease and the incubation continued for another 10 min at 37°. The reaction mixture was then rapidly chilled and centrifuged at 7,000 x g to remove insoluble material[1]. Twenty microliters of the supernatant was precipitated with CCl_3COOH (20 min at 90°C) and the radioactivity in the precipitate determined (total incorporation). The remainder of the incubation mixture (50 µl) was subjected to immunoprecipitation with EF-Tu antiserum (see below). The synthesis of EF-Tu could be separated into transcription and translation stages (10) by first synthesizing EF-Tu mRNA in the absence of amino acids and tRNA, followed by the addition of 2 µg of rifampicin (to inhibit initiation of RNA synthesis) and the [^{14}C]amino acids and tRNA to initiate translation. The transcription phase (first incubation) was carried out at 37° for 25 min and translation at 37° for an additional 60 min.

Assay for EF-Tu by immunoprecipitation. To 50 µl of the ribonuclease-treated reaction mixture were added 2 µg of [^{3}H]EF-Tu (300 cpm/µg) and 300 µl of EF-Tu antiserum. This solution was made to 0.5 ml in buffer A (0.05 M Tris-HCl, pH 7.4, 0.5 M NaCl, 1% Triton X-100) (11) and incubated at 37° for 5 hr and then further incubated at 4° for an additional 15 min. After centrifugation, the immunoprecipitate was washed four times in 0.5 ml of buffer A and dissolved in 50 µl of 4 M urea containing 50% acetic acid. An aliquot, generally 20 µl, was assayed for radioactivity in a Beckman liquid scintillation counter and the remainder analyzed by gel electrophoresis. The [^{3}H]EF-Tu served as an internal standard for estimation of the recovery of the synthesized product after immunoprecipitation and subsequent washings. The recovery of [^{3}H]EF-Tu averaged about 30% and the results have been normalized to 100% recovery.

Analysis of synthesized EF-Tu by gel electrophoresis. Aliquots of the solubilized immunoprecipitates were lyophilized and then dissolved in 25 µl of 1% sodium dodecyl sulfate (SDS). SDS gel electrophoresis was carried out as described by Laemmli (12) using 15% polyacrylamide gels (7). The gels were sliced into two mm sections (Gilson Aliquogel Fractionator), extracted with 0.7 ml of 0.1% SDS for 1 hr at 80°, and 5 ml Instabray (Yorktown Research, New Jersey) was added and the radioactivity was determined. Molecular weights were estimated as described previously (7).

[1] It was found that although the centrifugation removed about 50% of the CCl_3COOH precipitable counts this step was necessary to remove any insoluble radioactive material that would interfere with the immunoassay.

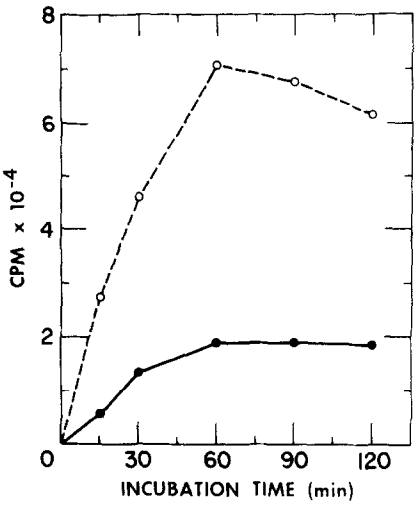

Figure 1: Time course of the synthesis of total proteins and EF-Tu immunoprecipitable material. Details are described in the text.
--- total protein
—— EF-Tu immunoprecipitate

EF-Tu activity. [^{14}C]EF-Tu was partially purified from a typical *in vitro* reaction mixture by gel filtration. In order to identify the small quantity of [^{14}C]labeled EF-Tu synthesized in the reaction, 5 mg of purified EF-Tu·GDP were added to the reaction mixture at the end of the incubation and the mixture was chromatographed on an Ultrogel Ac44 column (LKB) (1.6 x 55 cm). The column was eluted with a buffer containing 0.05 M Tris·Cl (pH 7.8), 0.01 M $MgCl_2$, 0.01 β-mercaptoethanol, 10% glycerol and 1 x 10^{-5} M GDP (buffer B). Fractions (1.3 ml) were collected and assayed for radioactivity and EF-Tu activity. The activity of EF-Tu was measured by its ability to bind GDP (13).

To examine whether the *in vitro* synthesized EF-Tu can react with EF-Ts to form an EF-Tu·EF-Ts complex, the fractions from the preceding chromatography which contained EF-Tu activity were pooled, concentrated by ultrafiltration using a Minicon B15 concentrator (Amicon Corp.) and incubated with 4 mg of EF-Ts. This mixture was rechromatographed on the Ultrogel column and eluted with buffer B except that GDP was omitted. To show that the *in vitro*-synthesized EF-Tu was also capable of reacting with GDP, the EF-Tu·EF-Ts containing fractions from this chromatography were reconcentrated and rechromatographed on the Ultrogel column equilibrated with buffer B containing 8 x 10^{-5} M GDP.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (14) with bovine serum albumin as standard.

RESULTS: Kinetics of synthesis of EF-Tu. Figure 1 shows the time course for the λrif^{d}18 DNA-directed incorporation of radioactivity into total protein and protein immunoprecipitated by antiserum against EF-Tu. Synthesis is nearly linear for up to 60 minutes and is complete by 90 minutes. About

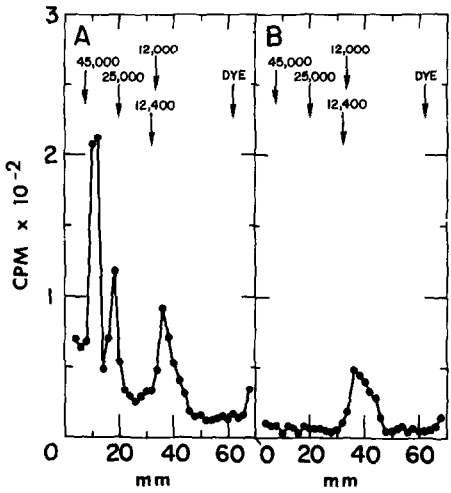

Figure 2: SDS gel electrophoresis of immunoprecipitated products synthesized from (A) λrif^{d}18 DNA and (B) λdlac DNA. An aliquot of the synthesis mixture was immunoprecipitated, solubilized and analyzed by electrophoresis in SDS. The gels were sliced and assayed for radioactivity. Details are described in the text. Marker proteins are ovalbumin (MW 45,000), chymotrypsinogen (MW 25,000), cytochrome C (MW 12,400) and ribosomal protein L12 (MW 12,000).

30% of the total protein synthesized was immunoprecipitable by EF-Tu antiserum and in the absence of DNA no radioactivity was immunoprecipitated (data not shown). When λdlac DNA was used as template in place of λrif^{d}18 DNA, a small amount of immunoprecipitated radioactivity was found but, as shown below, the product was not EF-Tu.

Characterization of the immunoprecipitates. The solubilized immunoprecipitates were analyzed by SDS gel electrophoresis. With λrif^{d}18 DNA as template, the synthesized immunoprecipitable material showed three radioactive peaks with molecular weights of 42,000, 29,000 and 11,000, respectively (Figs. 2A). The largest polypeptide (peak A) comigrated with authentic [^{3}H]EF-Tu. When λdlac DNA was used as a control template, the solubilized immunoprecipitate migrated as one radioactive peak (peak C) with a molecular weight of about 11,000 (Fig. 2B), suggesting

that the smallest polypeptide in the immunoprecipitate may be a gene product of the λ DNA.

The polypeptide of 29,000 daltons (peak B) could have resulted from proteolytic degradation of the in vitro -synthesized EF-Tu. In an attempt to determine this, 5 mM phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor, was added at the beginning of the incubation. It was found, however, that PMSF did not reduce the amount of polypeptide B synthesized relative to the amount of the polypeptide that comigrated with EF-Tu.

Interaction of in vitro-synthesized EF-Tu with EF-Ts and GDP. In order to purify the in vitro-synthesized [^{14}C] labeled EF-Tu sufficiently to permit a study of its functional properties, carrier EF-Tu was added and this mixture was subjected to gel filtration. Because the number of proteins synthesized in this in vitro system is small, this chromatographic procedure separated [^{14}C]EF-Tu from most other in vitro synthesized products. As Figure 3A shows, several [^{14}C]-containing peaks emerge from the column with one of the peaks (centered about fractions 55-56) comigrating with authentic EF-Tu. The specific activity of the [^{14}C]labeled EF-Tu was calculated to be 0.16 cpm/pmol.

The radioactive product which comigrated with authentic EF-Tu (Fig. 3A) behaved like EF-Tu in its reactions with EF-Ts and GDP. Figure 3B shows that in the presence of a stoichiometric amount of EF-Ts most of the [^{14}C]labeled EF-Tu can combine with EF-Ts to form an EF-Tu·EF-Ts complex, which now emerges in the peak centered around fraction 47. The small amount of radioactive material emerging in fractions 55-60 may be unreactive EF-Tu, or impurities not removed by the first chromatography. The specific activity of the [^{14}C]labeled EF-Tu in the EF-Tu·EF-Ts complex is 0.12 cpm/pmol, similar to the initial specific activity.

It has been shown that when EF-Tu·EF-Ts is chromatographed in the

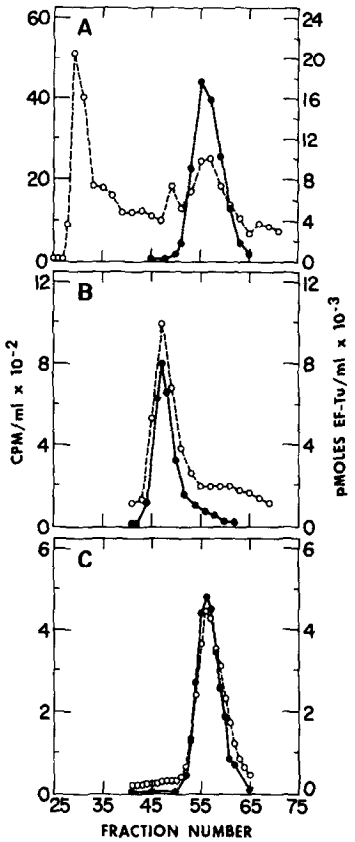

Figure 3: Panel A: Gel filtration of a reaction mixture labeled with [^{14}C] amino acids. Details are described in Methods. 0—0, [^{14}C]labeled protein; ●—● EF-Tu activity.
Panel B: Gel filtration of the EF-Tu·EF-Ts complex. 0—0, [^{14}C]labeled protein; ●—● EF-Tu activity.
Panel C: Gel filtration of the EF-Tu·EF-Ts complex recovered from Panel B. Column equilibrated with 8 x 10^{-5} M GDP. 0—0, [^{14}C] labeled protein; ●—● EF-Tu activity.

presence of GDP, the complex dissociates forming EF-Tu·GDP and EF-Ts (8,13). Similarly, the putative [^{14}C] EF-Tu·EF-Ts complex isolated from the preceding experiment dissociates in the presence of GDP and emerges in the elution volume where an EF-Tu·GDP complex would appear (Fig. 3C). The results in Figure 3 demonstrate that EF-Ts and GDP can interact with the EF-Tu synthesized in vitro.

Table I

Effect of ppGpp on the In Vitro Synthesis of EF-Tu

Time	$\frac{\text{cpm in immunoprecipitate}}{\text{cpm in total protein}}$ X 100	
	- ppGpp	+ppGpp
60 min	26.9	15.1
90 min	31.4	13.2
180 min	36.0	18.8

EF-Tu was synthesized in vitro in the presence or absence of 200 μM ppGpp and was then isolated by immunoprecipitation as described in Methods. The results are expressed as the percent of radioactivity in the immunoprecipitate compared to total protein synthesis. It was found that 72,190 cpm and 81,980 cpm were incorporated into protein in the presence and absence of ppGpp, respectively.

Effect of ppGpp on EF-Tu synthesis. It is known that in vivo, ribosomal RNA and ribosomal proteins are synthesized coordinately (15,16). Since it has recently been shown in vitro that the synthesis of ribosomal RNA from E. coli total DNA (17,18) and that of ribosomal protein L12 from $\lambda rif^{d}18$ DNA (7) are inhibited by ppGpp, the effect of ppGpp on the in vitro synthesis of EF-Tu was determined. Table I shows the effect of 200 μM ppGpp on EF-Tu synthesis (total immunoprecipitable cpm) at three different times of incubation. Although the nucleotide depressed total protein synthesis by less than 15%, it decreased total EF-Tu synthesis by more than 50%. It is to be noted that the concentration of ppGpp used in these studies are within the physiological range since during amino acid starvation of a stringent strain of E. coli the intracellular concentration of ppGpp increases to about 1-2 mM (19).

The effect of ppGpp on the synthesis of the three polypeptides precipitated by EF-Tu antiserum was also determined by measuring the amount of radioactivity in each polypeptide peak after SDS gel electrophoresis. As shown

Table II

Effect of ppGpp on the Synthesis of the Three Polypeptides Precipitated by Antiserum to EF-Tu

Polypeptide (mol. wt.)	cpm	
	-ppGpp	+ppGpp
A (42000)	4500	2800
B (29000)	2070	1350
C (11000)	2830	3080

The DNA-dependent *in vitro* synthesis was carried out using λrif^{d}18 DNA as template in the presence of [^{14}C]amino acids, and the [^{14}C]EF-Tu synthesized was isolated by immunoprecipitation. The solubilized immunoprecipitates were analyzed by electrophoresis in 15% polyacrylamide gels containing 1% SDS. Details are described in Methods. The amount of each polypeptide synthesized was determined by quantitating the radioactivity under each peak.

in Table II, the presence of 200 μM ppGpp in the incubation significantly reduced peaks A and B, whereas peak C was virtually unchanged.

In an attempt to elucidate the site of action of ppGpp, transcription and translation were uncoupled by the use of rifampicin (see Methods). ppGpp (200 μM) was added either at the beginning or end of transcription and the relative amounts of EF-Tu synthesized were determined by immunoprecipitation. Table III summarizes the results of such a study. Inhibition of EF-Tu synthesis was observed only when ppGpp was added at the beginning of transcription, indicating that the nucleotide primarily inhibits the transcription process.

DISCUSSION: A previous report has described the DNA-dependent *in vitro* synthesis of EF-Tu using λrif^{d}18 DNA as template (2). The synthesized EF-Tu was identified by its immunological properties but no evidence of homogeneity, biological activity or regulation was presented. In the present study, the synthesized EF-Tu was shown to be biologically active. In addition,

Table III

Effect of ppGpp on the Transcription and Translation of EF-Tu

Transcription	Translation	total protein	$\frac{\text{cpm in immunoprecipitate}}{\text{cpm in total protein}}$ X 100
		cpm	
1. ---	---	16,170	10.0
2. ppGpp	---	14,500	5.0
3. ---	ppGpp	14,020	10.0

EF-Tu was synthesized *in vitro* in an uncoupled system (see Methods), then quantitated by immunoprecipitation as described in Methods. The results are expressed as the percent of radioactivity in the immunoprecipitate as compared to total protein synthesis.

two other immunoprecipitable polypeptides were found to be synthesized (Fig. 2) with molecular weights corresponding to 29,000 and 11,000. It should be noted that the protein of 11,000 daltons was also synthesized when λdlac DNA (which does not contain the Tu gene) was used as template. This finding and the lack of effect of ppGpp on the synthesis of the smallest polypeptide (Table II) suggest that this polypeptide may be a nonrelated λ phage protein which crossreacts with EF-Tu antiserum. The relationship between the polypeptides of molecular weights of 42,000 (EF-Tu) and 29,000 is not clear at present. It is possible that premature release of EF-Tu mRNA during transcription or EF-Tu fragments during translation could give rise to polypeptides smaller than 42,000 daltons. In this case, analysis of the NH_2 and COOH termini of the fragment should clarify this point.

The *in vivo* synthesis of both elongation factors Tu and G has been shown to be depressed in a stringent, but not in a relaxed organism during amino acid starvation (20) and it is known that ppGpp accumulates under

this condition. In addition, it has been reported previously that the *in vitro* synthesis of ribosomal RNA and ribosomal protein L12 are inhibited by ppGpp (7,17,18). The present communication provides direct evidence that the *in vitro* synthesis of EF-Tu, a non-ribosomal protein required for translation is also depressed by ppGpp and that the nucleotide acts at the transcription stage (Table III). Preliminary results (unpublished data) suggest that ppGpp also inhibits the synthesis of ribosomal protein L12 at the transcription level of protein synthesis. During the time that this manuscript was in preparation, Lindahl *et al.* also observed a similar inhibitory effect of ppGpp on the *in vitro* synthesis of EF-Tu, EF-G and several ribosomal proteins, using either $\lambda rif^{d}18$ DNA or $\lambda fus3$ DNA as template (21). Thus, it appears that the synthesis of a large number of macromolecules may be affected by ppGpp in a coordinate manner.

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