JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 240–250
0021-9193/93/010240-11$02.00/0

Vol. 175, No. 1

A Single Amino Acid Substitution in Elongation Factor Tu Disrupts Interaction between the Ternary Complex and the Ribosome

IOANNIS TUBULEKAS AND DIARMAID HUGHES*

Department of Molecular Biology, Box 590, The Biomedical Center, Uppsala University, S-751 24 Uppsala, Sweden

Received 30 March 1992/Accepted 2 November 1992

Elongation factor Tu (EF-Tu) · GTP has the primary function of promoting the efficient and correct interaction of aminoacyl-tRNA with the ribosome. Very little is known about the elements in EF-Tu involved in this interaction. We describe a mutant form of EF-Tu, isolated in *Salmonella typhimurium*, that causes a severe defect in the interaction of the ternary complex with the ribosome. The mutation causes the substitution of Val for Gly-280 in domain II of EF-Tu. The in vivo growth and translation phenotypes of strains harboring this mutation are indistinguishable from those of strains in which the same *tuf* gene is insertionally inactivated. Viable cells are not obtained when the other *tuf* gene is inactivated, showing that the mutant EF-Tu alone cannot support cell growth. We have confirmed, by partial protein sequencing, that the mutant EF-Tu is present in the cells. In vitro analysis of the natural mixture of wild-type and mutant EF-Tu allows us to identify the major defect of this mutant. Our data shows that the EF-Tu is homogeneous and competent with respect to guanine nucleotide binding and exchange, stimulation of nucleotide exchange by EF-Ts, and ternary complex formation with aminoacyl-tRNA. However various measures of translational efficiency show a significant reduction, which is associated with a defective interaction between the ribosome and the mutant EF-Tu · GTP · aminoacyl-tRNA complex. In addition, the antibiotic kirromycin, which blocks translation by binding EF-Tu on the ribosome, fails to do so with this mutant EF-Tu, although it does form a complex with EF-Tu. Our results suggest that this region of domain II in EF-Tu has an important function and influences the binding of the ternary complex to the codon-programmed ribosome during protein synthesis. Models involving either a direct or an indirect effect of the mutation are discussed.

The prokaryotic translation factor elongation factor Tu (EF-Tu) mediates the productive interaction of aminoacyl-tRNA (aa-tRNA) with the ribosome. The ternary complex EF-Tu · GTP · aa-tRNA interacts with the ribosome such that the anticodon region of aa-tRNA is properly positioned in the ribosomal A site on the 30S subunit, while the aa region remains bound to EF-Tu outside the A site (43). EF-Tu on the ribosome protects bases in the universally conserved α-sarcin loop of 23S rRNA on the 50S ribosomal subunit, suggesting that this may form at least part of its ribosomal binding site (20, 44). This conclusion is supported by data showing that a base substitution mutation in the α-sarcin loop can affect the binding of ternary complex to the ribosome (62). If the tRNA codon and the mRNA anticodon in the 30S A site match, there is a high probability that the GTP on EF-Tu will be hydrolyzed. Whether the probability of GTP hydrolysis depends mainly on the length of time that the ternary complex spends on the ribosome or on signals transmitted to EF-Tu after correct codon-anticodon interaction is an open question. However, after GTP hydrolysis, EF-Tu · GDP leaves the ribosome and is recycled, via interaction with the nucleotide exchange factor EF-Ts, to EF-Tu · GTP and is again capable of forming a ternary complex with aa-tRNA and participating in translation (30, 41). According to the data and model of Moazed and Noller (43), it is only after EF-Tu · GDP has left the ribosome that aa-tRNA is able to occupy the A site on the 50S ribosomal subunit. Moazed and Noller (43) have proposed that this can provide a physical basis for how kinetic proofreading occurs (21, 46) by showing that aa-tRNA selection in the A site is a multistep process. There is experimental support from in vitro translation for the kinetic proofreading of aa-tRNA selection (53, 63). Thus, according to the model of Moazed and Noller (43), an important function of EF-Tu is to facilitate the possibility of codon-anticodon interactions while at the same time preventing peptide bond formation until after the GTP on EF-Tu has been hydrolyzed and EF-Tu has left the ribosome.

One of the aims of current studies of EF-Tu is to understand at the molecular level the relationships between its structure and functions. The crystal structure of *Escherichia coli* EF-Tu in complex with GDP reveals three distinct domains with the guanine nucleotide bound to the N-terminal domain (13, 33, 35, 47). We have studied *Salmonella typhimurium* EF-Tu, which is identical in sequence to *E. coli* EF-Tu with the exception of a Leu→Ile change at residue 189 (65), a residue which is not conserved (14, 38). Thus, data on structure-function relationships should apply equally to EF-Tus from both species. *S. typhimurium*, besides being closely related to *E. coli*, has the advantage that each of its two genes for EF-Tu is individually dispensable for growth (23), facilitating mutant analysis. For reasons that are currently unclear, the *E. coli tufA* gene cannot be inactivated (69).

The molecular interactions of EF-Tu with the guanine nucleotides have been studied in most detail, but some progress has also been made in defining the elements involved in interactions with the nucleotide exchange factor EF-Ts and with aa-tRNA. Conserved amino acid loops in the

* Corresponding author.

TABLE 1. *S. typhimurium* strains used in this study

Strain	Genotype
TH488	*trpE91 hisG3720 tufA8*
TH506	*trpE91 hisG3720 tufA8 tufB414*
JT506	*trpE91 hisG3720 tufA8 tufB414 argH1823*::Tn*10*
TH195	*trpE91 tufA8*
JT620	*trpE91 tufA8 proB1657*::Tn*10*
JT608	*trpE91 tufA8 tufB414*
JT627	*trpE91 tufA8 tufB414 proB1657*::Tn*10*
JT640	*trpE91 tufB414 argH1823*::Tn*10*
JT642	*trpE91 tufB414*
JT631	*trpE91 tufB414 proB1657*::Tn*10*
JT863	*trpE91 proBΔ1 tufB441*::MudJ(*lacZ950*::Tn*10*)
JT848	*trpE91 proBΔ1 tufA8 tufB414*::MudJ(*lacZ950*::Tn*10*)

immediate neighborhood of the guanine nucleotide (10, 15) were shown by mutagenesis to be involved in determining the relative affinity of GDP and GTP for the molecule, its hydrolysis, and the nature of the nucleotide which is bound (2, 25, 26). EF-Ts binding is influenced by a mutation close to the guanine nucleotide in domain I (27), but the primary binding site for EF-Ts may be on domain III (8, 49, 56). The binding of tRNA to EF-Tu, studied by cross-linking (16, 40, 68) and chemical protection (5, 28), indicates that residues in domains I and II of EF-Tu interact with aa-tRNA and that the aa moiety can be crosslinked to His-66 in domain I. In EF-1α (the eukaryotic homolog of EF-Tu), which lacks the equivalent of His-66 in domain I, the amino acid has been cross-linked to the extreme edge of domain II (31). Evidence from biochemical (11) and genetic (6, 34, 57) assays indicates that EF-Tu interacts with the amino acid, the acceptor stem, and the TψC helix of aa-tRNA.

Once formed, the EF-Tu · GTP · aa-tRNA complex must interact with the ribosome. That EF-Tu is directly involved in interacting with the ribosome is suggested by the binding of EF-Tu · GDP to the ribosome in the presence of kirromycin (70). EF-Tu, bound to the ribosome in the form EF-Tu · GDP · aa-tRNA · kirromycin, protects bases in the α-sarcin loop on the large ribosomal subunit against chemical attack (44); this suggests that this region of the ribosome may be at least part of its binding site. In addition, there is genetic evidence that EF-Tu's interaction with the ribosome is influenced strongly by protein S12 on the small ribosomal subunit (7, 60, 62, 64). There is currently very little information on the residues in EF-Tu which are important for the interaction with the ribosome. On the basis of the area of homology between EF-Tu and EF-G, with which it shares an overlapping binding site on the ribosome (37, 44), one would predict that the ribosome interaction should involve domain I of EF-Tu, and there is evidence supporting this prediction (48, 50). In this article, we report experiments showing that an amino acid substitution at residue 280, a conserved residue in a loop in domain II, disrupts ternary complex interaction with the ribosome. The effects of the alteration must be relatively specific to the ternary complex-ribosome interaction because we do not detect changes in the other molecular interactions of EF-Tu which we have tested.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used in this study are listed in Table 1. All of the strains are derivatives of the wild-type strain *S. typhimurium* LT2. Transductions were made with P22 HT105/1 *int*-201 (55). The selection of the mutation studied here, *tufB414*, has been described previously (1). Briefly, beginning with strain TH488 (which carries a kirromycin-resistant *tufA* allele and a wild-type kirromycin-sensitive *tufB* gene and is thus sensitive to the antibiotic), mutants resistant to kirromycin were selected. Among these mutant strains were a number in which the *tufB* gene was no longer able to support cell growth in the absence of an active *tufA* gene. DNA sequencing of the *tufB* regions of these mutants identified a number of amino acid substitutions, including the change at position 280 studied here.

The *tufB414* mutation was introduced into different strains by cotransduction with the linked marker *argH1823*::Tn*10*, selecting for tetracycline resistance. This marker could be subsequently removed by a transduction selecting for arginine prototrophy. The cotransduction of *tufB414* into the *tufA8* background was confirmed by the appearance of a kirromycin-resistant phenotype. The cotransduction of *tufB414* into the *tufA*$^+$ background was confirmed both by our ability to select kirromycin-resistant colonies at a high frequency and by the appearance of a kirromycin-resistant phenotype after the subsequent introduction of *tufA8* by transduction. We confirmed that the *tufB414* mutation is inviable by our inability to introduce *tufA*::MudJ alleles (23) by cotransduction with the linked marker *zhb-736*::Tn*10*. The marker *proB1657*::Tn*10* was introduced by selection for tetracycline resistance and used for the subsequent selection and maintenance of F′ factors carrying the *E. coli lac* operon and *proAB* region by selection for proline prototrophy.

Media. Media and antibiotic concentrations have been described previously (64). Kirromycin (mocimycin) was a gift from Gist-Brocades NV, Delft, The Netherlands.

Determinations of growth rate and suppression. Growth rates were measured in liquid M9 salts supplemented with glucose (0.4%, wt/vol) and tryptophan (10 mM) by inoculating 100 μl of an overnight culture into 20 ml of fresh medium in a 300-ml flask, aerating the mixture by vigorous shaking, and measuring the increase in optical density of the culture as a function of time. Suppression of the *trpE91* and *hisG3720* mutations was measured as the time taken to form colonies on M9 minimal medium agar (24). Suppression of nonsense mutations in the *lacI* part of a *lacIZ* fusion was determined by measuring β-galactosidase activity and normalizing it to the activity from a nonmutated fusion in the same strain (22). The translation elongation rate in vivo was estimated by measuring the step time for β-galactosidase synthesis after induction of a wild-type *lac* operon as described by Andersson et al. (4). β-Galactosidase activity was measured as described by Miller (42).

In vitro translation assays. EF-Tu was purified according to the method of Leberman et al. (36) with the following modifications. Fractions containing EF-Tu from the DEAE-Sepharose and Ultrogel AcA44 (AcA) (Sepracor, France) columns were not ammonium sulfate precipitated but were instead pooled and loaded directly onto a small (10-ml) column of Q Sepharose Fast Flow (Pharmacia) previously washed with buffer A (DEAE) or phosphate buffer (AcA) as appropriate. Samples from the DEAE column were washed from the Q Sepharose by the application of buffer A containing 0.4 M NaCl and loaded directly onto the AcA column. Samples from the AcA column were washed from the Q Sepharose by the application of a phosphate gradient from 50 to 250 mM. The final EF-Tu-containing fractions from the AcA column (about 20 ml) were concentrated 10-fold with Aquacid and then dialyzed against polymix buffer containing GDP and stored at −80°C. These additional steps with Q Sepharose have the effect of rapidly concen-

trating the EF-Tu samples and simultaneously giving a significantly improved purification over that achieved by the traditional method. All other factors, components, and chemicals for in vitro translation were purified and prepared by the method of Ehrenberg et al. (18).

Translation assays in vitro were done at 37°C with 100-μl volumes in polymix buffer (final ion concentrations as follows: 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 95 mM KCl, 5 mM NH_4Cl, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate [pH 7.3], and 1 mM dithioerythritol). To assay translation elongation, two separate mixes of components were prepared on ice: an initiation mix (70S mix) and a factor mix. Each mix was prepared with appropriate volumes of water, 10× polymix buffer, 20× potassium phosphate, and 50× dithioerythritol to balance the final polymix buffer. The 70S mix contained (per 50 μl) 10 pmol of active ribosomes, 20 μg of poly(U), and [^{3}H]NAc (*N*-acetyl-Phe-tRNA^{Phe}) in a 30% excess over the total number of 70S ribosomes. The factor mix contains (per 50 μl) 10 μl of A/P (10 mM ATP and 100 mM phosphoenolpyruvate in water at pH 7.0), pyruvate kinase (5 μg), myokinase (0.3 μg), [^{14}C]Phe (30 nmol), Phe synthetase (100 U), tRNA^{Phe} (250 pmol), EF-Tu (600 pmol), EF-G (250 pmol), and EF-Ts (150 pmol). All translation experiments were carried out by the methods and principles detailed by Ehrenberg et al. (18) and Bohman et al. (9), with the exception of the variations noted below. After the EF-Tu · GDP concentration was measured by a nucleotide exchange assay (18, 19), different preparations were routinely compared by gel electrophoresis and quantified with an LKB-Pharmacia laser densitometer. Translation assays as a function of kirromycin concentration were carried out as described by Hughes (23). The ability of kirromycin to bind EF-Tu on ribosomes was assayed by measuring the translation rate at fixed amounts of EF-Tu (30 pmol) and kirromycin (100 pmol) and with a titration of active ribosomes from 30 down to 1 pmol (from approximately 3-fold over to 10-fold under the concentration of kirromycin-sensitive EF-Tu). The translation incubation times were from 15 to 120 s for EF-TuA8 B$^+$ and from 20 to 25 s for EF-TuA8 B414 to obtain precipitable poly(Phe) chains of 20 to 25 amino acids. Other components were present in nonlimiting amounts. Measurements of the k_{cat}/K_m of the interaction of the ternary complex with the ribosome were made as described by Tubulekas et al. (64). Briefly, we measured the rate of translation of poly(U) as a function of the amount of EF-Tu (titrated from 20 to 600 pmol) in a 100-μl translation cocktail containing 10 pmol of active ribosomes and an excess of all other components to achieve a maximal elongation rate. Reaction times were from 4 to 20 s to obtain precipitable poly(Phe) chains of 15 to 24 amino acids. The assay of translation rate as a function of aa-tRNA^{Phe} concentration was done as described by Tapio et al. (59). Briefly, the translation rate as a function of the amount of Phe-tRNA^{Phe} (titrated from 100 to 2,000 pmol) was measured at small and rate-limiting amounts of EF-Tu (15 pmol) and ribosomes (50 pmol, of which about 25% was active in translation). The reaction times were varied from 10 to 20 s to obtain precipitable poly(Phe) chains of about 20 amino acids. Nucleotide exchange on EF-Tu by EF-Ts was stimulated as described by Tapio et al. (59).

EF-Tu · GTP · aa-tRNA^{Phe} complex formation. The ability of EF-Tu preparations to form a ternary complex with aa-tRNA was assayed by a nondenaturing polyacrylamide gel electrophoresis (PAGE) system. The details of this method were developed by N. Bilgin, T. Gluick, and M. Ehrenberg, Department of Molecular Biology, Uppsala University (7a). The electrophoresis buffer consisted of 50 mM Tris-HCl (pH 6.5), 10 mM Mg acetate, 65 mM NH_4 acetate, 1 mM disodium EDTA, 10 μM GTP, and 1 mM dithioerythritol. The vertical slab gels (5 mm thick by 20 cm long) were made with electrophoresis buffer containing 5% acrylamide-bisacrylamide (19:1), 1 mg of ammonium persulfate per ml, and 8.3 mM TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine). The gels were prerun for 90 min at 40 mA and 100 V and after sample loading were run for a further 3 h at 60 mA and 150 V (with fresh buffer and a further buffer change during the run). Electrophoresis was performed in a cold room (4°C) with fan cooling and buffer circulation. Each sample was prepared as a 50-μl translation factor mix (18) containing 200 pmol of EF-Tu, from 0 to 300 pmol of tRNA^{Phe}, and no EF-Ts or EF-G. This mix was incubated at 37°C for 10 min (during which the tRNA was fully charged and the ternary complex formed) and cooled on ice, and then 20 μl was immediately applied to the gel after the addition of 0.1 volume of 50% glycerol in water with a dash of bromophenol blue as a marker. The remainder of each sample was used to measure tRNA charging levels. The gels were fixed and stained for 20 min in 40% methanol–10% acetic acid–0.1% Coomassie brilliant blue. The gels were destained in 12% 2-propanol–5% acetic acid and air dried in a frame between two cellophane sheets. For relative quantification, the gels were scanned with an LKB-Pharmacia laser densitometer.

Protein sequencing. Cyanogen bromide (CNBr) fragments of EF-Tu (3,000 pmol) were electrophoresed and transferred by blotting onto a polyvinylidine difluoride membrane as described by Matsudaira (39). The fragment containing position 280 (29) was extracted from the membrane and directly loaded into the sequencing machine. Automated amino acid sequencing was performed on an Applied Biosystems 477A Protein Sequencing System equipped with an on-line 120A phenylthiohydantoin analyzer.

RESULTS

We have isolated and sequenced a series of spontaneous mutations in *S. typhimurium tufB* which cause single amino acid substitutions individually lethal to some essential function of EF-Tu (1). A mutation was defined as lethal if no viable cells with an insertionally inactivated *tufA* in the presence of the *tufB* mutation could be isolated. Neither *tufA* nor *tufB* is individually essential for viability in *S. typhimurium*, and either one can be inactivated by Mud insertions (23). In this study, we investigated the functional basis of the lethality of one of these mutations, Gly-280→Val, which is a mutation at a conserved position in a loop in domain II of EF-Tu (Fig. 1).

Phenotypes of *tufB414* in vivo. The mutation *tufB414* was initially isolated on the basis that it conferred a kirromycin-resistant phenotype on TH488 (a strain which already harbored a kirromycin-resistant and error-prone allele of *tufA* and a wild-type copy of *tufB* and is thus sensitive to the antibiotic). It was noted that *tufA8*-mediated suppression of both a nonsense mutation (*hisG3720*) and a frameshift mutation (*trpE91*) present in the strain was reduced, as judged by colony growth in the absence of the appropriate amino acid. An obvious possibility was that suppression was reduced because of an increased efficiency of termination by release factors competing against a reduced amount of active EF-Tu. To quantify these effects and extend our analysis, we constructed strains carrying *lac* operon constructs which facilitate the measurement of nonsense read-through and

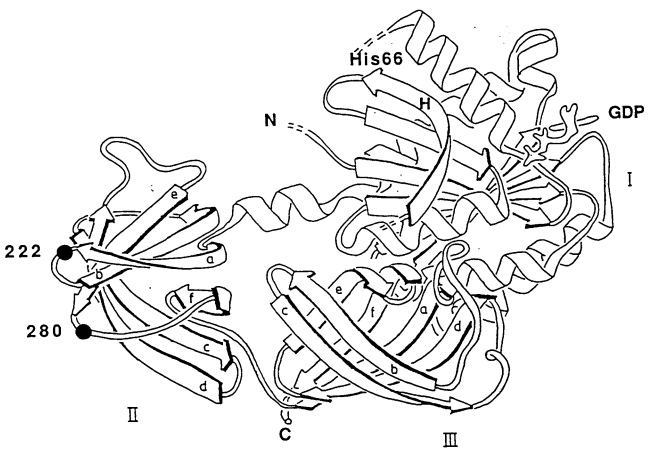

FIG. 1. Ribbon diagram of the X-ray crystallographic structure of a mildly trypsinized form of EF-Tu · GTP lacking amino acids 45 to 58, based on the model of Clark et al. (13). The domains of EF-Tu referred to in the text are labelled I, II, and III. The approximate positions of the amino acid substitution mutations referred to in the text (Gly-280→Val and Gly-222→Asp [Bo]), the site of cross-linking of the amino acid on aa-tRNA (His-66), and the site of the guanine nucleotide (GDP) are indicated.

translation elongation rate (see Materials and Methods). In addition, we measured the growth rates of these strains. As a useful comparison, we measured each of these parameters for strains carrying a MudJ-inactivated *tufB* gene. The results of these measurements were that all three parameters (growth rate, step time for β-galactosidase synthesis [elongation rate], and nonsense read-through) were reduced significantly and to equivalent degrees by the *tufB* mutation in each set of strains (Table 2). Thus, the *tufB414* mutation, in both the *tufA*$^+$ and *tufA8* backgrounds, has an in vivo phenotype which is similar to that of an insertionally inactivated *tufB* gene.

The mutant species EF-TuB Val-280 is present. We confirmed that the mutant EF-Tu was present in vivo by purifying EF-Tu from a *tufA*$^+$ *tufB414* strain and sequencing the relevant part of the protein. EF-Tu was cleaved with cyanogen bromide and blotted onto polyvinylidine difluoride, and the relevant fragment was excised and sequenced as described in Materials and Methods. The amino acid sequence was as expected for this part of the protein (29) and showed the presence of both Gly and Val at position 280 (Fig. 2). This confirms that both EF-Tu species were present in the cells and in the preparations we used for in vitro translation analysis. The ratio of Gly to Val at position 280 in our protein preparations, as estimated from protein sequencing, was approximately 10:6. This estimate is derived from the increase in the area of each of the amino acid peaks compared with the area of the same peak in the previous sequencing cycle (thus, the peak sizes in Fig. 2 are not representations of the amount of each amino acid at that position in the protein). We performed control experiments to show that our sequencing result is from a pure fragment and is not contaminated by other peptides from EF-Tu. The sequencing reaction gives results which are more qualitative than quantitative, and our primary purpose in this experiment was to confirm the existence of both types in our preparation. However, our estimate that approximately one-third of the EF-Tu is the mutant EF-TuB species is in agreement with a variety of data showing that the amount of *tufB* product in *E. coli* and *S. typhimurium* is normally close to one-third of the total EF-Tu (3, 23, 51, 67).

TABLE 2. Growth rate, translation elongation rate, and nonsense read-through phenotypes of *tufB414*

Strain	Genotype	Suppression (10^4) at UGA codon[a]: 189	220	Elongation (amino acids/s)	Growth rate (doublings/h)
JT619	*tufA*$^+$ *tufB*$^+$	18.9	66.0	13.1	1.31
JT620	*tufA8 tufB*$^+$	76.7	139.8	13.1	1.18
JT631	*tufA*$^+$ *tufB414*	7.3	32.6	10.7	1.08
JT627	*tufA8 tufB414*	35.3	58.1	9.3	0.83
JT863	*tufA*$^+$ *tufB*::MudJ	6.4	30.0	10.5	1.20
JT848	*tufA8 tufB*::MudJ	40.0	80.0	10.2	0.88

[a] Nonsense read-through at position 189 or 220 in the *lacI* part of a *lacIZ* fusion on an F′ factor. Suppression is measured in arbitrary units, as defined by Miller (42).

In vitro characterization of the *tufB414*-encoded EF-TuB Val-280. The *tufB414* mutation cannot support cell growth in the absence of an active *tufA* gene, so we did all of our assays with the natural mixture of A and B EF-Tu species. This has the disadvantage that in each assay we measured a change in the magnitude of a signal against a background of active EF-TuA. Conversely, however, using the natural mixture of A$^+$ and B414 has the advantage of providing an internal control for the activities of our EF-Tu preparations.

EF-Tu concentration determination by nucleotide exchange. The concentration of EF-Tu · GDP preparations was measured by a nucleotide exchange assay (19) in which [^{3}H]GDP released from EF-Tu in the absence of EF-Ts is enzymatically converted into [^{3}H]GTP. The ratios of the nucleotides as a function of time are then measured after thin-layer chromatography and scintillation counting. The experiment is run in two halves, with and without a known amount of extra cold GDP. The EF-Tu concentration is determined as described in detail by Ehrenberg et al. (19). Concentrations determined by this method (for various preparations of wild-type and mutant EF-Tu) were tested for their protein content by sodium dodecyl sulfate (SDS)-PAGE and Coomassie blue staining followed by quantification by laser densitometry (see Materials and Methods). We used as a standard an EF-Tu preparation whose concentration was also independently measured by amino acid analysis. For each of the preparations of EF-Tu tested, there was a very good correlation between the two methods. Figure 3 shows the results of a typical experiment, in which the concentrations estimated from nucleotide exchange assays (Fig. 3A and B) were used to apply equal amounts of EF-Tu for SDS-PAGE (Fig. 3C), which was followed by densitometric quantification, which revealed no significant difference. These EF-Tu preparations are fully active in GDP binding. The slope of the lines in Fig. 3A and B is the spontaneous exchange-and-dissociation rate of GDP on EF-Tu (K_d). The K_d values of each of these EF-Tu preparations are identical and are very close to the published wild-type value of 0.011 s^{-1} (52). Furthermore, the slopes for each EF-Tu preparation are monophasic, showing that there is no heterogeneity with respect to the exchange rate of GDP. We conclude that EF-Tu Val-280 is fully competent in GDP binding and has a normal spontaneous dissociation rate.

Stimulation of nucleotide exchange on EF-Tu by EF-Ts. The stimulation of the spontaneous nucleotide exchange rate on

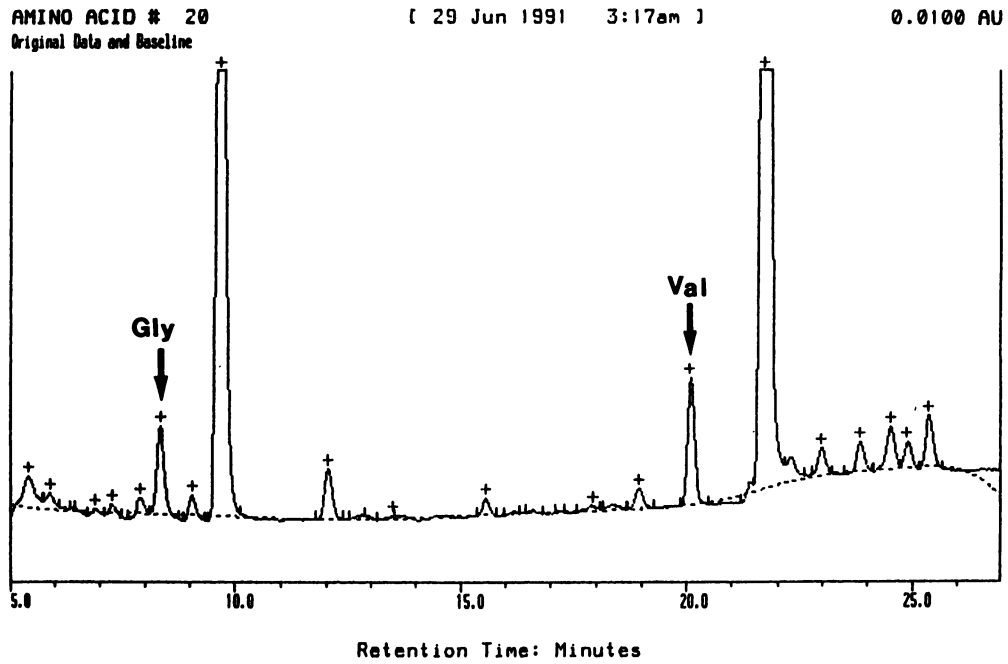

FIG. 2. Amino acid sequence analysis of a cyanogen bromide-generated fragment showing that at the expected position (20 min) both the wild-type Gly and the mutant Val are present. The two large peaks are internal standards.

EF-Tu by EF-Ts was measured in a nucleotide exchange assay as described by Tapio et al. (59). The concentration of EF-Ts was 1.2 pmol per 100-μl assay mixture, and the amount of EF-Tu was varied from 0 to 1,000 pmol. No significant differences between the stimulation of nucleotide exchange on wild-type EF-Tu and that on the wild-type/mutant (*tufA*$^+$ *tufB414*) species of EF-Tu were observed (Fig. 4). We conclude that EF-Ts can stimulate the exchange of guanine nucleotide on both mutant EF-Tu and wild-type EF-Tu.

EF-TuB Val-280 binds kirromycin. We used the method of Anborgh et al. (3) to test whether EF-TuB414 can bind kirromycin. Briefly, the natural mixture of EF-TuA8 (kirromycin resistant) and EF-TuB414 was incubated with kirromycin and chromatographed as described previously (3), and the fractions from the column were tested for the presence of EF-Tu and kirromycin. Under the experimental conditions used, EF-Tu · GDP in complex with kirromycin is retarded on the column and elutes as a separate peak from the kirromycin-resistant EF-Tu · GDP (3). From our EF-Tu mixture, we obtained two separate peaks which clearly indicated that EF-TuB414 binds kirromycin and that it elutes at the same position as wild-type kirromycin-sensitive EF-Tu (Fig. 5). In control experiments, wild-type EF-Tu elutes as a single peak at either of two positions, depending on the presence or absence of kirromycin. In contrast, pure EF-TuA8 elutes as a single peak at one position irrespective of whether kirromycin is present. From the peak areas, we

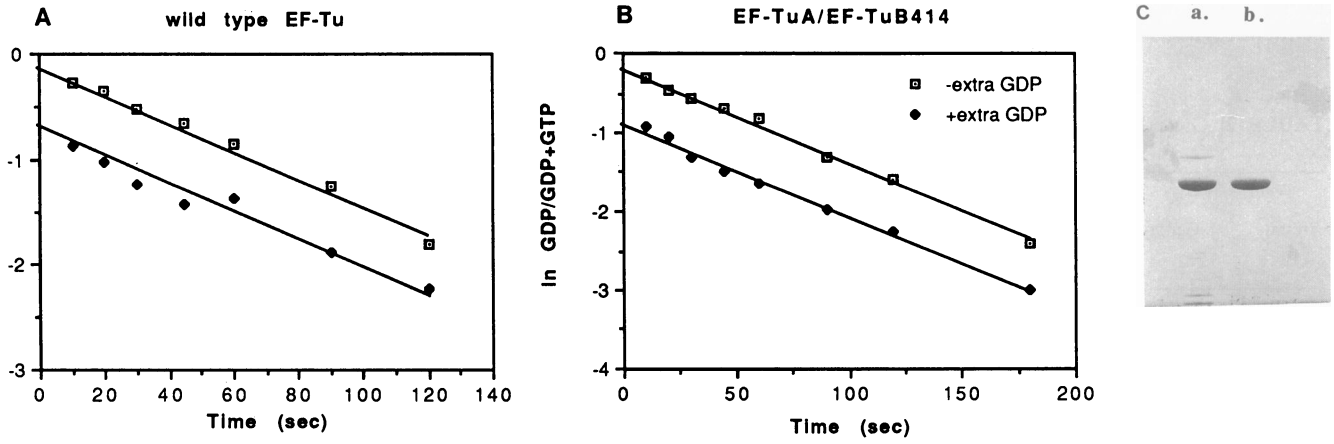

FIG. 3. Determination of EF-Tu concentration by nucleotide exchange assay for EF-Tu isolated from mutant (*tufA*$^+$ *tufB*$^+$) (A) and wild-type (*tufA*$^+$ *tufB414*) (B) strains. (C) Two hundred fifty picomoles of each of these EF-Tu preparations, as determined by the nucleotide exchange assay (18, 19), was electrophoresed. Lanes: a, wild-type strain (*tufA*$^+$ *tufB*$^+$); by, mutant strain (*tufA*$^+$ *tufB414*). Quantification of the EF-Tu bands by laser densitometry revealed no difference in amount.

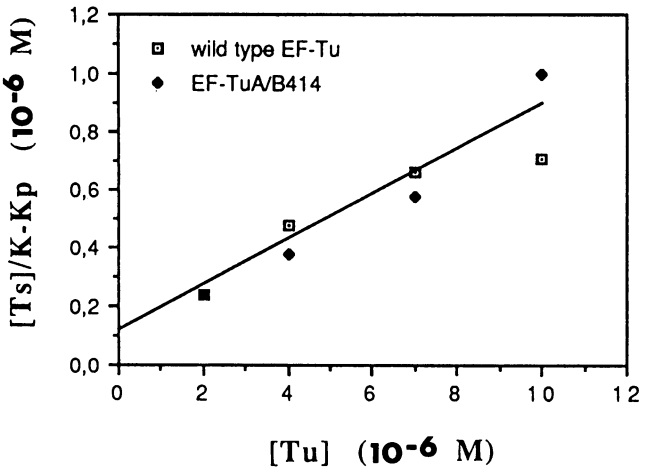

FIG. 4. Stimulation of the nucleotide exchange rate on EF-Tu by EF-Ts. The dissociation rate constants for the release of GDP from EF-Tu in the absence (K_p) and in the presence (K) of EF-Ts were measured and calculated as described by Ruusala et al. (52) and Tapio et al. (59). The [Ts]/($K - K_p$) ratio is plotted as a function of [Tu].

were able to measure the relative amounts of each of the EF-Tu species. Measurements of several different preparations of EF-TuA8 B414 and EF-TuA8 B^+ indicated that about 30% of each preparation is EF-TuB, in agreement with the protein sequencing data.

Kirromycin does not immobilize EF-TuB Val-280 on ribosomes. EF-Tu purified from JT642 (*tufA*$^+$ *tufB414*) and the kirromycin-resistant strain JT608 (*tufA8 tufB414*) was used

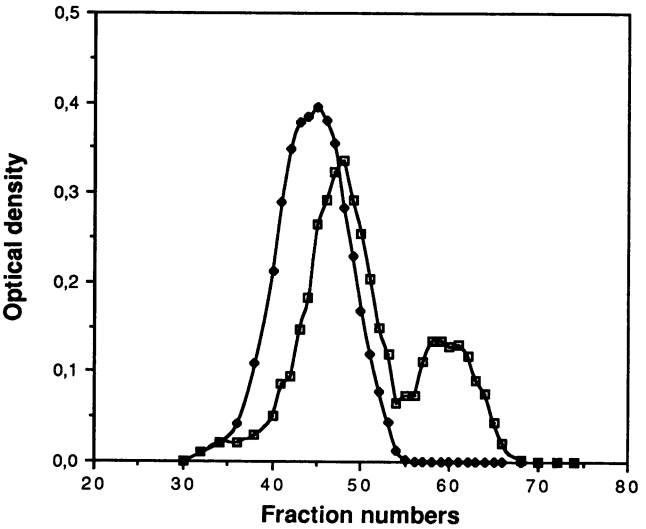

FIG. 5. Chromatography on DEAE-Q Sepharose Fast Flow of EF-TuA8 B414 in the presence (⊡) and absence (◆) of kirromycin by the method of Anborgh et al. (3). The A_{595} of the protein in column fractions was measured spectrophotometrically by the Bradford (12) assay. The A_{325} of kirromycin was measured. In the absence of kirromycin, EF-Tu elutes as a single peak. In the presence of kirromycin, EF-Tu separates into two peaks and kirromycin coelutes with the second (smaller) peak, which can thus be identified as EF-TuB414.

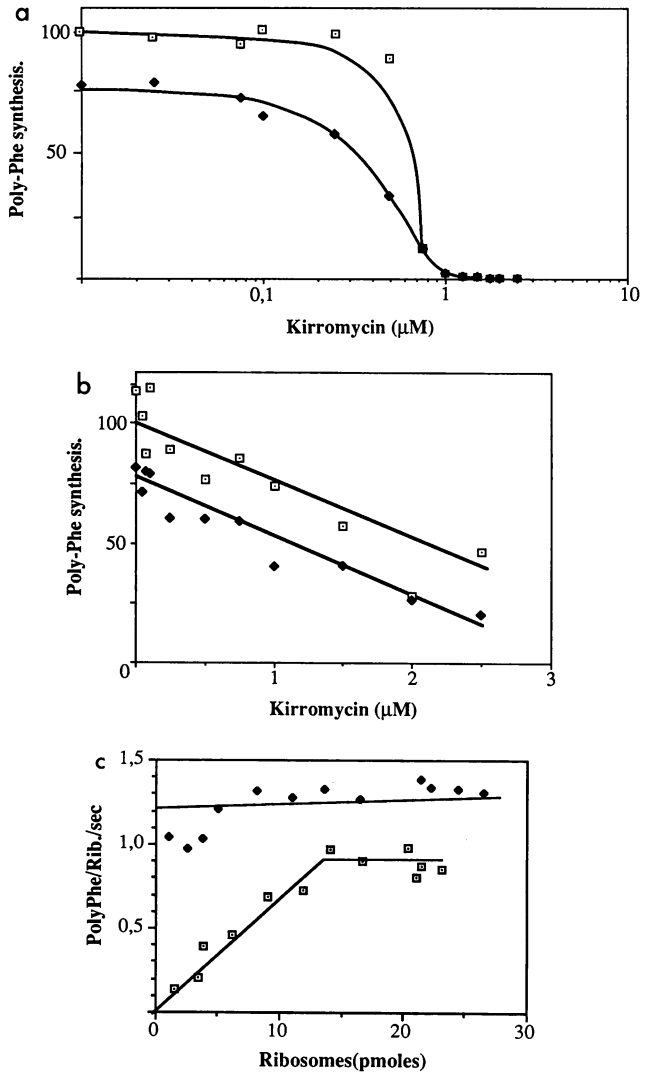

FIG. 6. Poly(Phe) synthesis as a function of the amount of kirromycin. (a) Symbols: ⊡, EF-TuA$^+$ B^+; ◆, EF-TuA$^+$ B414. (b) Symbols: ⊡, EF-TuA8 B^+; ◆, EF-TuA8 B414. In panels a and b, the results are normalized to a 0 μM kirromycin concentration for the EF-TuB^+ preparation set at 100%. (c) Translation at fixed EF-Tu and kirromycin concentrations with a titration of active ribosomes (Rib.) from 10-fold under to approximately 3-fold over the concentration of kirromycin-sensitive EF-Tu. Symbols: ⊡, EF-TuA8 B^+; ◆, EF-TuA8 B414.

to support translation in vitro in the presence of different amounts of kirromycin. Translation was carried out with an excess of active ribosomes over EF-Tu so that EF-Tu blocked on ribosomes by kirromycin (70) would not prevent other EF-Tu molecules from participating in translation (23). As controls, pure wild-type and pure kirromycin-resistant EF-Tu preparations were also used in these experiments. The results (Fig. 6a and b) show that the EF-TuA$^+$ B414 preparation responds to kirromycin as a pure sensitive population, whereas the EF-TuA8 B414 preparation responds as a pure resistant population. Thus EF-TuB414 has no phenotype in this experiment except to reduce the overall amount of protein synthesis. Given that we know, on the basis of protein sequencing and column chromatography, that the EF-TuB414 species is present, we conclude that it

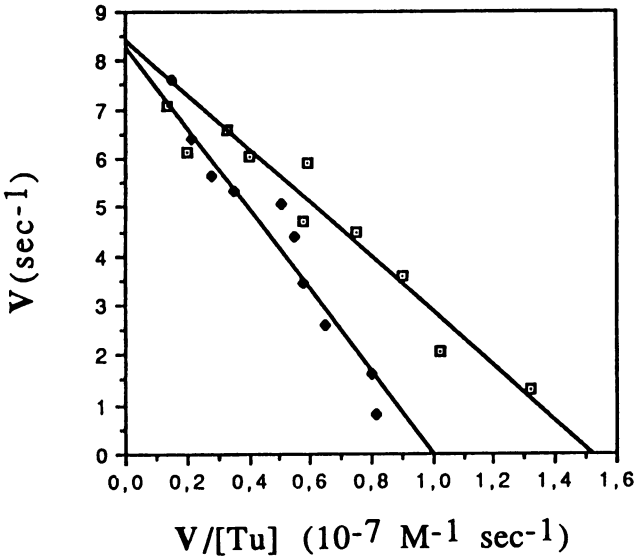

FIG. 7. In vitro translation as a function of the concentrations of wild-type EF-TuA$^+$ B$^+$ (⊡) and the natural mixture of wild-type and mutant EF-TuA$^+$ B414 (◆). Experimental details are described in Materials and Methods and in a previous report by Tubulekas et al. (64).

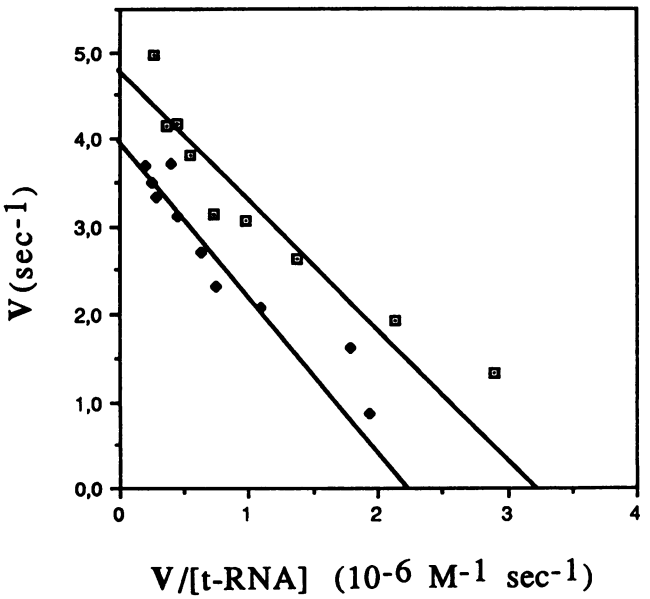

FIG. 8. In vitro translation as a function of the concentrations of Phe-tRNA^{Phe} with wild-type EF-TuA$^+$ B$^+$ (⊡) and the natural mixture of wild-type and mutant EF-TuA$^+$ B414 (◆). Experimental details are described in Materials and Methods and in a previous report by Tapio et al. (59).

does not detectably participate in translation in vitro, although it does bind kirromycin.

We tested whether EF-TuB414 is bound on ribosomes by kirromycin, using translation assays as described above but with a ribosome titration at fixed EF-Tu and kirromycin concentrations (see Materials and Methods). Under these conditions, wild-type kirromycin-sensitive EF-Tu is bound on ribosomes and does not significantly contribute to protein synthesis. As the ribosome concentration is lowered below the concentration of EF-Tu, this sensitive EF-Tu population competes with the active kirromycin-resistant EF-Tu for ribosomes and progressively prevents it from participating in protein synthesis. We compared the effects on translation of mixtures containing the kirromycin-resistant EF-TuA8 and either wild-type EF-TuB or the mutant EF-TuB414. The results (Fig. 6c) clearly show that EF-TuB414 does not inhibit translation under conditions of ribosome limitation, indicating that it is not bound on the ribosome by the antibiotic. The small difference in the translation rates supported by each preparation is due to a small difference in the amount of EF-Tu used.

Kinetics of the interaction between the ternary complex and the ribosome. We have determined the k_{cat}/K_m ratio of the interaction between wild-type ribosomes and EF-Tu species isolated from *tufA*$^+$ *tufB*$^+$ or *tufA*$^+$ *tufB414* strains. The translation rate in this type of experiment is strictly limited by the concentration of EF-Tu and the efficiency of its interaction in the ternary complex with ribosomes (all other components, including aa-tRNA, are present in a vast excess). The results presented in Fig. 7 show that the mixed EF-Tu population (A$^+$ B414) has a k_{cat}/K_m ratio about 30% lower (average of five experiments) than that of the pure wild-type EF-Tu. The degree of k_{cat}/K_m reduction, taken in the context of the relative amounts of mutant and wild-type EF-Tu in the preparation, indicates complete (or nearly complete) inactivity in translation associated with EF-TuB Val-280. A reduced k_{cat}/K_m ratio for the ternary complex-ribosome interaction is compatible with the reduced growth and elongation rates seen in vivo. Our interpretation of this experiment is that the mutant ternary complex is defective in its interaction with the ribosome. However, an alternative possibility is that the formation of the ternary complex is defective because of a poor interaction between EF-Tu and aa-tRNA (this would give the same result as that shown in Fig. 7). To test for this possibility, we performed the experiments on the EF-Tu interaction with aa-tRNA detailed in the next two sections.

Interaction between EF-Tu and aa-tRNA^{Phe} during in vitro translation. We asked whether the mutant EF-Tu was inactive in translation because of a defect in its interaction with aa-tRNA. To test this, we performed a translation assay with 10 pmol of active ribosomes, rate limited by the amount of EF-Tu (15 pmol), and with varying amounts of aa-tRNA^{Phe} in the range of 100 to 2,000 pmol. If the mutant EF-Tu is defective in forming the ternary complex, its activity should be enhanced at higher aa-tRNA concentrations. Thus, our expectation was that the lines would converge at high aa-tRNA concentrations if the defect was compensated by excess aa-tRNA. On the basis of the slopes of the lines in Fig. 8, we conclude that there is no significant difference between the different EF-Tu species in their interactions with aa-tRNA. Both the k_{cat}/K_m ratio and the k_{cat} for translation are lower with the mutant EF-Tu mixture, as in the previous experiment. In a control experiment (data not shown) using only 50% of the normal amount of wild-type EF-Tu, we plotted a line parallel to and slightly below that seen with the natural mutant/wild-type mixture. This again is consistent with the mutant EF-Tu making up only 30% of the EF-Tu mixture. We conclude that excess aa-tRNA cannot compensate for the mutant EF-Tu defect in translation and thus that this defect is unlikely to be due to low efficiency in forming a complex between aa-tRNA and EF-Tu. However, this conclusion would not be valid if the defect in ternary

complex formation were so severe as to almost completely prevent the interaction. In other words, if it had been necessary to titrate aa-tRNA to much higher concentrations than we had titrated it to before the defect was overcome, we might have obtained the result shown in Fig. 8. To test this possibility, we performed a physical assay of the ability of the mutant EF-Tu to form the ternary complex with GTP and aa-tRNA as detailed in the next section.

Interaction between EF-Tu and aa-tRNA^{Phe} by native PAGE. We performed a physical assay of the ability of the mutant EF-Tu to form a ternary complex with aa-tRNA (using a method developed by N. Bilgin, T. Gluick, and M. Ehrenberg [7a] [see Materials and Methods]). Complexes were formed in solution with a constant amount of EF-Tu and a titration of aa-tRNA applied to gels and electrophoresed under nondenaturing conditions, and we analyzed the results by densitometry. The gels shown in Fig. 9A and B are typical, and the densitometric data from these gels plotted in Fig. 9C reveal no significant difference in the abilities of the wild-type EF-Tu and the mutant/wild-type EF-Tu mixture to form ternary complexes. Both curves plateau at the same level, corresponding to 200 pmol of EF-Tu, indicating that all of the input EF-Tu is in a complexed form. Control experiments show that complex formation is absolutely dependent on aa-tRNA and on GTP. Thus, the translation defect associated with EF-TuB Val-280 in vitro is not due to an inability to form a complex with aa-tRNA. Furthermore, complex formation in this assay proceeds with the same kinetics for wild-type and wild-type/mutant EF-Tu species. We conclude from this experiment that our EF-Tu preparations are fully active in binding aa-tRNA. These results, showing no obvious defect in the EF-Tu–aa-tRNA interaction, allow us to make a clear interpretation of the k_{cat}/K_m reduction in Fig. 7 as being due to a reduced efficiency of interaction between the mutant ternary complex and the ribosome.

EF-TuB Val-280 does not influence translational errors in vitro. We have measured missense error in vitro (data not shown) for the natural mixtures of wild-type EF-TuA$^+$ and EF-TuB Val-280 and of error-prone EF-TuA8 and EF-TuB Val-280 in parallel with pure wild-type and pure error-prone EF-Tu. Translational accuracy in this assay is very sensitive to perturbations, yet we measured in each natural mixture only the error level typical of the wild-type or error-prone EF-TuA species alone (23). Thus, EF-TuB Val-280 does not influence the error level of the other EF-Tu.

GTP hydrolysis during ternary complex-ribosome interaction. The data presented in the previous sections show that the mutant EF-TuB Val-280 is competent and apparently normal with respect to its interactions with the guanine nucleotides, EF-Ts, and aa-tRNA. By elimination, and as shown by the assays of the ternary complex-ribosome interaction (reduced k_{cat}/K_m ratio and failure to block translation when complexed with kirromycin), this suggests that the defect of EF-TuB Val-280 in translation is related to its inability to interact productively with ribosomes. We performed another assay of the interaction of the mutant EF-Tu species with the ribosome by measuring the hydrolysis of GTP on EF-Tu associated with this interaction. We used the method developed by Ehrenberg et al. (18, 19) to measure the amount of GTP hydrolyzed on EF-Tu per peptide bond formed. This assay uses a translation elongation system that is complete except for the absence of EF-Ts. Thus, upon mixing, preformed ternary complexes take part in an initial rapid burst of translation and GTP hydrolysis followed by a very slow translation phase rate limited by the spontaneous

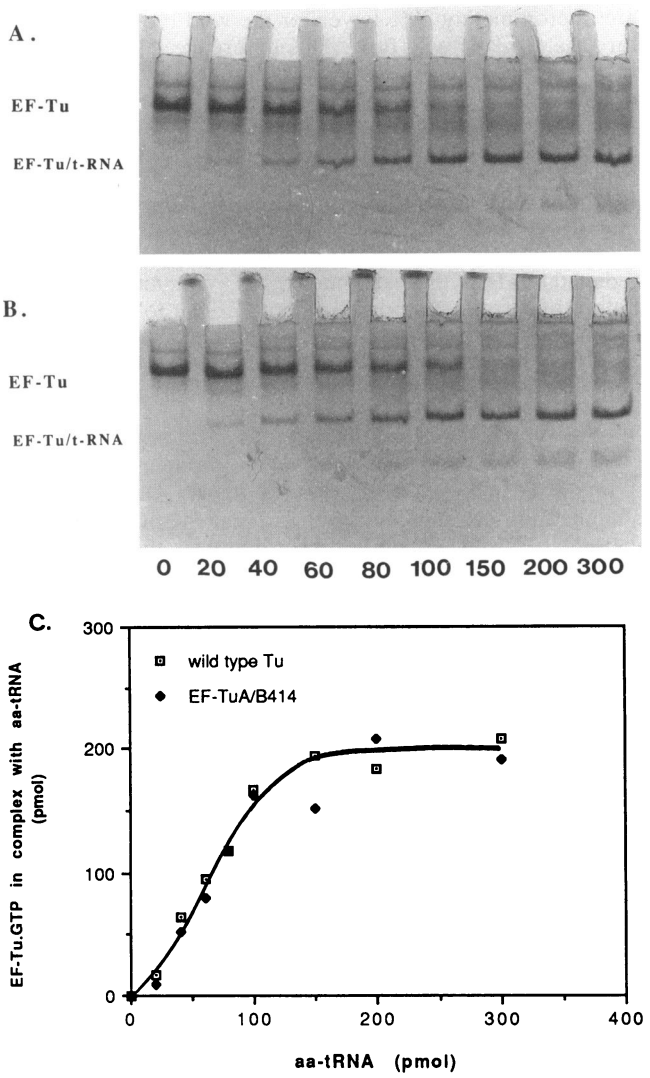

FIG. 9. Formation of a complex between EF-Tu and Phe-tRNA^{Phe} visualized by nondenaturing PAGE. (A) Wild-type EF-TuA$^+$ B$^+$; (B) natural wild-type/mutant mixture of EF-TuA$^+$ B414. The amount of EF-Tu in each lane was 200 pmol, and Phe-tRNA^{Phe} was titrated from 0 to 300 pmols (amounts shown below lanes). (C) Plot of densitometric data for formation of ternary complex derived by scanning gels A and B.

nucleotide exchange rate on EF-Tu in the absence of EF-Ts. The intrinsic GTPase level of EF-Tu (which is enhanced in some EF-Tu mutants although not detectably in EF-TuB Val-280) is taken into account and subtracted as a background level. In the first few seconds of this assay, there is an initial burst of protein synthesis during which virtually all the GTP bound to the EF-Tu · aa-tRNA complex during preincubation is hydrolyzed. Our results with wild-type EF-Tu indicate that almost all of the GTP on EF-Tu (approximately 500 pmol) is hydrolyzed in this burst, with the concomitant synthesis of 300 pmol of poly(Phe). In contrast, an equivalent amount of the natural mixture of mutant and wild-type EF-Tu hydrolyzed approximately 400 pmol of GTP, and 250 pmol of poly(Phe) was synthesized. Thus, the levels of both GTP hydrolysis and poly(Phe) synthesis associated with the ternary complex-ribosome interaction

are approximately 20% lower for the wild-type/mutant mixture than for the wild type. This difference, although small, is reproducible. The maximum difference we expect if the mutant EF-TuB Val-280 is completely inactive in interacting with ribosomes is 30% on the basis of the amount of EF-TuB and the magnitude of the R-factor reduction for the ternary complex-ribosome interaction. We conclude that under the conditions of this assay, the ternary complex with the mutant EF-TuB Val-280 fails in most cases to interact with ribosomes, resulting in GTP hydrolysis. This result, showing reduced GTP hydrolysis, the k_{cat}/K_m reduction, and the failure of kirromycin-bound mutant EF-Tu to inhibit translation, is consistent with the translation defect caused by EF-TuB Val-280 being in the interaction of the ternary complex with ribosomes.

DISCUSSION

In this article, we have presented data showing that a single amino acid substitution in domain II of EF-Tu, Gly-280→Val, abolishes or severely reduces the activity of EF-Tu in translation. The severe effect of this mutation, *tufB414*, in vivo is similar to that of insertional inactivation of the same gene. Because of the inviability of strains with *tufB414* as the only active *tuf* gene, we have done all of our in vitro experiments with EF-Tu purified from strains which also carry an active *tufA* gene. Doing in vitro experiments with the natural mixture of EF-TuA and EF-TuB414 (our measurements show that about 30% of the mixture is EF-TuB414) means that we can identify the gross defects caused by this mutant but cannot exclude the possibility that there are additional defects of smaller magnitude in other interactions. We have shown that the disruptive effect of the mutation is associated with the ternary complex-ribosome interaction in vitro. However, mutant EF-TuB Val-280 is active and indistinguishable from wild-type EF-Tu in binding and exchanging guanine nucleotides, in stimulation of nucleotide exchange by EF-Ts, and in forming complexes with aa-tRNA. Results of three different in vitro assays are consistent with the mutant EF-Tu having a defect which severely reduces or abolishes productive ternary complex-ribosome interactions. The assay of the ternary complex-ribosome interaction, under conditions where the amount of EF-Tu strictly limits the rate of translation, shows little or no translation activity associated with the mutant. The assay of translation in the presence of kirromycin shows that the mutant EF-Tu is not bound on the ribosome by kirromycin, although it does bind the antibiotic. Finally, most of the GTP on the mutant EF-Tu remains unhydrolyzed during an assay of peptide bond formation.

Other data suggest the involvement of domain II of EF-Tu in the ternary complex-ribosome interaction. A mutation isolated in the *E. coli tufB* gene, *tufB0* (Gly-222→Asp) (17, 66) probably has a major effect on the correct interaction of the ternary complex with ribosomes (58). In vitro translation experiments suggest that this mutant also is inactive in translation (61) except at high, nonphysiological magnesium concentrations, at which a defect in binding to ribosomes is overcome (58). Together, the data on the Gly-222 and Gly-280 mutants support the suggestion that domain II of EF-Tu has a role in the ternary complex-ribosome interaction.

It is worth pointing out that the normal interaction is one of the ternary complex (rather than EF-Tu) with ribosomes and that when EF-Tu dissociates from aa-tRNA it also leaves the ribosome. There is no a priori reason to assume a direct EF-Tu–ribosome interaction. In principle, the interactions might all be dictated by the bound aa-tRNA molecule. An indication that in the ternary complex EF-Tu itself is directly involved in interacting with ribosomes is that it has a region of homology (in domain I) with EF-G, a factor which interacts directly with ribosomes. The GTPase activity of the isolated domain I of *E. coli* EF-Tu is activated by 70S ribosomes (48), while tryptic cleavage or enzymatic modifications to this region of either factor (EF-Tu/EF-1α or EF-G/EF-2) can interfere with the ribosome interaction in each case (32, 45, 50, 54), and each factor, when stabilized on ribosomes by an antibiotic, provides an overlapping pattern of protection of 23S rRNA against chemical and enzymatic attack (44). In summary, these data, although often based on gross alterations to the protein, support the expectation, based on homology, that domain I of EF-Tu contains a region involved in interacting with ribosomes.

Our data and those of Swart et al. (58) suggest that, in addition to a domain I-ribosome interaction, domain II is involved, either directly or indirectly, in the ternary complex-ribosome interaction. We shall present here alternative models which could describe in general terms this interaction and how it might be disrupted by the domain II mutations in EF-Tu.

Direct interactions. EF-Tu may have temporally and spatially different interactions with ribosomes. For example, an initial interaction (possibly involving domain II) leading to codon-anticodon interaction might be followed by a second interaction (involving domain I and the α-sarcin region of the 50S subunit) leading to GTPase and the subsequent dissociation of EF-Tu · GDP from the ribosome. Arguing against multiple sites on ribosomes at which EF-Tu–ribosome interactions occur are rRNA protection experiments (43, 44) which do not detect any EF-Tu-mediated protection outside of the α-sarcin region. However, these experiments depend on freezing EF-Tu on ribosomes by using kirromycin and hence binding the GDP form of EF-Tu, and thus they do not provide information on the initial interaction of the ternary complex with ribosomes. Furthermore, these experiments would not necessarily have detected protein-protein interactions. An alternative model for a direct interaction is that EF-Tu in the ternary complex may have a conformation which brings regions of domains I and II close together so that they form, in essence, one hybrid EF-Tu site for interaction with the ribosome. Suggestive of this possibility is the cross-linking of the aa moiety of aa-tRNA to domain I in EF-Tu (16) and to domain II in EF-1α (31). A reasonable prediction from each of the direct-interaction models is that it should be possible to select compensating mutations with alterations in the ribosome.

Indirect effects. The domain II mutation may exert an indirect effect by altering the tertiary structure of EF-Tu such that the interaction of domain I with the ribosome is severely affected. Given the position of the mutation in a loop at the edge of domain II (like the Gly-222 mutation) this seems unlikely, but it cannot be ruled out with the present data. A more likely alternative for indirect effects is suggested by the recent results of Kinzy et al. (31) who made extensive cross-links between EF-1α and several aa-tRNAs and used the data to model the interaction between the EF and the aa-tRNA. According to their model, the domain II mutations are each very close to the 5′ and 3′ ends of the tRNA molecule. If this model is correct, it seems reasonable that local changes in domain II might have a direct influence either on the conformation of the bound aa-tRNA, affecting its ability to interact with the codon-programmed ribosome,

or on the stability of the EF-Tu · aa-tRNA complex on the ribosome, affecting the probability that aa-tRNA will dissociate from the ribosome. A prediction of this model is that the perturbations in the ternary complex might cause measurable differences in the constants of binding of aa-tRNA with EF-Tu · GTP. Clearly, more data on the structure of the ternary complex and experiments using only the mutant EF-Tu would be useful in evaluating these models.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Natural Science Research Council to D. Hughes and to C. G. Kurland and from the Swedish Cancer Society to C. G. Kurland.

We thank Neşe Bilgin, Tom Gluick, and Måns Ehrenberg for allowing us to use their nondenaturing PAGE method prior to publication. We also thank Åke Engström for running the amino acid sequencing reactions, Måns Ehrenberg for frequent useful advice on in vitro translation assays, Neşe Bilgin for suggesting helpful modifications to the EF-Tu purification protocol, and Barbro Wiklund for excellent technical assistance in purifying EF-Tu. Tarmo Ruusala, Måns Ehrenberg, and C. G. Kurland are thanked for their helpful and critical reading of the manuscript.

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