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Novel RNA interactions with the elongation factor EF-Tu: consequences for protein synthesis and *tuf* gene expression

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The elongation factor EF-Tu, discovered more than 20 years ago, has not suffered from a lack of attention. Despite numerous investigations, this important protein continues to yield new and interesting information about its functions, its three-dimensional structure and the organization and regulation of its two encoding genes.

During recent years novel interactions between RNA and the elongation factor EF-Tu have come to light. It is common knowledge that EF-Tu forms a ternary complex with aminoacyl-tRNA and GTP and as such plays an essential role in protein synthesis. This multifunctional translational factor, however, possesses a second tRNA-binding site which opens up as soon as the ternary complex associates with the ribosome/mRNA complex. The second site can accommodate not only aminoacyl-tRNA but also peptidyl-tRNA and non-acylated tRNA with relatively high affinities. Mutant species of EF-Tu, altered in tRNA binding and GTPase activity, act as suppressors of nonsense codons. Evidence is accumulating that EF-Tu functions is an autogenous repressor of one of its two encoding genes, *tufB*, by binding to the mRNA coding for EF-Tub. These EF-Tu/RNA interactions are considered in the light of the crystal structure of EF-Tu.

A preliminary model of the three-dimensional structure of EF-Tu.GDP is depicted in Fig. 1. It is based on X-ray diffraction studies by LaCour, Nyborg, Clark and their colleagues[1]. Three structural domains can be discerned. The tracing of the polypeptide chain is given here for domain I only. Cys 81, located in the loop connecting β-strand 2 and α-helix I, is exposed in EF-Tu.GDP and EF-Tu.GTP and reacts with thiol agents like *N*-tosylphenylalanine chloromethyl ketone (TPCK) and, *N*-ethylmaleimide (NEM). Binding of aminoacyl-tRNA to EF-Tu.GTP shields Cys 81, thus preventing it from reacting with the alkylating agents[2]. This reaction with Cys 81 revealed two tRNA-binding sites on EF-Tu (Ref. 3). One is the classical binding site (site I) accessible only when GTP occupies the nucleotide binding site (also indicated in Fig. 1)[4,5]. Although there may be various areas of contact between aminoacyl-tRNA and site I, the 3′ terminal adenosine is probably somewhere between β-strand 1 and α-helix VI. This is suggested by our finding that tRNA oxidized with periodate, when bound to site I, can be crosslinked under certain conditions to the ϵ-NH_2 of Lys 237 (see Fig. 1).

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A second tRNA-binding site

The first indication for the second tRNA-binding site came from modification studies involving Cys 81. Whereas tRNA binding to site I prevents Cys 81 from reacting with thiol agents, binding to site II enhances the reaction. Under conditions where Cys 81 modification signals tRNA binding to site II, the 3′ terminal adenosine can be crosslinked to Lys 208. This crosslinking site is at the loop connecting α-helix V and β-strand 6 (Ref. 6). Other contact areas of site II and tRNA have not been identified yet.

Perhaps the tRNA-binding site II escaped the attention of previous investigators because it becomes apparent only when EF-Tu interacts with suitable effectors, such as the ribosome or the antibiotic kirromycin. These effectors alter the conformation of EF-Tu and act as inducers of site II. Kirromycin induces site II on both EF-Tu.GTP and EF-Tu.GDP. We localized the antibiotic by its crosslinking to Lys 375 (Ref. 6), which indicates that it has close contact to the back of domain II (as shown in Fig. 1).

Of particular interest is the kirromycin-induced high affinity of peptidyl-tRNA for tRNA-binding site II. It exceeds that of aminoacyl-tRNA for site I and suggests that, on the ribosome, peptidyl-tRNA also interacts with EF-Tu.GTP through site II. Accordingly, we found that peroxidized tRNA ($tRNA_{oxi}$) at the ribosomal P-site, crosslinks to Lys 208 of EF-Tu.GTP.Phe-tRNA that has entered the ribosome at the A site. No crosslinking to Lys 237 occurs under these conditions. When, however, $tRNA_{oxi}$ is positioned at the ribosomal A-site in the presence of EF-Tu.GTP and the ribosomal P-site is occupied by deacylated-tRNA, crosslinking to Lys 237 is observed. Crosslinking is exclusive and depends on the position of tRNA at either the ribosomal P- or A-site, irrespective of the presence of kirromycin[7]. Outside the ribosome, however, both crosslinking sites are accessible to tRNA when kirromycin acts as the allosteric effector. These data illustrate the specificity of the interaction between ribosome-bound tRNA and EF-Tu. They strongly suggest that the two tRNA-binding sites of EF-Tu are functionally and topographically distinct and fulfil an important role in polypeptide synthesis on the ribosome[8,9].

EF-Tu and aminoacyl-tRNA selection on the ribosome

Aminoacyl-tRNA selection on the ribosome is thought to occur in at least

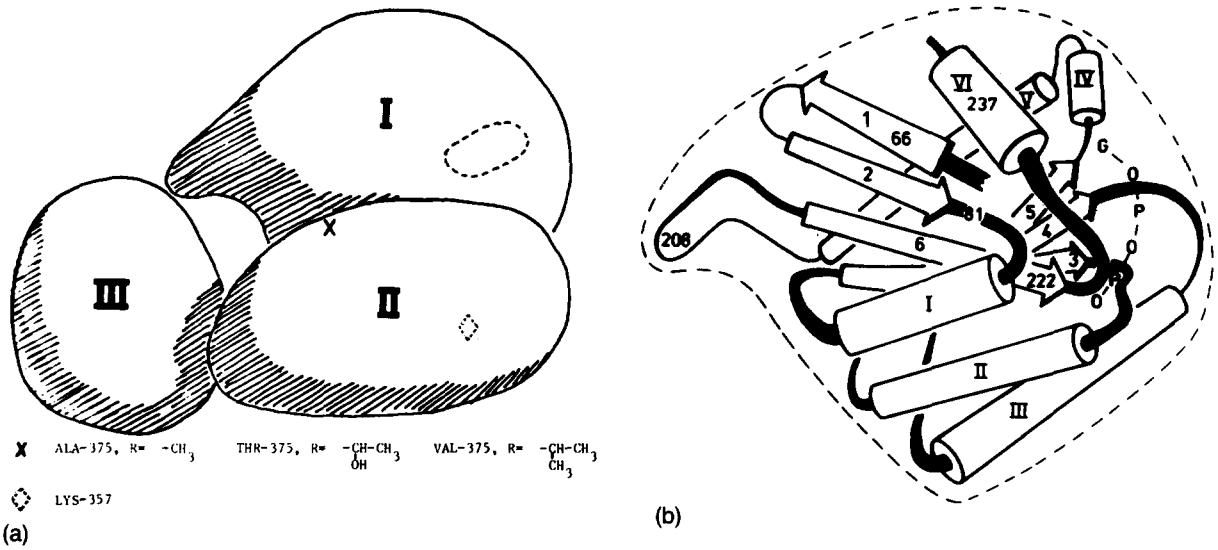

Fig. 1. (a) Three-dimensional representation of the overall shapes of the domains of EF-Tu. The position of GDP in domain I is shown by the dotted region. The dotted lines of the symbol for residue 357 in domain II indicate that this residue is located at the backside of the domain. (b) Structural cartoon of domain I with arrows representing β-strands and cylinders representing α-helices. The model is based on X-ray diffraction studies of mildly trypsinized EF-Tu. GDP by LaCour et al.[1]

two discriminating steps (for review see Ref. 10). The first involves initial recognition of the ternary complex EF-Tu.GTP.aminoacyl-tRNA and the second (after GTP hydrolysis), rejection or retention of aminoacyl-tRNA before peptide bond formation. The latter step is called proofreading.

Hydrolysis of GTP is mediated by EF-Tu (Ref. 11) and the GTPase center of EF-Tu is activated by the same effectors that induce tRNA-binding site II: the ribosome or kirromycin[12]. Activation in the presence of kirromycin is increased by adding tRNA. Very recently we found (van Noort *et al.*, unpublished) that this increase is due to binding of tRNA to site II of EF-Tu, whereas binding of aminoacyl-tRNA to site I does not influence the GTPase. Although the implications of this are not yet fully understood, they are probably highly significant for the selection of aminoacyl-tRNA on the ribosome.

Before entering the ribosome, aminoacyl-tRNA occupies the classical tRNA-binding site of EF-Tu and GTP hydrolysis does not occur. Only upon interaction with the ribosome, does EF-Tu undergo a conformational transition which opens up tRNA-binding site II; we do not yet know exactly when this occurs. There is evidence that codon/anticodon interaction alters the conformation of aminoacyl-tRNA (Ref. 13). This may force EF-Tu on the ribosome to adapt its own conformation, thereby disclosing tRNA-binding site II. Only then can peptidyl-tRNA bind to site II: whereupon GTP hydrolysis ensues, EF-Tu.GDP releases the 3′ end of aminoacyl-tRNA and eventually leaves the ribosome[4,5]. Non-cognate aminoacyl-tRNA does not change its conformation because codon/anticodon interaction does not occur and site II of EF-Tu remains closed. The residence of aminoacyl-tRNA in site I as such does not activate the GTPase and permits the ternary complex to leave the ribosome without expending energy through GTP hydrolysis. Obviously, the postponement of GTP hydrolysis until proper codon/anticodon interaction has taken place is energetically advantageous. This sequence of events is now open to further experimental verification.

Mutant EF-Tu and suppression of nonsense codons

We have seen that EF-Tu.GTP interacts on the ribosome with aminoacyl-tRNA through tRNA-binding site I and with peptidyl-tRNA through tRNA-binding site II (see Fig. 2). This dual interaction of EF-Tu with the two tRNAs may contribute significantly to the proper positioning of the latter on

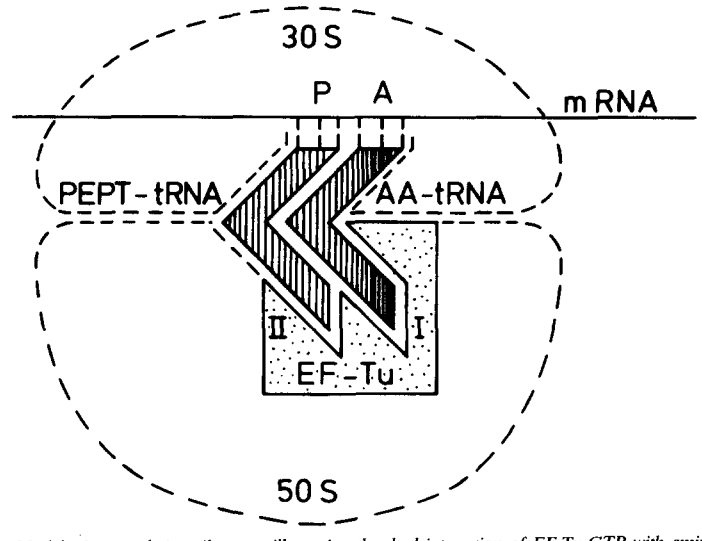

Fig. 2. Model of a translating ribosome illustrating the dual interaction of EF-Tu.GTP with aminoacyl-tRNA at the ribosomal A-site and with peptidyl-tRNA at the ribosomal P-site.

the ribosome. Interestingly, mutations altering tRNA binding of EF-Tu influence the reading of the messenger RNA. We found that *E. coli* cells harbouring mutations in both *tufA* and *tufB*, the two EF-Tu genes, suppress all three nonsense codons, UAG, UAA and UGA (Ref. 14) (for some representative experiments see Table I). The product of the mutant *tufA*, designated EF-TuA_R, has Ala 375 replaced by Thr (Ref. 15) (see Fig. 1). This replacement has lowered the binding constants of Phe-tRNA and Tyr-tRNA for site I of EF-TuA.GTP three- and six-fold, respectively[16]. We do not know whether site II is also affected. The GTPase centre is slightly influenced[17]. The product of the mutant *tufB*, designated EF-TuB_o, has Asp substituted for Gly 222 and has a defective tRNA-binding site II (Refs 15, 18). Its GTPase activity is drastically reduced (compare the next paragraph). Surprisingly, nonsense suppression requires the combined action of both EF-TuA_R and EF-TuB_o in the cell (Table I). This raises the interesting question whether translation of a sense codon also occurs by cooperation of two EF-Tu molecules on the ribosome. If so, this would shed new light on the mechanism of translation.

Table I. Suppression of nonsense codons by mutant EF-Tu, measured as β-galactosidase activity[a]

Strain[c]	Nonsense codon[b] UAG–189	UAA–189	UGA–189
EV2,A_sB_s	0.8±0.4	0.8±0.4	360±85
EV5,A_RB_o	7.4±0.4	6.1±0.4	1320±170
EV3,A_sB_o	1.7±0.4	1.3±0.4	150±26
EV8,A_RB_s	2.0±0.6	1.2±0.4	260±47

[a] Nonsense suppression has been measured by assaying the β-galactosidase activity of cells harbouring a chromosomal *lac proB* deletion and an F′ episome containing a fused *lacI* and *lacZ* gene. The latter episome carries one of the three nonsense codons in the I portion of the fusion at position 189. These nonsense codons cause termination of translation before the Z-encoded portion of the protein is formed. Suppression of the nonsense codons permits translation of the *lacZ* coding sequence. As a result, a hybrid repressor/β-galactosidase molecule is synthesized. The hybrid protein has a normal β-galactosidase activity. The latter activity is expressed in arbitrary units.

[b] After the dash the codon position is indicated. Similar experiments have been performed with nonsense codons at position 220 in the I portion of the *lacI/lacZ* fusion.

[c] The symbols A_s and B_s stand for wild-type *tufA* and *tufB* products (sensitive to kirromycin). A_R indicates a *tufA* product resistant to kirromycin; B_o indicates a product of mutant *tufB*, recessive to kirromycin resistant *tufA*. The F′ episome (compare a) has been introduced into the four strains indicated.

Mutant EF-TuB and the oncogene product p21 *ras*

A GTP-binding protein that has attracted much attention recently is the human bladder protein p21 *ras*. The encoding gene becomes an oncogene by a point mutation replacing Gly 12. Human tumor cell lines and chemically induced rodent tumors contain such activated *ras* genes, capable of inducing morphological and tumorigenic transformation when introduced into preneoplastic immortal cell lines (for review see Ref. 19). The p21 *ras* protein binds GDP and GTP with high affinities comparable to that of EF-Tu for GDP. Gly 12 is in a region that shares sequence similarity with the nucleotide-binding site of other proteins. Models for this region of p21 *ras* have been suggested. Normal p21 *ras* has GTPase activity but this is grossly impaired in the oncogene protein[20]. Neither the extent nor the specificity of nucleotide binding is altered by the mutation conferring transforming activity. It has been speculated that p21 *ras* acts as a transducer of signals to the adenylate cyclase and that loss of GTPase activity results in abnormally high intracellular cyclic AMP concentrations.

Interestingly, the effects of p21 *ras* and the EF-TuB_o(Gly 222→Asp) mutations are very similar; both mutations affect a Gly residue in the GTP-binding region (Fig. 1). The affinity of EF-TuB_o for GTP is hardly affected but the GTPase activity of the mutant protein is drastically reduced (Swart *et al.*, unpublished results).

EF-Tu regulates the expression of *tufB*

The two EF-Tu genes, *tufA* and *tufB*, are arranged in two different transcription units: the *str* operon with the genes coding for S12, S7, EF-G, EF-TuA at position 72 min of the *E. coli* chromosome; and the tRNA/*tufB* operon with the genes coding for $tRNA_4^{Thr}$, $tRNA_2^{Tyr}$, $tRNA_2^{Gly}$, $tRNA_3^{Thr}$ and EF-TuB at position 88 min (Ref. 21). Studies of the regulation of the expression of *tufA* and *tufB* are hampered because the corresponding gene products, EF-TuA and EF-TuB, differ only in the C-terminal amino acid[22]. Advantage can be taken of a mutation affecting one of the *tuf* genes that permits distinction and separation of the *tuf* products, provided it does not upset gene regulation. A mutation in *tufB*, substituting Asp for Gly 222 in domain I of EF-TuB_o (see Fig. 1), permits separate determination of the intracellular concentrations of EF-TuA and EF-TuB_o. Such determinations revealed that the ratio of EF-TuA and EF-TuB concentrations in the cell remain constant under varying steady-state growth conditions. This means that there is coordinate regulation of the expression of *tufA* and *tufB*. Another mutation affecting *tufA*, and substituting Thr for Ala 375 in domain II disturbs the regulation of *tufB*, indicating that EF-Tu itself is involved in the regulation of *tufB*. Altering *tuf* gene dosage and *in-vitro*

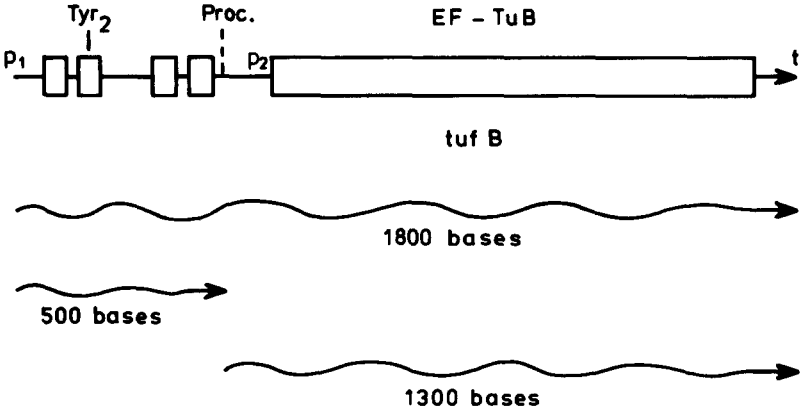

Fig. 3. Transcripts of the tRNA/tufB operon.

studies of EF-Tu in a coupled transcription/translation system, showed that EF-Tu acts as an autogenous repressor of *tufB* (not of *tufA*)[23,24].

TufB is cotranscribed with four upstream tRNA genes. Processing of the cotranscript of 1800 bases yields a *tufB* transcript of 1300 bases (Fig. 3). Transcription from the secondary promoter P2 is negligible. Assays of the intracellular concentration of tRNA encoded by the *Tyr 2* gene upstream of *tufB* and other studies did not reveal any effect of the autogenous repressor on transcription of the tRNA/*tufB* operon or on the processing of its cotranscript. Recent *in-vitro* studies revealed that EF-Tu acts as an autogenous repressor by feedback inhibition of translation. Deletion of the four tRNA genes of the operon results in a sharp increase of the intracellular EF-TuB/EF-TuA ratio (van Delft *et al.*, unpublished results).

The mutation Gly 222→Asp of EF-TuB_o does not affect autogenous repression (see above). More strikingly, EF-Tu that has undergone a deletion of α-helices I, II and III and β-strands 3 and 4 (see Fig. 1) is an active repressor. Obviously, this deletion radically mutilates domain I and destroys the nucleotide binding site (van Delft *et al.*, unpublished). We conclude that an intact tertiary structure of domain I is not required for autogenous repression, nor is the binding of GTP. The point mutation Ala 375→Thr in domain II (EF-TuA_R) significantly affects the repressor function[24]. This may implicate domain II rather than domain I in the binding of the cotranscript but it remains for further studies to define the interaction of EF-Tu with the cotranscript and to explain how this interaction interferes with the translation but not with the processing of the cotranscript.

EF-Tu is not the only regulator of *tufB* expression. Our recent experiments[25] indicate that transcription from the P1 promoter of tRNA/*tufB* operon (Fig. 3) is submitted to the same regulatory mechanism as transcription of the ribosomal RNA genes arranged in the *rrn* operons. Nomura and his colleagues demonstrated[26] that a product of the *rrn* operons represses transcription of rRNA and tRNA operons. Interestingly, the two regulatory mechanisms: repression of transcription and feedback inhibition of translation also govern the regulation of rRNA and ribosomal protein synthesis, albeit separate operons are involved. In EF-TuB synthesis, these two mechanisms act at one and the same operon. Additional mechanisms are not excluded, however.

Viral RNA replicase

Beside its role in protein synthesis, EF-Tu functions in viral RNA replication as a host-donated subunit of the replicase. We do not know whether EF-Tu directly interacts with the viral RNA template. Modification of Cys 81 with TPCK, which prevents tRNA binding to site I (but not to site II, van Noort *et al.*, unpublished results), does not abolish the replicase subunit function of EF-Tu (Ref. 27). EF-Tu.GTP, crosslinked at site I via His 66 to ε-*N*-Br-acetyllysyl-tRNA, still exhibits poly(A)-dependent binding to ribosomes and successfully performs GTP hydrolysis. However, the complex does not allow subsequent transpeptidation in the protein chain elongation cycle. On the other hand, the crosslinked complex can be reconstituted into active viral replicase. Finally EF-Tu can be covalently linked to EF-Ts without loss of ability to work in Qβ replicase[27].

All these data can be readily interpreted by excluding binding of EF-Tu to viral RNA. However, the discovery of a second tRNA-binding site on EF-Tu and the binding of the latter protein to the cotranscript of the tRNA/*tufB* operon call for caution in this respect. These novel RNA interactions might share common recognition sites with a potential viral RNA binding to this three-domain protein.

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