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Mutants of the elongation factor EF-Tu, a new class of nonsense suppressors

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Read-through of nonsense codons has been studied in wild-type *Escherichia coli* cells and in cells harbouring mutant species of the elongation factor EF-Tu. The two phenomena differ essentially. Readthrough of UGA in wild-type cells is reduced by inactivation of *tufB* but is restored to the original level by introducing into the cell plasmid-borne EF-Tu. This shows that the natural UGA leakiness is dependent on the intracellular concentration of EF-Tu. Strains of *E. coli* harbouring mutant species of the elongation factor EF-Tu suppress the nonsense codons UAG, UAA and UGA. Suppression shows a codon context dependence. It requires the combined action of two different EF-Tu species: EF-TuA_R(Ala 375 → Thr) and EF-TuB_0(Gly 222 → Asp). Cells harbouring EF-TuA_R(Ala 375 → Thr) and wild-type EF-TuB, or wild-type EF-TuA and EF-TuB_0(Gly 222 → Asp) do not display suppressor activity. These data demonstrate that mutated *tuf* genes form an additional class of nonsense suppressors. The requirement for two different mutant EF-Tu species raises the question whether translation of sense codons also occurs by the combined action of two EF-Tu molecules on the ribosome.

Key words: nonsense suppression/elongation factor EF-Tu/mutated *tuf* genes

Introduction

Intergenic suppression of nonsense mutations can be mediated by suppressor genes coding for a tRNA or a ribosomal protein (Ozeki *et al.*, 1980; Gorini, 1974). In the former case the suppressor tRNA reads one of the three stop signals as if it were a signal for a specific amino acid, in the latter ribosomes containing a mutated ribosomal protein misread all three nonsense codons in the complete absence of suppressing tRNAs.

So far no nonsense suppression by translational factor mutations has been reported with the possible exception of the *uar*-1 mutation, which causes enhanced misreading of UAG and UAA and which may affect a protein involved in translation termination at these stop codons (Ryden and Isaksson, 1984). Mutations affecting the elongation factor EF-Tu (Bosch *et al.*, 1983) are of interest since this important translational factor can reduce translation errors in a poly(U)-directed polypeptide synthesizing system (Gavrilova and Perminova, 1982).

EF-Tu mediates the binding of aminoacyl-tRNA to the ribosome/mRNA complex during protein synthesis. Under these conditions a complex is formed between aminoacyl-tRNA, EF-Tu and GTP in a 1:1:1 molar ratio (Miller and Weissbach, 1977; Kaziro, 1978). Previous investigations in our laboratory demonstrated that EF-Tu possesses a second tRNA-binding site which is induced upon interaction of EF-Tu with the ribosome. This second site can bind peptidyl-tRNA, aminoacyl-tRNA and non-acylated tRNA with relatively high affinity (van Noort *et al.*, 1982). Cross-linking of periodate oxidized tRNA, residing either in the ribosomal P-site or A-site to Lys-208 and Lys-237 of EF-Tu.GTP, respectively, indicated that EF-Tu.GTP interacts on the ribosome with aminoacyl-tRNA through its classical tRNA-binding site and with peptidyl-tRNA through its second site (van Noort *et al.*, 1984, 1985). This suggested to us that the relative positioning of the two tRNAs on the ribosome is determined to a large extent by this dual interaction with EF-Tu (Kraal *et al.*, 1983).

In the present investigation we have asked the question whether correct positioning is also secured in the presence of mutant species of EF-Tu, particularly those with an altered tRNA binding. We show that mutant EF-Tu can act as a suppressor of UAG, UAA and UGA and conclude that mutated *tuf* genes form an additional class of nonsense suppressor genes. Surprisingly suppression of all three nonsense codons requires the combined action of two different mutant EF-Tu species.

Results

Mutant species of EF-Tu act as nonsense suppressors

Mutant strains used in the present investigation are listed in Table I. Besides mutations of the *tuf* genes they harbour a chromosomal *lac proB* deletion and an F′ episome containing a fused *lacI* and *lacZ* gene. The latter episome (compare Figure 1) carries one of the three nonsense codons in the I portion of the fusion, either at position 189 or 220. 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 which lacks the last four residues of *lac* repressor and the first 23 residues of β-galactosidase. The hybrid protein has a normal β-galactosidase activity. The activity of the read-through product gives a measure of suppression of UAG, UAA and UGA in two different codon contexts.

Measurement of β-galactosidase activity of the strains of Table I revealed that suppression of all three nonsense codons in position 189 and of UGA in position 220 occurs in the strain EV5,A_RB_0 (Table II). The latter strain harbours a kirromycin-

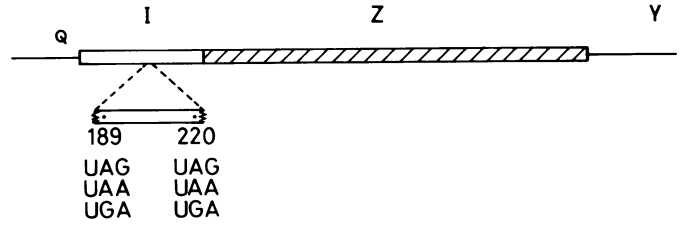

Fig. 1. The *lacI-Z* fusion carrying UAG, UAA or UAG mutations in position 189 or 220. For details see Müller-Hill and Kania (1974), Brake *et al.* (1978) and Miller *et al.* (1978).

Table I. Strains of *E. coli* used in this investigation

Designation	Sex; extra-chromosomal markers	Chromosomal markers	Relevant characters	Origin
193[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)	carries ochre mutations in I part at position 189	J. Miller
194[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)	carries amber mutation in I part at position 189	J. Miller
195[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)	carries UGA mutation in I part at position 189	J. Miller
196[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)	carries ochre mutation in I part at position 220	J. Miller
197[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)	carries amber mutation in part at position 220	J. Miller
198[a]	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Arc *gyrA* *rpoB argE* (amber)	carries UGA mutation in I part at position 220	J. Miller
XAc/△14	F′ *lacIZ* fusion *proA*$^+$, *B*$^+$	△(*pro lac*) Ara *gyrA* *rpoB argE* (amber)		Brake *et al.* (1978)
KMBL 1164	F$^-$	△(*pro lac*) *supE thi*		[b]
LBE 1001, A_sB_s[c]	F$^-$	wild-type		[b]
LBE 2019, A_RB_o[c]	F$^-$	△(*pro lac*) *xyl tufA tufB*	van Klundert (1978)	
LBE 2020, A_sB_o[c]	F$^-$	*xyl rpoB tufB*	van Klundert (1978)	
PM 816, A_RB_s[c]	F$^-$	*fus tufA*	van der Meide (1982)	
PM 505, A_s[c]	F$^-$	*rpoB tufB::(Mu)*	van der Meide (1982)	
PM 455, A_R[c]	F$^-$	*rpoB tufA tufB::(Mu)*	van der Meide (1982)	
EV2, A_sB_s[c]	F$^-$	△(*pro lac*)	This paper	
EV3, A_sB_o[c]	F$^-$	△(*pro lac*) *rpoB tufB*	This paper	
EV4, A_s[c]	F$^-$	△(*pro lac*) *rpoB tufB::(Mu)*	This paper	
EV5, A_RB_o[c]	F$^-$	△(*pro lac*) *tufA tufB*	This paper	
EV8, A_RB_s[c]	F$^-$	△(*pro lac*) *fus tufA*	This paper	
EV9, A_R[c]	F$^-$	△(*pro lac*) *rpoB tufA tufB::(Mu)*	This paper	

[a]The chromosomal markers are identical to those of strain UD132, Andersson *et al.* (1982). These strains were generously provided by Dr. D.I. Andersson, Uppsala, Sweden.
[b]Obtained from the Department of Molecular Genetics, State University, Leiden, The Netherlands.
[c]The symbols A_s, A_R, B_s, and B_o refer to wild-type *tufA*, kirromycin-resistant *tufA*, wild-type *tufB* and mutant *tufB* recessive to kirromycin-resistant *tufA*.

Table II. Suppression of nonsense codons *in vivo* by mutant EF-Tu

Strain[c]	B-Galactosidase activity[b]					
	Nonsense codon[a]					
	UAG-189	UAG-220	UAA-189	UAA-220	UGA-189	UGA-220
EV2,A_sB_s[d]	0.8±0.4	17±2	0.8±0.4	13±2	360± 85[e]	200±42
+pGp82	n.d.	n.d.	n.d.	n.d.	370± 36	180±27
EV5,A_RB_o[d]	7.4±0.4	16±1	6.1±0.4	10±1	1320±170[f]	830±32
EV3,A_sB_o	1.7±0.4	22±2	1.3±0.4	15±2	150± 26	130±22
EV8,A_RB_s	2.0±0.6	14±5	1.2±0.4	8±1	260± 47	180±21
EV4,A_s	1.1±0.2	19±2	0.9±0.2	15±2	88± 4	81± 9
+pGp82	n.d.	n.d.	n.d.	n.d.	250± 32	190±16
EV9,A_R	0.9±0.4	16±3	0.8±0.4	14±2	100± 23	110± 8
+pGp82	n.d.	n.d.	n.d.	n.d.	311± 49	160±31

n.d. = not determined
[a]After the dash the codon position is indicated (compare Figure 1).
[b]β-Galactosidase activity was determined and expressed in arbitrary units (average of five determinations) as described in Materials and methods.
[c]For the genotype of these strains see Table I.
[d]Similar results were obtained after introduction of the *rpoB* mutation (see Materials and methods).
[e] and [f]Corresponding to 3.6% and 13% of the activities of EV2 and EV5 cells with a F′ episome harbouring a *lacI/Z* fusion without a nonsense codon, respectively.

resistant *tufA* (symbol A_R) and a kirromycin-sensitive *tufB* recessive to kirromycin resistance (symbol B_o). In this strain, read-through of UAG and UAA in position 189 is ~5–6 times that in the wild-type strain (symbols A_sB_s). Read-through of UGA in the same position is ~3–4 times higher in the mutant strain than in the wild-type strain. About the same result is obtained with UGA in position 220. No read-through above wild-type level is observed in position 220 with UAG and UAA. We conclude that mutant species of EF-Tu can suppress all three nonsense mutations.

Suppression requires the combined action of two different mutant EF-Tu species

Since strain EV5,A_RB_o harbours two different mutations (Table I), the question arises which of the two *tuf* products, EF-TuA_R or EF-TuB_o, is responsible for the nonsense suppression. To answer this question we first introduced the F′ episome into cells which do not produce EF-TuB due to inactivation of *tufB* by insertion of bacteriophage Mu (compare strains EV4,A_s and EV9,A_R in Table I). As can be seen in Table II, strain EV9,A_R does not display read-through of nonsense codons above the level of strain EV4,A_s or that of strain EV2,A_sB_s. These experiments suggest that either EF-TuB_o is the true nonsense suppressor or that suppression requires the combined action of EF-TuA_R and EF-TuB_o. A direct test of the former possibility is not feasible since we have no strain available which produced EF-TuB_o as the sole *tuf* product. We therefore have studied read-through of nonsense codons in strains harbouring either mutant *tufA* and wild-type *tufB* (strain EV8,A_RB_s) or wild-type *tufA* and mutant *tufB* (strain EV3,A_sB_o). Neither of these strains, however, displayed β-galactosidase activities exceeding that of the wild-type strain EV2,A_sB_s (compare Table II). These experiments lead to the conclusion, therefore, that the presence in the cell of both EF-TuA_R and EF-TuB_o is a requisite for nonsense suppression.

Suppression and codon context.

Suppression of UGA in strain EV5,A_RB_o occurs in both positions: 189 and 220, that of UAA and UAG however is restricted to position 189. Apparently suppression of the latter two codons depends on codon context, that of the former is affected somewhat but to a much lesser extent. Suppression does not follow the same rules as formulated by Miller and Albertini (1983) and Bossi (1983) for tRNA suppressors since an AGU codon is found at the 3′ side of both position 189 and position 220 (Miller *et al.*, 1978). Wild-type strain EV2,A_sB_s shows a rather high leakiness in recognizing UGA in either position, a low leakiness of UAG and UAA in position 189 and an intermediate one of UAG and UAA in position 220. This has also been observed by other investigators (Andersson *et al.*, 1982). This leakiness may be due to re-initiation but other causes cannot be excluded (Miller and Albertini, 1983; Files *et al.*, 1974).

UGA leakiness is affected by tuf gene dosage

Leakiness of UGA in wild-type cells (EV2,A_sB_s) is significantly higher than that in cells lacking a functional *tufB* gene (strain EV4,A_s; compare Table II). This is true for UGA in both position 189 and position 220. Inactivation of *tufB* causes a reduction in intracellular EF-Tu level of ~43% (van der Meide *et al.*, 1982), suggesting that UGA read-through depends on the EF-Tu concentration in the cell. Confirmation of this suggestion was sought by re-elevating the EF-Tu concentration with plasmid-borne EF-Tu. As can be seen in Table II cells from the strain EV4,A_s transformed with the multicopy plasmid pGp82 harbouring *tufA* show a UGA read-through which is restored to approximately wild-type level. Transformation of strain EV9,A_R also enhances UGA read-through. This demonstrates that the UGA leakiness is dependent on the intracellular EF-Tu level. However, elevation of the EF-Tu concentration above that of wild-type does not further increase UGA leakiness. This can be concluded from Table II showing that UGA read-through in cells from the wild-type strain EV2,A_sB_s transformed with pGp82 is equal to that of the parental strain (see also Discussion). UGA leakiness in strain EV3,A_sB_o is reduced ~2-fold as compared with that of wild-type EV2,A_sB_s (compare Table II). An explanation of this phenomenon has to await *in vitro* translation experiments with purified EF-TuB_o and wild-type EF-Tu. These experiments have recently been initiated.

Discussion

Three major conclusions emerge from the present investigation: (i) certain mutant species of EF-Tu act as suppressors of nonsense codons; (ii) suppression requires the combined action of two different EF-Tu mutant species: these species are EF-TuA_R(Ala 375→Thr); and EF-TuB_o(Gly 222→Asp.); (iii) UGA leakiness in wild-type cells is dependent on the intracellular level of EF-Tu.

In the Introduction we pointed out that EF-Tu interacts on the ribosome with aminoacyl-tRNA residing at the ribosomal A-site and with peptidyl-tRNA residing at the ribosomal P-site. For this dual interaction EF-Tu makes use of two tRNA-binding sites (van Noort *et al.*, 1984, 1985). It is noteworthy that one of the suppressor EF-Tus: EF-TuB_o(Gly 222→Asp) is defective in its second tRNA-binding site (Bosch *et al.*, 1983; unpublished results).

It is clear from the present data, however, that we cannot explain the suppression of nonsense codons by the EF-TuB_o defect alone. Cells harbouring EF-TuB_o and wild-type EF-Tu do not suppress. Surprisingly, suppression is only observed when the defective EF-TuB_o(Gly 222→Asp) is complemented by another mutant factor: EF-TuA_R(Ala 375→Thr). Apparently, the replacement of Ala 375 by Thr enables the latter factor to complement EF-TuB_o but we do not know how this comes about. The replacement has lowered the apparent binding constants of Phe-tRNA and Tyr-tRNA tRNA-binding site I of EF-TuA_R.GTP by a factor of approximately 3 and 6, respectively (Sam, 1983). Whether the second tRNA-binding site of EF-TuA_R is also affected remains to be investigated. Evidently, the suppression phenomenon can only be understood by taking the striking requirement for two different mutant species of EF-Tu into account.

Nonsense codons are recognized by termination factors (Caskey, 1977). It may be assumed, therefore, that a decisive event in the read-through of a stop codon is the competition between these factors and aminoacyl-tRNA complexed to EF-Tu.GTP. Our findings (Table II) show that the normal leakiness of UGA in wild-type cells is dependent on the level of EF-Tu bound to aminoacyl-tRNA. In these cells almost all EF-Tu.GTP is taken up in complexes with aminoacyl-tRNA (Furano, 1975). Inactivation of *tufB* lowers the intracellular EF-Tu level by ~43% (van der Meide *et al.*, 1982) and consequently raises the level of free aminoacyl-tRNA. As a result, UGA leakiness is reduced but can be restored to almost normal wild-type values upon introduction into the cell of a plasmid harbouring a *tuf* gene. Introduction of such a plasmid into cells with two functional chromosomal *tuf* genes, however, does not lead to increased leakiness. This is plausible since the EF-Tu.GTP/aminoacyl-tRNA ratio is now raised above one and the excess of free EF-Tu.GTP is not expected to compete with termination factors. Previous EF-Tu assays in cells harbouring EF-TuA_R and EF-TuB_o (van der Meide *et al.*, 1982, 1983a) do not support the idea that nonsense suppression in these cells can be ascribed to increased concentrations of ternary complexes. On the contrary, this type of suppression is due to the nature rather than to the intracellular concentration of EF-Tu. The mechanism of suppression by mutant EF-Tu may differ therefore from that underlying leakiness in wild-type cells.

The findings presented in Table II demonstrate that codon context plays a significant role in nonsense suppression by mutant EF-Tu. This suggests that suppression of specific nonsense codons is accompanied by the incorporation of a specific amino acid in-

to the nascent polypeptide chain. Possibly codon context contributes to the discrimination between aminoacyl-tRNAs at the site of the nonsense codon.

It is clear from the data discussed so far that they do not lend themselves to a ready explanation of the suppression mechanism. The most striking observation is the requirement for two different mutant EF-Tu species. A better insight may be gained from *in vitro* studies dealing with polypeptide synthesis mediated by EF-TuB_0, by EF-TuA_R and by combinations of the two. Preliminary results present suggestive evidence for cooperative effects exerted by the two mutant factors on polypeptide synthesis which cannot be explained by the activities of each factor separately (Swart *et al.*, unpublished results).

A final question but of major importance is, whether translation of a sense codon also requires the combined action of two EF-Tu molecules. It is possible of course that read-through of a stop codon makes specific demands on the elongation factor which can only be met by the cooperation on the ribosome of two EF-Tu molecules each with a specific but different defect. Sense codons, which are not recognized by termination factors, may then be translated using one EF-Tu molecule only. The possibility has to be given serious consideration, however, that translation of a sense codon only occurs by the cooperation of two EF-Tu molecules on the ribosome. This would mean that some essential features are lacking in our understanding of the translation mechanism.

Materials and methods

Genetic procedures

The *E. coli* K12 strains used in this study are listed in Table I. Strains were constructed by phage P1 transduction as described by Miller (1972). The *pro lac* deletion was transduced into strains harbouring various combinations of mutant and wild-type *tuf* genes using a P1 lysate on strain KMBL 1164. Recipient strains were LBE 1001, LBE 2020, PM 505, LBE 2019, PM 816 and PM 455 (compare Table I). After two successive penicillin treatments, colonies were screened for *pro lac* genotype. The strains obtained (EV2,EV3,EV4,EV5,EV8 and EV9, respectively) are isogenic except for *rpoB* and *fus*. In order to exclude that the presence or absence of *rpoB* affects read-through, spontaneous rifampicin-resistant mutants were selected on rich medium (LC) plates supplemented with 1 mM EDTA and 30 μg/ml rifampicin. LC medium contained per liter: 10 g bactotryptone, 8 g NaCl, 5 g yeast extract, pH 7.0. For plates, 16 g Difco agar per liter was added. F′ factors harbouring the *lac I-Z* fusion (compare Figure 2) were introduced into EV2, EV3, EV4, EV5, EV8 and EV9 by liquid matings (Miller, 1972). Strains UD 132 carrying F′ factors with *lac I-Z* fusion and a nonsense codon in the *lac I* part of the fusion were generously provided by D.I. Andersson and C.G. Kurland. EV2, EV4 and EV9 were transformed with the *tufA* containing plasmid pGp82 (van der Meide *et al.*, 1983b) according to Lederberg and Cohen (1974).

Media and growth conditions

The strains used for the *in vivo* determination of the nonsense suppression were grown with good aeration at 37°C for at least four generations in minimal medium (Vogel and Bonner, 1956) supplemented with 0.5% glucose. Cells were harvested at mid-exponential phase.

Preparation of S30 extracts and β-galactosidase assay

To prepare the S30 extracts, cells were harvested in mid-exponential phase, rapidly cooled in ice and centrifuged. The cells were resuspended in 1 ml of 10 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 10 mM $MgAc_2.4H_2O$, 6 mM β-mercaptoethanol 0.1 mM PMSF and broken by ultrasonication. From this 'crude extract', cell wall components and other large structures were removed by centrifugation at 30 000 *g*. The supernatant (S30) was used in the β-galactosidase assay and kept at 4°C until used. To determine the β-galactosidase activity an aliquot of the S30 extract was mixed with Z-buffer (Miller, 1972) up to a volume of 1 ml and 200 μl of a 4 mg/ml ONPG solution was added. This mixture was incubated at 37°C. The reaction was stopped by the addition of 500 μl of a 1 M $NaCO_3$ solution, the mixture was centrifuged for 5 min in an Eppendorf centrifuge and the optical density of the supernatant was read at 420 nm. The β-galactosidase activity was expressed in arbitrary units defined by the following equation.

$$\text{units } \beta\text{-galactosidase} = \frac{\text{OD}}{v \times t \times c \times q}$$

with v = volume in μl
t = time in min
c = protein concentration in mg/ml
q = β-galactosidase activity in the same strain with a F′ factor harbouring a *lacI-Z* fusion without a nonsense codon.

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard.

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