MISCHARGING IN MUTANT TYROSINE TRANSFER RNAs

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1. Introduction

The su_{III} gene of *E. coli* offers a good system for the isolation of mutant tyrosine transfer RNAs [1–4]. Alteration by mutation of the amino-acid acceptor specificity of a tRNA would provide a method of investigating the nature of the site on a tRNA recognised by its cognate aminoacyl-tRNA synthetase. We here describe properties of two previously isolated su_{III} mutants which strongly suggest that their acceptor specificity is thus altered, and the isolation of two new mutants having similar properties. The sites of all four mutations are tightly clustered in stem (a) of the cloverleaf structure.

2. Materials and methods

E. coli strains MB93, MB93A2, MB93A2P and MB93A81 carrying the su_{III} alleles su^+_{III}, A2, A2P and A81 have been described [2]. MB100 is the lac ∇ parent of MB93. CA1 (HfrC prototroph), CA274 (HfrC lac^-_{am125} $tryp^-_{am}$) and CA161 (HfrC lac^-_{amY14} su^+_{II}) were from the Cambridge collection, and 1000BT (F^- lac^-_{am1000}) from D. Zipser. Transducing phage $\phi 80psu^+_{III}$ and its derivatives carrying mutant su_{III} genes have been described [5, 1, 2].

BC1G dye plates, on which phages suppressing the host lac_{amber} form blue plaques, are described in [5]. β-Galactosidase was measured in phage-infected cells after induction with 2 mM IPTG by treating with chloroform and assaying the rate of ONPG hydrolysis as described in [6]; thiogalactoside transacetylase was measured in sonicated extracts as described in [7].

For fingerprinting, bacteria were infected with $\phi 80psu$ phage and labelled with ^{32}P-orthophosphate as described in [1]. Tyrosine tRNA was separated by polyacrylamide gel electrophoresis [2], and oligonucleotide fingerprinting carried out by the method of Sanger et al. [8].

3. Results

In order to search for variants of the su^+_{III} tyrosine tRNA which might accept other amino acids, we first sought amber mutations of phage $\phi 80$ which were suppressible by other amber suppressors but not by su^+_{III}. We reasoned that such mutations should affect sites in proteins where other amino acids, such as serine or glutamine, are acceptable but where tyrosine is not; hence derivatives of su^+_{III} tyrosine tRNA which can suppress these mutations might well have acquired the ability to accept other amino acids, albeit perhaps at a low efficiency.

We mutagenised a non-defective derivative of phage $\phi 80$, carrying a single non-suppressor copy of the su_{III} tyrosine tRNA gene ($\phi 80psu^-_o$) as follows. Bacterial strain MB100 was grown at 37° in B broth to ~6 × 10^8/ml, centrifuged, and the pellet resuspended in 1/10 vol of M9 + 0.01 M $MgSO_4$. To 1.0 ml was added 0.3 ml of phage at a titer of 1 × 10^{11}/ml, and adsorption was allowed to proceed for 30 min without aeration at 37°. Tris-maleate [5] was then added to bring the vol to 10 ml, and the infected cells were centrifuged and resuspended in 10 ml Tris-maleate supplemented with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) at

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100 μg/ml. After an additional 65 min at 37°, the infected, mutagenized cells were once again centrifuged and resuspended in 20 ml B broth and bubbled until the culture cleared (90 min) at 37°. This admittedly harsh procedure yielded only about 0.1 progeny phage per initial cell, and the frequency of amber mutants among them was about 1%.

These progeny were plated on strain CA161 (su_{II}^+) and individual plaques were tested by picking and stabbing to CA161 and CA274 (su^-). Fifty-nine amber mutants were obtained from 5537 stabbed plaques, and 3 of these, *am*21, *am*78 and *am*83 turned out to be suppressible by su_{II}^+ but not by su_{III}^+. As would be expected, all 3 were suppressible by the ochre suppressor su_B^+ but not by the ochre suppressor su_C^+; *am*21 and *am*83, but not *am*78 were suppressible by su_I^+.

We had intended to construct a phage φ80 derivative carrying 2 of these mutations as well as su_{III}^+, to permit direct selection of su_{III}^+ variants which could suppress these mutations. However, the 3 amber mutants were first tested individually for suppression on a number of bacterial hosts carrying already isolated mutant derivatives of su_{III}^+ which had been selected for their partial suppressor character. The tRNA sequence changes resulting from these mutations had already been determined [2] and are designated by the base at the mutant site and its residue number from the 5′-terminus (fig. 1). Surprisingly, 3 such derivatives, MB93A2, MB93A2P (both of which produce the same species of tRNA, viz A2, see [2]) and MB93A81, suppressed both *am*21 and *am*83. That suppression in these cases was due to the su_{III}^+ derivative, and not to some other unsuspected suppressor, was shown by the fact that these phages could, at a frequency of about 0.1%, pick up the su_{III}^+ derivative genes of these hosts (presumably in exchange for their own original su_{III}^- gene) and thereby acquire the ability to grow on non-suppressor strains. Furthermore, reversion of one of the su_{III}^+ derivatives, MB93A2, back to full suppressor activity by a second-site mutation within the su_{III} gene (A2U80, [2]), destroyed its ability to suppress *am*21 and *am*83.

The nature of the su_{III}^+ derivatives with these unusual suppression properties is discussed below. We should perhaps mention here that their initial detection was a fortunate consequence of the availability of *am*21 and *am*83; had we used only *am*78, or other specifically su_{II}^+-suppressible mutants such as sus3 and sus20 of

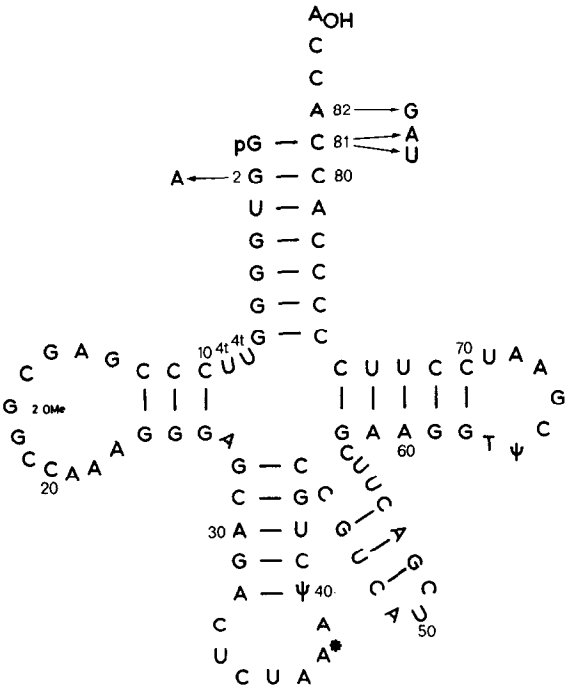


Fig. 1. Su_{III} mutants exhibiting mis-supression. The isolation of A2 and A81 is described in [2]; that of U81 and G82 is described in the text.

phage λ, they would have been missed.

Our initial attempts to detect charging of su_{III} tRNA isolated from cells infected with A2, A2P or A81 phage, with an amino-acid other than tyrosine, were unsuccessful. This could be explained in the following ways:

(a) The level of mischarging is so low as to be undetectable in the *in vitro* system.

(b) Mischarging is more sensitive to the difference in conditions between the cell and the *in vitro* assay mixture than is normal charging.

(c) Our assumptions about the mechanism of the *in vivo* effect are wrong. An alternative explanation for the observed suppression pattern is as follows: the efficiency of transmission of an amber mutation is influenced by the codon environment [9, 10]. It is possible that the extent of this is different for different suppressors: for instance, one might imagine that some feature of the structure of su_{III}^+tRNA is incompatible with binding to a ribosome carrying a certain tRNA in its 'P' site, and that this feature is not shared by

Table 1
Quantitation of mis-suppression.

E. coli strain	Infected with phage	Mean β-galactosidase induction rate
CA1	φ80	100
CA274	none	< 0.5
CA274	$\phi 80 psu^{+}_{III}$	88
1000BT	none	< 0.5
1000BT	$\phi 80 psu^{+}_{III}$	< 0.5
1000BT	φ80psuA2P	15
1000BT	φ80psuA2	3.8
1000BT	φ80psuA81	7.5
1000BT	φ80psuU81	4.5
1000BT	φ80psuG82	53

Bacteria grown to 2×10^8/ml in LP medium [12] with glycerol as carbon source at 30° were concentrated 10-fold by centrifugation, $MgSO_4$ added to 0.02 M, and aliquots infected with phage at a multiplicity of 10; uninfected controls received medium without phage to give the same total volume. After 20 min adsorption, all suspensions were diluted 10-fold into fresh LP medium with glycerol at 30° and aerated. IPTG was added to 2 mM and samples withdrawn at 0, 30, 60 and 90 min after induction were treated with chloroform and assayed as described in Materials and methods.

su^{+}_{II} tRNA, or by mutant tRNAs A2 and A81. Lack of suppression of *am*21 and *am*83 by su^{+}_{III} would thus be attributed not to inacceptability of tyrosine but to lack of transmission.

We have been able to distinguish between these possibilities with the aid of the β-galactosidase amber mutant lac^{-}_{1000}, described by Michels and Zipser [11], which is not suppressed by su^{+}_{III}. Determination of the ability to give lac^{+} transductants with various $\phi 80 psu_{III}$ phages showed that this mutant, like *am*21 and *am*83 was suppressed by A2, A2P and A81 alone of the su_{III} alleles available (in this system it was possible to extend the measurements to those su_{III} alleles available only as phage derivatives which could not be tested with *am*21 and *am*83: none of these, which included the recombinant U80 [2], suppressed lac^{-}_{1000} (compare the behaviour of A2U80 above). The amber lac^{-}_{1000} thus behaves like *am*21 and *am*83, so that by measuring β-galactosidase activities in this strain a semi-quantitative estimate can be made of the efficiency of mis-suppression (table 1, which also includes data on mutants U81 and G82 whose isolation is described below). If one assumes that the specific activity of the suppressed lac^{-}_{1000} product is not greater than that of

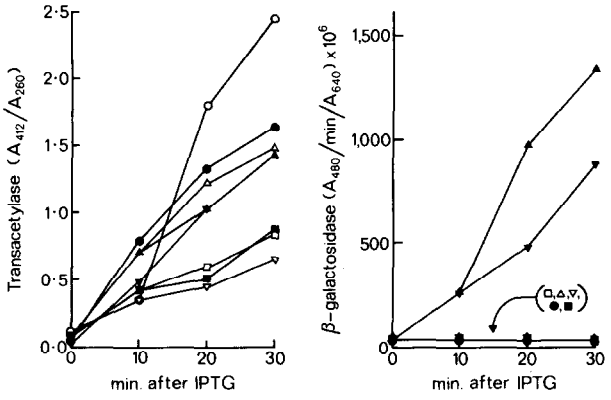


Fig. 2. Relief of polarity of lac^{-}_{1000} by su_{III} alleles at 30°. *E. coli* 1000BT growing exponentially in LP Medium [12] with glycerol as carbon source were concentrated to 2×10^9/ml by centrifugation. $MgSO_4$ was added to 20 mM and the cells infected with phage at a multiplicity of 10. After adsorption for 20 min at 30° the suspension was diluted 10-fold into prewarmed LP medium with glycerol and aerated for 45 min at 30° to allow synthesis of su_{III} tRNA. 2 mM IPTG was then added to induce the lac operon and samples withdrawn at 0, 10, 20 and 30 min after induction were assayed for β-galactosidase and thiogalactoside transacetylase as described in Materials and methods. Curves refer to (○–○–○): CA1; (□–□–□): 1000BT, uninfected; (△–△–△): 1000BT/$\phi 80 psu^{+}$; (▽–▽–▽): 1000BT/$\phi 80 psu^{-}_{O}$; (▲–▲–▲): 1000BT/φ80psuA2P; (▼–▼–▼): 1000BT/φ80psuA81; (●–●–●): 1000BT/φ80psuA2U80; (■–■–■): 1000BT/φ80psuA25.

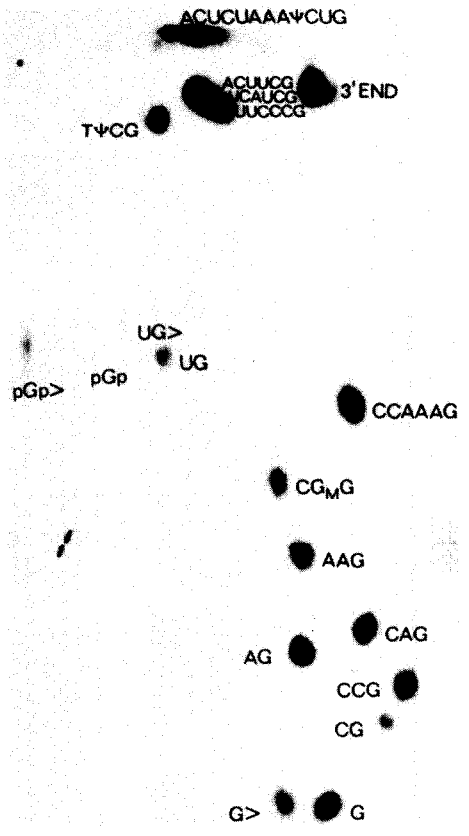
wild-type β-galactosidase, then in the case of the lac^{-}_{1000}/A2P system, at least 15% of initiated β-galactosidase chains are transmitted to give an active product. This makes it unlikely that our failure to observe mischarging *in vitro* is for quantitative reasons (i.e. explanation (a) above). Similar conclusions hold for A81 and probably also A2.

The availability of the mutant lac^{-}_{1000} also allowed a test to be made of explanation (c), viz, that failure of su^{+}_{III} to suppress is due to failure to transmit. The lac^{-}_{1000} mutation exerts a strong polar effect [13]. Nonsense suppressors relieve polarity to an extent which is a quantitative measure of the efficiency of transmission [14, 15]. Thus if su^{+}_{III} tRNA inserts tyrosine to give an inactive gene product it should partially relieve polarity, while if it fails to insert tyrosine, no relief of polarity should be seen. Fig. 2 shows that in uninfected 1000 BT, the polar effect of lac^{-}_{1000} reduces the rate of induction of transacetylase

activity to about 30% of that of the lac$^+$ control strain CA1. Infection with phage carrying either of the alleles su_o^- or A25 (which has no detectable suppressor activity for any amber mutation) has no effect on this rate, while in cells infected with A2P or A81 phage it is increased to 60% of the rate for CA1. Infection with phage carrying either su_{III}^+ or A2U80, while leading to induction of no measurable β-galactosidase activity, relieves polarity to an extent at least equal to that observed with A2P and A81. It is clear therefore that explanation (c) can be ruled out, leaving us with explanation (b). For this reason, our subsequent experiments to demonstrate mischarging and identify the amino-acid(s) accepted have been carried out *in vivo*. These experiments will be the subject of a further communication (J. Celis, M.L.H. and J.D.S., manuscript in preparation).

Strain 1000BT provided a convenient selective system for the isolation of more mis-suppressing mutants. $\phi 80psu_{III}^+$ was mutagenised with NG as described above except that exposure to the mutagen was for 30 min at 37°. It was then plated with 1000BT on minimal lactose plates at 30°. Transductant colonies were purified and on spontaneous induction yielded phage from which those forming blue plaques on a 1000BT seeded BClG-dye plate were picked. All such strains whose $tRNA^{tyr}$ has so far been fingerprinted were derived from a single mutagenesis and each was identified as one of two mutants, U81 and G82 as described below.

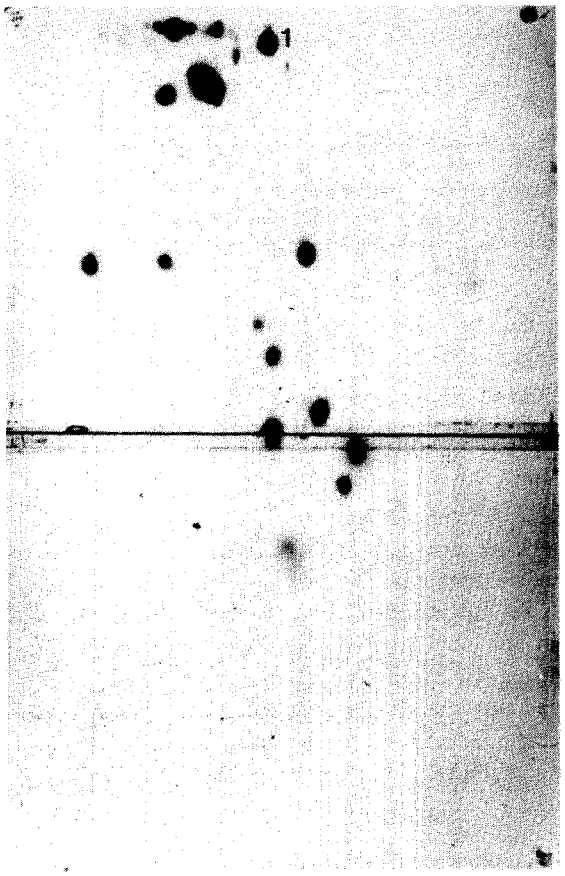
Fig. 3(a) and (b) show T1 RNAse fingerprints of $tRNA^{tyr}$ from cells infected with $\phi 80psu_{III}^+$ and $\phi 80psuU81$, respectively. They are identical except that in (b) the normal 3′-terminal oligonucleotide $AAUCCUUCCCCCACCACCA_{OH}$ is missing and a new



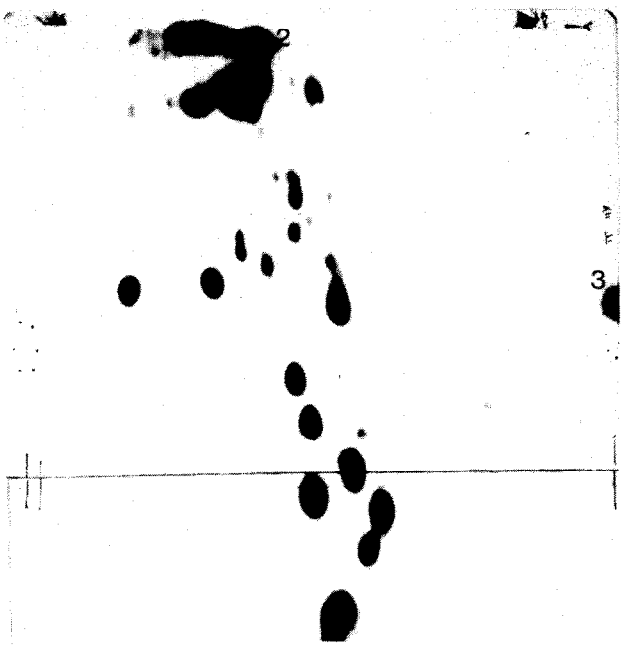
a)

Table 2
Pancreatic RNAse digestion products of spots 1 and 2 of fig. 3

	su_{III}^+ 3′ end	Molar yields 1	2
AAU	1.1	1.1	1.2
AU	0	0	0
AC	1.8	2.1	1.0
G	0	0	1.0
C	9.0	8.1	8.0
U	1.9	2.6	1.5



b)



c)

Fig. 3. T1 RNAse fingerprints of $tRNA^{tyr}$ from cells infected with $\phi 80psu_{III}$ carrying the following alleles (a) su^+; (b) U81; (c) G82. In (a) the 3'-end oligonucleotide is $AAUCCUUCCCCCACCACCA_{OH}$. Separation is by electrophoresis from right to left on cellulose acetate in 7 M urea, pH 3.5; followed by electrophoresis from top to bottom on DE81 paper in 7% formic acid.

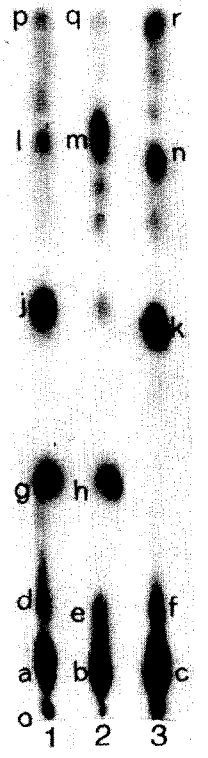


Fig. 4. U2 ribonuclease digestion products of: 1) spot 1 of fig. 3(b); 2) the 3′-terminal T1 product of su^{+}_{III} tRNA; 3) spot 2 of fig. 3(c). The eluted oligonucleotide without added carrier RNA was incubated with 10 μl .05 M sodium acetate pH 4.5, 0.002 M EDTA containing 0.1 unit/ml RNAse U2 and 0.1 mg/ml crystalline bovine serum albumin for 2 hr at 37°. Under these conditions essentially complete splitting of ApPy bonds is achieved but digestion between adjacent A residues is incomplete. The digestion products were applied to Whatman DE81 paper and subjected to electrophoresis in pH 1.9 buffer (2.5% formic acid, 8.75% acetic acid, v/v) at 50 V/cm for 2 hr. Spots a–r were characterised by alkali digestion (table 3).

spot 1 is present, whose position suggests that it is derived from the normal 3′-end by a C → U change. This is confirmed by pancreatic RNAse digestion of the eluted spots (table 2), which shows that the residue affected is one of the nine found as the mononucleotide after digestion rather than one of the two found as AC. Ribonuclease U2 digestion of 1 (fig. 4)

Table 3
Alkali digestion products of spots from fig. 4.

Spot	Products	Deduced composition
a, b, c,	U, C, A	UCCUUCCCCCA*
d, e, f,	U, C	UCCUUCCCCC*
g, h	C	CCA_{OH}*
j	C, U, A	(C, U)A
k	C, G	CCG
l, n	A	AA
m	C, A	AA + CCA
p, q, r	A	A

* Deduced from wild-type sequence. Other deductions made solely on basis of digestion products, mobility and specificity of RNAse U2.

gives no CCA but a spot j identified as (U, C)A (table 3). This must be CUA rather than UCA since AU is absent from the pancreatic RNAse digest of 1. This information is sufficient to define the mutation as C81 → U. (table 4, fig. 1).

Fig. 3(c) shows the T1 RNAse fingerprint of $tRNA^{tyr}$ from cells infected with ϕ80psuG82. Again the 3′-terminal spot of su^{+}_{III} tRNA is missing, this time being replaced by two spots 2 and 3. Pancreatic RNAse digestion of 2 showed that G was present and that one mole of AC and probably one mole of C were missing. U2 digestion gave no CCA or CCA_{OH} but instead a product k characterised as CCG. Spot 3 gave C as the only labelled product on treatment with pancreatic RNAse, and pC and pA on treatment with snake venom phosphodiesterase. These data establish that the mutation is A82 → G, giving rise to T1 products AAUCCUUCCCCCACCG (2) and CCA_{OH} (3) (table 4).

4. Discussion

The sites of the 4 mutations giving rise to mis-suppression are tightly clustered in stem (a) of the cloverleaf (fig. 1). The results presented here argue strongly that the mechanism of mis-suppression involves mischarging. However, proof of this mechanism requires identification of the amino-acid(s) inserted. It will then be necessary to identify which amino-acyl tRNA synthetase is involved, and to determine whether the effect on recognition is direct or indirect, before def-

inite conclusions can be drawn about the nature of the synthetase recognition site on a tRNA.

Su_{III} mutants with anomolous suppression properties have also been isolated by Aono et al. [16] and by Ghysen et al. [17]. It would be very interesting to know whether these mutant sites are also in stem (a).

Acknowledgements

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Table 4
Expected digestion products of su^+_{III}, U81 and G82 tRNA's

tRNA	su^+	U81	G82
3'-Terminal sequence	GAAUCCUUCCCCCACCACCA$_{OH}$	GAAUCCUUCCCCCACUACCA$_{OH}$	GAAUCCUUCCCCCACCGCCA$_{OH}$
T1 digestion products	AAUCCUUCCCCCACCACCA$_{OH}$	AAUCCUUCCCCCACUACCA$_{OH}$	AAUCCUUCCCCCACCG + CCA$_{OH}$
U2 ribonuclease products from T1 oligonucleotide	A(A); UCCUUCCCCCA;CCA;CCA$_{OH}$	A(A); UCCUUCCCCCA;CUA; CCA$_{OH}$	A(A); UCCUUCCCCCA;CCG
Pancreatic ribonuclease products from T1 oligonucleotide	AAU; 9C; 2U; 2AC	AAU; 8C; 3U; 2AC	AAU; 8C; 2U; AC; G