The EMBO Journal vol.12 no.6 pp.2559-2566, 1993

Functional tRNAs with altered 3′ ends

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Communicated by J.F.Atkins

The CCA trinucleotide is a universally conserved feature of the 3′ end of tRNAs, where it serves as the site of amino acid attachment. Despite this extreme conservation, we have isolated functional mutants of $tRNA^{His}$ and $tRNA_1^{Val}$ with altered CCA ends. A mutant that leads to de-repression of the histidine biosynthetic operon in *Salmonella typhimurium* has been characterized and found to have the CCA end of the sole $tRNA^{His}$ species mutated to UCA. However, constructed mutants of $tRNA^{His}$ with ACA or GCA ends appeared to be non-functional *in vivo*. Mutants of *Escherichia coli* $tRNA_1^{Val}$ with GCA or ACA ends were isolated on the basis of their ability to promote frameshifting at a specific sequence. These same $tRNA_1^{Val}$ mutants also caused read-through of stop codons that were one, or in some instances two, codons downstream of the valine codon decoded by the mutant tRNA. A startling implication of these data is that disruption of interactions between the CCA end of the tRNA and the large ribosomal subunit promotes these aberrant codon-anticodon interactions on the small ribosomal subunit.

Key words: protein synthesis/tRNA CCA end

Introduction

The CCA trinucleotide at the 3′ end of tRNAs is an invariant feature of all known tRNAs examined to date and the terminal nucleotide is the site of aminoacylation. The ultimate function of this trinucleotide is to place the attached amino acid in the peptidyl-transferase centre of the ribosome. During the course of protein synthesis, the 3′ end of a tRNA must interact with several translational components. As well as being involved in the interaction with tRNA synthetases, it is important for peptide bond formation, when the 3′ ends of two adjacent tRNAs must come into close proximity to allow transpeptidation to occur. Crystallographic studies, as well as chemical probing and nuclease protection experiments, have suggested that the CCA end is also important for the formation of ternary complexes of tRNA, elongation factor Tu (EF-Tu) and GTP. The successive interactions of the 3′ end of tRNAs with these components may be facilitated by the single stranded, flexible nature of the CCA end.

An aminoacylated CCA trinucleotide alone can interact with the large ribosomal subunit and donate its attached amino acid to an acceptor molecule located in the ribosomal A site (Munro and Marcker, 1967; Munro *et al.*, 1968). This suggests that the CCA trinucleotide of the tRNA contains most, or all, of the structural elements required for interaction with the peptidyl-transferase centre of the large ribosomal subunit. In several models of tRNA-ribosome interactions the CCA end interacts with the large subunit ribosomal RNA via canonical base pairing (Chladek and Sprinzl, 1985). In the most appealing of these models, the sequential movement of the tRNA from the A site to P to the E site during the translation cycle is initiated by the dissolution and formation of base pairing interactions between the flexible CCA end and successive series of complementary nucleotides in the large subunit rRNA (Moazed and Noller, 1989). Thus, interactions between the 3′ end of the tRNA and the ribosome may occur at all stages of the translation cycle.

The exact pathway followed by tRNAs through the ribosome has not been well described. Nevertheless, mutations in both components that perturb normal tRNA-ribosome interactions can be readily isolated using a variety of genetic selections. tRNA-derived informational suppressors that permit non-standard decoding have been useful in developing our understanding of the structural features of tRNAs that are important for interaction with synthetases, ribosomes and other translational components (Atkins *et al.*, 1991). The level of expression of many amino acid biosynthetic operons that are regulated by attenuation mechanisms is affected by the intracellular concentration of various tRNAs. In *Salmonella typhimurium*, the expression of the histidine operon is determined by the rate at which a ribosome translates seven tandem histidine codons in a leader region preceding the first structural gene of the operon (Johnson *et al.*, 1980). Mutations affecting both the level and the structure of $tRNA^{His}$ lead to de-repression of the operon (Brenner and Ames, 1972; Bossi and Smith, 1984).

In this study, selections for informational suppressors and characterization of a previously described, histidine de-repression mutant have uncovered tRNAs with altered 3′ ends. Mutants of $tRNA_1^{Val}$ with GCA or ACA 3′ ends are described which were isolated as suppressors of a −1 frameshift mutation and which were also found to suppress a wide variety of nonsense mutants. A previously described mutant of $tRNA^{His}$ that led to de-repression of the histidine operon was sequenced and found to contain a CCA → UCA change at the 3′ end. It is proposed that these effects are due to aberrant interactions between the 3′ end of the tRNA and (i) the large subunit rRNA in the case of misreading and (ii) the histidinyl-tRNA synthetase in the case of de-repression of the histidine operon.

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Results

Isolation of suppressor derivatives of tRNA$_1^{Val}$

The −1 frameshift mutation, *trpE91* (Atkins *et al.*, 1983), can be suppressed by a variety of altered translational components. Among such suppressors are mutants of EF-Tu (Hughes *et al.*, 1987; Tuohy *et al.*, 1990) and release factor 2 (Atkins and Ryce, 1974). In addition, a number of mutants of tRNA$_2^{Gly}$ have been isolated as suppressors of *trpE91*, at least some of which permit doublet decoding of GG (O'Mahony *et al.*, 1989; Pagel *et al.*, 1992). Previous work has described the isolation and characterization of mutants of tRNA$_1^{Val}$ that have insertions in the anticodon loop that permit dissociation and re-pairing of tRNA and message on the ribosome (O'Connor *et al.*, 1989). These mutants of tRNA$_1^{Val}$ are relatively efficient. However, a number of the mutants isolated at that time are much weaker mediators of frameshifting and, though also encoding variants of tRNA$_1^{Val}$, are quite distinct. These alleles, which are the subject of the current study, have been designated *valU11, 15, 16* and *530* (Hughes *et al.*, 1989).

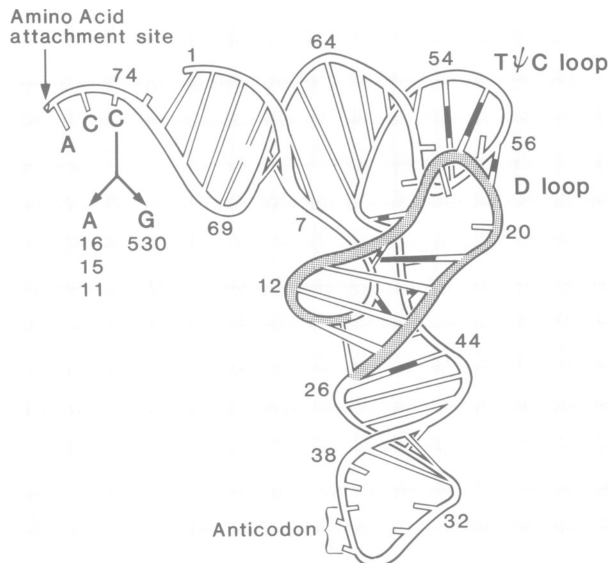

Fig. 1. Mutants of tRNA$_1^{Val}$ shown in a tertiary structure derived from that of yeast tRNA^{Phe}.

All four of these weak suppressor alleles were cloned into low copy-number vectors and sequenced. Comparison of their sequences with that of the wild-type *valU* operon (O'Connor *et al.*, 1990) revealed that in each case the mutation was a base substitution in the encoded CCA end, at the position corresponding to nucleotide 74 of the mature tRNA. In the case of *valU11, 15* and *16*, the change was from CCA to ACA, whereas in *valU530*, the change was from CCA to GCA (Figure 1). Since the sole change in each of the suppressor alleles is at nucleotide 74 in just one of the four identical copies of the gene encoding tRNA$_1^{Val}$ in *E.coli* (Komine *et al.*, 1990), it follows from their activity that these mutational alterations result in the synthesis of mature tRNAs with non-standard 3′ ends which can be aminoacylated and take part in protein synthesis in competition with wild-type tRNA$_1^{Val}$. These considerations make unlikely the possibility that the mutations cause a deficiency of charged wild-type tRNA$_1^{Val}$ which, by analogy with other situations (Weiss and Gallant, 1983), might have led to the suppressor phenotype.

Table I. Effects of stop codon read-through and +2 frameshifting by tRNA$_1^{Val}$ mutants

	mRNA sequence stop codons	tRNA$_1^{Val}$ CCA	tRNA$_1^{Val}$ GCA	tRNA$_1^{Val}$ ACA
p400	GUU UAG GCC	272	256	928
p401	GUG UAG GCC	58	50	438
p402	CUU UAG CUA	133	132	191
p410	GUG UGA UCC	28	67	449
p411	GUG UGA GCC	166	358	1167
p412	GAU UGA CCG	12	15	18
p415	GUG AAA UGA GCC	347	1140	974
p100	GUG UAA GUU	48	138	357
p141	GUG UAA CUG	21	24	73
p142	GUG UAA CUA	13	19	65
p181	GUG AAA UAA GUU	25	38	47
p183	GUG GAA UAA GUU	2	1	2
p184	GUG CAA UAA GUU	3	4	4
p120	AUG UAA GUU	6	8	8
	+2 Frameshifting			
p201	GUGUA CUA	19	37	25
p202	GUGUU CUA	31	35	58
p210	GUUUC CUA	16	26	26
p211	GUAUC CUA	52	87	114
p212	GUCUC CUA	4	5	5
p270	GUAAU AAC	25	20	26

Values for stop codon read-through and frameshifting are expressed in Miller units. Each value is the average of 3−6 independent determinations; standard errors were <20% in all cases.

Mutants of tRNA$_1^{Val}$ with altered 3′ ends cause frameshifting and read-through of stop codons

The tRNA$_1^{Val}$ mutants described above were isolated on the basis of their ability to cause frameshifting at a specific position within the *trpE* gene. In order to analyse the decoding properties of these novel tRNA mutants, a series of *lacZ* mutants was constructed in the extreme 5′ part of the gene where the identity of the encoded amino acids is unimportant for β-galactosidase activity. Each of these *lacZ* mutants requires that a ribosome bypass a stop codon or frameshift in order to make active, full-length β-galactosidase. The effects of the tRNA mutants on read-through and frameshifting were tested by co-transforming each *lacZ* mutant with a plasmid encoding either a wild-type or a mutant tRNA.

The data presented in Table I show that not only do the tRNA$_1^{Val}$ mutants cause frameshifting (p201, p202, p210 and p211) but, surprisingly, they also allow high levels of read-through of nonsense codons (p400, p401, p410, p411, p415, p100, p141, p142 and p181). The basic requirement for nonsense read-through is that the nonsense codon be preceded by a valine codon (compare p100 and p120). The exceptions to this general pattern are seen in p181 (GUG AAA UAA GUU) and p415 (GUG AAA UGA GCC) where the GUG valine codon is two codons upstream from the stop codon. However, when the lysine codon separating the valine and stop codons in these two constructs is replaced either by a glutamine codon (p184, GUG CAA UAA GUU) or by a glutamate codon (p183, GUG GAA UAA GUU), no read-through is observed.

Considerable variation exists both in the level of suppression of the various *lacZ* mutants and in the efficiency of suppression of a *lacZ* mutant by either of the two tRNA mutants. In general, most UAA codons were rather poorly read through (p141, p142 and p181) whereas high levels of read-through of both UGA and UAG codons were observed (p400, p401, p410, p411 and p415). The tRNA mutant with the CCA → ACA change at the 3′ end is considerably more efficient than the mutant with the CCA → GCA substitution; the latter mutant failed to suppress any of the UAG stop codon constructs tested (p400–p402). The identity of the codons 5′ and 3′ to the (suppressed) stop codon was found to influence the level of read-through seen with both wild-type and mutant tRNAs. Previous analyses in several laboratories have described analogous 5′ and 3′ context effects on nonsense read-through and suppression (Ayer and Yarus, 1986; Kopelowitz *et al.*, 1992).

Suppression by +2 frameshifting (constructs p201–p270) is considerably less efficient that the levels of nonsense read-through elicited by the same tRNA mutants. The GUC valine codon is decoded by two minor tRNA$_2^{Val}$ species whereas GUU, GUG and GUA are decoded by tRNA$_1^{Val}$. Comparison of p212 (GUCUC) with the remaining constructs in this series indicates that alteration of the 3′ end of the tRNA does not promote frameshifting at a GUC codon. The identity of the codon following the valine codons in these frameshift constructs also influences the level of frameshifting; UAC, UUC and UCC codons (p201, p202 and p211 respectively) permit frameshifting by both mutant tRNAs whereas an AUA codon in the same position (p270) abolishes any suppression.

No tRNA$_1^{Val}$ mutants with CCA → UCA changes at the 3′ end were found among the suppressors of *trpE91* isolated in the course of this study. A tRNA$_1^{Val}$ UCA mutant was constructed and expressed in the IPTG-inducible vector, pKK223-3 (Brosius and Holy, 1984). The constructed tRNA$_1^{Val}$ UCA mutant was viable but failed to suppress *trpE91* or any of the *lacZ* nonsense mutants tested (data not shown).

Stop codon read-through by tRNA$_1^{Val}$ mutants: induction of miscoding at the 3′ codon tRNA$_1^{Val}$

The ability of tRNA$_1^{Val}$ mutants to cause read-through at all three stop codons suggests that this occurs by some mechanism not involving codon–anticodon pairing between the valine tRNA and the stop codon. To investigate the mechanism by which this read-through occurs, β-galactosidase from strains containing a *lacZ* nonsense mutant and a mutant tRNA$_1^{Val}$ was isolated and sequenced. The protein sequencing data presented in Figures 2 and 3 (and sequence analysis of a *lacZ* UAG mutant isolated from a tRNA$_1^{Val}$ ACA-containing strain, data not shown) indicate

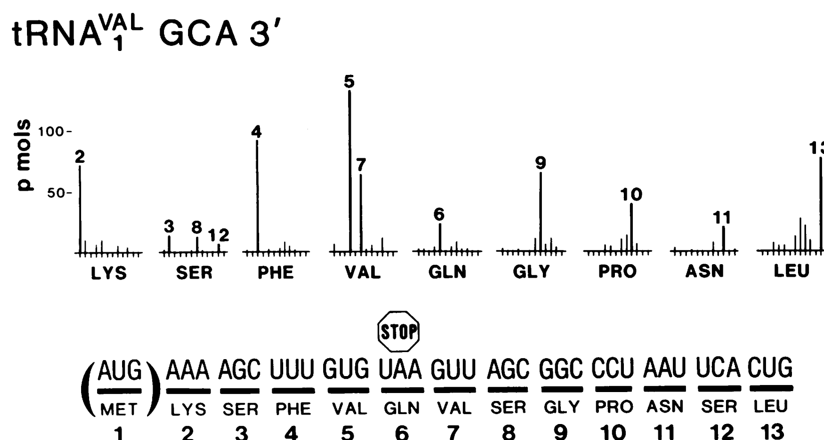

Fig. 2. Amino-terminal sequencing to determine the mechanism of read-through of UAA stop codons effected by tRNA$_1^{Val}$ GCA. Each panel shows the yield in picomoles of selected phenylthiodantoin-amino acids analysed at sequencing cycles 2–13. The aligned mRNA sequence is displayed underneath.

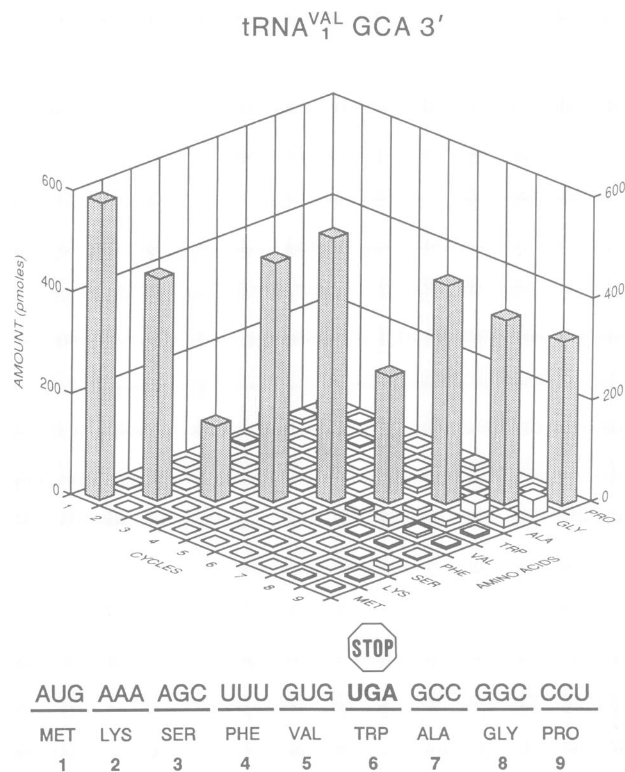

Fig. 3. Amino-terminal sequencing to determine the mechanism of read-through of UGA stop codons in strains containing tRNA$_1^{Val}$ GCA. Shown are the amounts in picomoles of selected phenylthiodantoin-amino acids derived from cycles 1–9. Underneath is displayed the aligned mRNA sequence with the (suppressed) in-frame UGA stop codon shown in bold type.

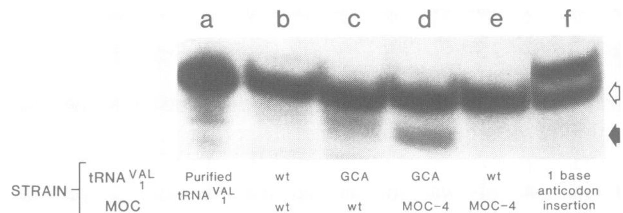

Fig. 4. Northern blot analysis of purified tRNA$_1^{Val}$ (a) and total tRNA isolated from *moc*^{wt} and *moc-4* strains containing either wt tRNA$_1^{Val}$ (lanes b and e) or tRNA$_1^{Val}$ GCA (lanes c and d). Lane f contains tRNA isolated from a strain containing a mutant of tRNA$_1^{Val}$ with a single base insertion in the anticodon. The filter was probed with labelled single-stranded DNA complementary to tRNA$_1^{Val}$.

that read-through occurs by a similar mechanism in each case. In the UAG and UAA mutants, glutamine is inserted at the stop codon and in the UGA mutant, tyrptophan is inserted. As wild-type tRNA^{Trp} can misread UGA codons *in vivo* while both UAA and UAG codons can be decoded at a low level by tRNA^{Gln} (Hirsch and Gold, 1971; Davies *et al.*, 1966; Weiss *et al.*, 1987b), changes at the 3′ end of tRNA$_1^{Val}$ thus increase the frequency of these naturally occurring miscoding events. These novel findings suggest that tRNA$_1^{Val}$ mutants decode valine codons accurately but alter the ribosome's ability to discriminate between cognate and near-cognate tRNAs at the codons downstream of the valine codon.

In an analogous manner, we have attempted to characterize the frameshifting events affected by tRNA$_1^{Val}$ ACA/GCA mutants by protein sequencing. Despite several attempts, it has not been possible to obtain unambiguous protein sequence of β-galactosidase isolated from strains carrying both a *lacZ* frameshift and a mutant valyl-tRNA. Invariably, several different signals were obtained from such analyses. Sequences obtained from strains carrying p211 (GUAUC) showed that quintuplet decoding of GUAUC, doublet decoding of GU as well as doublet decoding of UC occurred, both in the presence of the mutant tRNA and in the wild-type strain. Each of these polypeptides represents a very low-level decoding event. The relative contribution of the mutant tRNA to each or any of these events is unknown because of the inadequate resolution of protein sequencing.

Modifiers of tRNA$_1^{Val}$ suppressors

Both tRNA$_1^{Val}$ ACA and GCA cause a reduction in cell growth and are lost when cells are grown under non-selective conditions or acquire secondary mutations that restore rapid cell growth. This effect is more pronounced in the ACA mutants which are the stronger suppressors. The nature of one of these secondary mutations (termed *moc* for modifier of CCA) was investigated by mapping one of the alleles. This particular mutant, *moc-4*, arose in the tRNA$_1^{Val}$ GCA strain during growth on minimal medium at 37°C and was easily distinguished from the parental strain by its faster growth. Hfr mapping experiments showed that the mutation mapped to the 30′ region of the chromosome, distant from the *valU* operon (52′). Transductional crosses with transposon insertions in the 30′ region showed that the *moc-4* mutation was 86% co-transducible with *trg*::Tn*10* and 2% co-transducible with *zdd230*::Tn*9*. These data place the *moc* gene at ~31′ on the *E.coli* genetic map (Bachmann, 1990). No genes involved in translation have been mapped to this region of the chromosome.

The tight linkage of *trg*::*Tn*10 and *moc-4* has facilitated the transfer of the *moc-4* mutation into wild-type strains or strains containing either of the tRNA$_1^{Val}$ mutants. Strain MC134 carries both *trpE91* and the *moc-4* mutation and is phenotypically Trp$^-$. This demonstrates that while *moc-4* may modify the activity of a tRNA, it does not have any suppressor activity of its own. The effects of the *moc-4* mutation on the tRNA$_1^{Val}$ ACA and GCA mutants were compared by transforming MC134 with plasmids carrying either of the mutant tRNAs. The *moc-4* mutation increased the growth rate of both suppressor containing strains, indicating that it acted on both ACA and GCA 3′ ends.

The effects of the *moc-4* mutation on suppression efficiency have been examined by transforming *moc-4*, tRNA$_1^{Val}$ mutant strains with *lacZ* UAG or UGA constructs and measuring β-galactosidase in the double mutants. The level of UGA read-through observed with the p411 construct (GUG UGA GCC) in the presence of wild-type tRNAs was relatively unaffected by the presence of the *moc-4* mutation (139 versus 161 units). However, the *moc-4* mutation caused at least a halving in the level of UGA read-through directed by either of the two tRNA$_1^{Val}$ mutants (179 units in a *moc-4*, tRNA$_1^{Val}$ GCA strain compared with 358 units in the absence of the *moc-4* mutation and 315 units in a *moc-4*, tRNA$_1^{Val}$ ACA strain compared with 1167 units in an isogenic, *moc*^{wt} strain). A similar pattern was observed with the *LacZ* UAG mutant, p400 (data not shown).

Direct evidence for modification of the mutant tRNAs in a *moc-4* strain was provided by Northern blot analysis of

bulk tRNA extracted from *moc-4* and *moc*^{wt} strains (Figure 4). The tRNA extracted from a *moc-4* tRNA$_1^{Val}$ GCA strain contained, in addition to the mature tRNA$_1^{Val}$ species, a lower molecular weight species which hybridized to a tRNA$_1^{Val}$-specific probe. This shorter species was not observed in strains containing either mutant or wild-type tRNAs but lacking the *moc-4* mutation, or in a *moc-4* strain containing only wild-type tRNAs. Thus, modification of mutants of tRNA$_1^{Val}$ in the *moc-4* containing strain is restricted to tRNAs with altered 3′ ends.

The observation that *moc-4* directly modifies tRNAs with altered 3′ ends provides an explanation for the decreased levels of stop codon read-through and the higher growth rates observed in tRNA$_1^{Val}$ ACA/GCA, *moc-4* mutants. Shortening of the tRNAs, observed in the *moc-4* strains, is expected to render these tRNAs inactive in translation. Consequently, less (full-length) mutant tRNA is available to cause stop codon read-through, frameshifting and any other deleterious effect of these tRNAs, resulting in the restoration of rapid cell growth.

The map position of the *moc-4* mutation does not correspond to that of any of the known ribonucleases or tRNA processing enzymes. Thus, *moc-4* defines a new gene somehow involved in tRNA synthesis or processing. The effect of the *moc-4* mutation could arise through activation of a cryptic nuclease or alteration of the target specificity of any of several ribonucleases known to be involved in tRNA processing (Deutscher, 1990).

The coding capacity of the 30′ region of the *E.coli* chromosome has recently been analysed by Moir *et al.* (1992), who have cloned 44 kb of DNA from this segment of the chromosome onto high copy number plasmid vectors. None of the genes in this region are essential since this entire segment of the chromosome can be deleted (Henson *et al.*, 1984). We have used the clones constructed by Moir *et al.* (1992) to carry out complementation analyses of the *moc-4* mutant. Of this collection of plasmid clones, only pPM2001 which contains a 10.5 kb *Hind*III insert, restored the low growth rate characteristic of *moc*^{wt}, tRNA$_1^{Val}$ ACA/GCA strains to *moc-4* tRNA$_1^{Val}$ ACA/GCA strains. This identifies the product of the *moc* gene as one of the unidentified gene products encoded by pPM2001.

Mutants of tRNA^{His} with altered 3′ ends

In both *E.coli* and its close relative *Salmonella typhimurium*, expression of the histidine operon is controlled by an attenuation mechanism. A number of mutants affecting the expression, primary sequence and modification of tRNA^{His} (encoded by the *hisR* gene) have been obtained by selection for de-repression of the histidine operon. The *S.typhimurium hisR1203* mutation leads to an 11-fold de-repression of histidine biosynthesis (Roth *et al.*, 1966).

The *hisR1203* mutant was cloned and sequenced. The sole difference between the wild-type gene and the *hisR1203* allele was a CCA → UCA change in the encoded CCA end, the same position altered in the tRNA$_1^{Val}$ mutants described above.

De-repression of the histidine operon by *hisR1203* could be due to either poor aminoacylation of the mutant tRNA, a poor association between the mutant tRNA^{His} and EF-Tu, or an alteration in the transit time of tRNA^{His} through the ribosome; the latter might favour formation of the antiterminator structure in the *his* operon leader (Johnson *et al.*, 1980).

Table II. Effects of tRNA^{His} mutants on histidine operon expression

Plasmid	MS28 (*hisR*$^-$)	MS29 (*hisR*^{wt})
None	730 ± 46	18 ± 4
pACYC184	620 ± 48	14 ± 1
ptRNA^{His} CCA (wt)	10 ± 2	9 ± 1
ptRNA^{His} UCA (1203)	21 ± 2	12 ± 1
ptRNA^{His} ACA	895 ± 173	29 ± 2
ptRNA^{His} GCA	933 ± 150	22 ± 4

MS28 and MS29 are isogenic strains containing a *hisD−lacZ* fusion; in addition, MS28 carries the *hisR1203* mutation whereas MS29 is *hisR*^{wt}. Values are Miller units of β-galactosidase activity and each represents the average of 4−6 independent determinations.

In an attempt to distinguish between these possibilities, the *S.typhimurium* strains MS28, which carries both the *hisR1203* mutation and a *hisD−lacZ* fusion, and MS29, an isogenic strain with a wild-type *hisR* gene, were transformed with plasmids carrying either a wild-type or mutant *hisR* operon. The level of β-galactosidase in these strains is proportional to the extent of de-repression of the histidine biosynthetic operon. The data presented in Table II show that de-repression of the histidine operon due to *hisR1203* mutation is virtually abolished in the presence of a plasmid carrying a wild-type tRNA^{His}. Furthermore, no significant de-repression of the histidine operon is seen in a wild-type strain (MS29) in the presence of a plasmid carrying the *hisR1203* allele. When the concentration of tRNA^{His} UCA in the cell is increased by transforming the *hisR1203* mutant with a plasmid containing the same tRNA^{His} mutant, the histidine operon is now fully repressed (Table II). The simplest explanation of these results is that tRNA^{His} UCA is partially defective in its interaction with the synthetase, EF-Tu or ribosomes; supplying either wild-type tRNA^{His} or increasing the level of the defective species (tRNA^{His} UCA) provides sufficient charged tRNA in ternary complex to restore repression of the operon.

The effects of CCA → ACA/GCA changes on tRNA^{His} function (as measured by the effect on histidine operon expression) have also been analysed. These mutants were constructed by site-directed mutagenesis. The data presented in Table II show that the presence of tRNA^{His} ACA or GCA either in a wild-type strain (MS29) or a tRNA^{His} UCA mutant (MS28), does not lead to any significant alteration in the level of expression of the *his* operon. The inability of either of these mutant tRNAs to complement the histidine de-repression phenotype of the tRNA^{His} UCA mutant suggests that these tRNAs are even more defective in interaction with the synthetase or the ribosome than the tRNA^{His} UCA mutant. This finding parallels the situation found with the tRNA$_1^{Val}$ mutants: the tRNA$_1^{Val}$ UCA mutant had little or no effect on tRNA−ribosome interaction whereas both the GCA and ACA mutants had significant effects on tRNA function.

Analysis of mutants of tRNA$_1^{Val}$ with altered 3′ ends indicated that they affected the fidelity of decoding. The effects of mutations in the CCA end of tRNA^{His} were analysed in an analogous experiment. Each of these tRNA mutants (carried on high copy number vectors) was transformed with a *lac* −1 frameshift mutant (p739, CACAC) or either of two *lacZ* nonsense mutants (p347, CAU UGA UCC and p3411, CAC UGA UCC). In each

of these *lacZ* mutants (by analogy with the *lacZ* mutants used in the $tRNA_1^{Val}$ study described above) a read-through or frameshift event must occur at or directly after the CAU or CAC histidine codon. No difference between wild-type and mutant $tRNA^{His}$ was observed with any of the *lac* constructs used. This lack of effect is due, at least in part, to a defect in the ability of these $tRNA^{His}$ mutants to be aminoacylated and/or bind to the ribosome. Thus, an unequivocal answer to the effect of mutations in the 3′ end of $tRNA^{His}$ on translational fidelity is not possible from these experiments.

Discussion

During a single round of translation, at least two distinct regions of a tRNA, the CCA end and the anticodon loop, must interact with the ribosome. Chemical protection experiments have indicated that only the anticodon stem and loop are required for protection of 16S ribosomal RNA residues (Moazed and Noller, 1991) in the 30S subunit. Biochemical analyses of the minimal features required for peptide bond formation indicate that the CCA end and its attached amino acid account for most of the interactions between the 50S ribosomal subunit and the tRNA. Despite the implicit functional importance of the CCA terminus in ribosome–tRNA interactions and the universal conservation of this feature of tRNAs, this study has demonstrated that tRNAs with some alterations of the CCA end are fully competent in translation. Nonetheless, these changes affect tRNA–ribosome interactions and also in the case of $tRNA^{His}$, the interaction with the histidyl-tRNA synthetase, EF-Tu or the ribosome.

The clearest consequence of changing the CCA end of $tRNA_1^{Val}$ is to cause misreading at a stop codon 3′ to a valine codon (Figures 2 and 3). The startling implication from this observation is that disruption of the interaction between the 3′ end of tRNA and the large ribosomal subunit affects the interaction, on the small subunit, of the next incoming tRNA with the 3′ codon. Thus, the protein sequencing data suggest that when a tRNA with a mutant 3′ end is bound to its cognate codon in the ribosomal P site, the ability of the ribosomal A site to discriminate between the binding of cognate and near-cognate tRNAs is reduced. This suggests a coupling between A and P sites operates to achieve the high accuracy of translation characteristic of wild-type ribosomes. Some evidence in support of this prediction derives from the work of Bergemann and Nierhaus (1984), who showed that occupation of the P site by a non-cognate tRNA reduced the accuracy of tRNA selection at downstream codons. Furthermore, an effect of the P site tRNA on the decoding efficiency at the A site has recently been demonstrated by Kato *et al.* (1990). The finding that the same mutant valine tRNAs can promote read-through of both UAA and UGA codons located two codons downstream of the valine codon (p181, GUG AAA UAA GUU and p415, GUG AAA UGA GCC, Table I) indicates that misreading at the A site can also occur when the mutant tRNA is located in the exit (E) site of the ribosome. *In vitro* experiments indicate that the A and E sites of the ribosome are linked allosterically; occupation of the A site reduces the affinity of the E site for tRNAs and vice versa (Nierhaus, 1990). The contribution of E site occupancy to tRNA selection at the A site has been addressed by Geigenmuller and Nierhaus (1990). Ribosomes containing a vacant E site or an E site occupied by a non-cognate tRNA displayed high levels of misreading at the A site. Taken together, both sets of results suggest that distortion of the interactions between the 3′ end of the tRNA and the E site affects the conformation of the allosterically coupled A site and allows binding of near-cognate tRNAs.

Several models can be formulated to explain the effects of a mutant 3′ end on decoding at downstream codons. In the first model, changes at the 3′ end may affect the overall structure of the tRNA molecule both at the 3′ end and in the anticodon loop region, resulting in a distortion of ribosome–tRNA–mRNA interactions in the P site. This distortion might then induce a perturbation of codon–anticodon interactions at the adjacent A site. The observation that both mutant valine tRNAs compete effectively with an excess of wild-type $tRNA_1^{Val}$ to cause frameshifting and nonsense read-through strongly suggests that the structure of these mutant valine tRNAs is not grossly distorted. All of the mutant tRNAs are processed correctly and so these changes at the 3′ end must not affect interactions between processing enzymes and the tRNA. Furthermore, since we have not observed any misreading of valine codons by the mutant tRNAs in the protein sequencing experiments, the mutant valine tRNAs must interact correctly with the valine synthetase and maintain correct codon–anticodon pairings in the A site.

The second model proposes that only the interaction between 23S rRNA and the 3′ end of the mutant tRNA is altered by mutations in the CCA terminus. This altered interaction then affects the conformation of 23S rRNA in the neighbouring A site (or the allosterically coupled E site), resulting in a loss of discrimination against near-cognate tRNAs.

Both phylogenetic analyses and chemical protection experiments using either intact tRNAs or tRNAs with successively shorter 3′ ends or NACCA fragments have suggested that base pairing occurs between the CCA trinucleotide and 23S rRNA (Moazed and Noller, 1991). All of the protected bases are found in a region of 23S rRNA (the central loop region of domain V) which many lines of evidence have implicated in peptide bond formation. Several residues in this loop are protected from chemical attack or have been cross-linked to the CCA end of both A and P site-bound tRNAs (Noller, 1991). Thus, loss of a base pairing interaction between C74 of a P site-bound tRNA and 23S rRNA might well be expected to change the conformation of the rRNA in this region which comprises part of both the P site and the adjacent/overlapping A site.

Different ribosomal proteins are closer to the CCA end of tRNA in the E site than are those in the A and P sites (Wower *et al.*, 1993). Consistent with this, chemical probing experiments of ribosomal complexes using an E site-bound tRNA have identified specific and highly conserved 23S rRNA residues protected by this tRNA. Again, some of these protected bases (and/or adjacent bases) have the potential to pair with the CCA end of the tRNA. Further evidence for the importance of the CCA end in E site tRNA binding comes from the work of Wintermeyer and co-workers (Lill *et al.*, 1988) who demonstrated that tRNA binding to the E site is drastically reduced by changes at the 3′ end. An effect of alterations at the CCA end on the rate of translocation and on EF-G function has also been observed (Lill *et al.*, 1989). The effect of modifications at the CCA

end on translocation may be related to the ability of $tRNA_1^{Val}$ ACA/GCA mutants to cause frameshifting *in vivo* as was observed in this study.

The influence of changes in the CCA end on tRNA–ribosome interactions at the A and P sites has been investigated by Chladek and co-workers (Tezuka and Chladek, 1990; Quiggle *et al.*, 1981). Their analyses suggest that A site binding probably does not involve CCA–23S rRNA base pairing whereas the base pairing potential of the CCA end of a P site-bound tRNA was found to be a major determinant of P site function. These data are consistent with our findings; both GCA and ACA mutant valine tRNA can compete with wild-type $tRNA_1^{Val}$ for binding to the A site, the effects of these mutant tRNAs are observed only when bound in the P or E sites. The results obtained with the $tRNA_1^{Val}$ mutants are also consistent with the existence of base-pairing between the CCA terminus and 23S rRNA. Mutations which distorted this pairing (CCA → ACA/GCA) affected decoding. Intriguingly, the constructed $tRNA_1^{Val}$ UCA mutant which still allowed (G-U) pairing to rRNA had no effect on decoding.

Analysis of mutants of $tRNA^{His}$ suggests that alteration of C74 in this tRNA affected its association either with the histidinyl-tRNA synthetase, EF-Tu or the ribosome. This is in contrast to the $tRNA_1^{Val}$ mutants which were not affected in their interactions with either the valyl-tRNA synthetase or EF-Tu and competed effectively with wild-type $tRNA_1^{Val}$ for binding to the ribosomal A site. While the interactions between a tRNA and EF-Tu (or the ribosome) are likely to be common to all tRNAs, the interactions between a tRNA and its cognate synthetase are likely to be particular to that tRNA–synthetase pair. This reasoning suggests that it is the interaction between the histidinyl-tRNA synthetase and $tRNA^{His}$ that is affected by the CCA → UCA change. Both crystallographic analyses and mutagenesis studies have indicated a crucial role for the acceptor stems of tRNAs in synthetase recognition and these acceptor stem determinants differ between aminoacyl-tRNA synthetases (Francklyn *et al.*, 1992). Aminoacyl-tRNA synthetases have been divided into two classes based on the presence of common sequence motifs. Valyl- and histidinyl-tRNA synthetases belong to different classes of aminoacyl-tRNA synthetases (classes I and II respectively) and consequently, are expected to have differing interactions with their cognate tRNA substrates (Eriani *et al.*, 1990). Thus, the CCA end may very well be important for the interaction between a tRNA and its synthetase in the context of a particular aminoacyl stem–synthetase interaction and consequently, changes in the CCA terminus may affect differentially the association of $tRNA^{His}$ and $tRNA_1^{Val}$ with their cognate aminoacyl-tRNA synthetases. Consistent with this view, preliminary experiments suggest that the defect in the $tRNA^{His}$ UCA mutant can be reversed by mutations in the histidinyl-tRNA synthetase gene that alleviate the aminoacylation defect in this tRNA.

Evidence gleaned from several diverse sources has led to the proposal that tRNA-like molecules, which minimally consisted of a simple stem–loop structure with a 3′ CCA, served to 'tag' the ends of ancient linear RNA genomes for replication by acting as initiation sites (Weiner and Maizels, 1987; Maizels and Weiner, 1993). The sequence CCA might have been selected in an RNA world as an efficient initiation site because the initiating guanosines would stack more strongly and have the potential for more hydrogen bonding interactions than adenosines or uridines. Furthermore, the ability to aminoacylate a CCA end may also have been selected for replication purposes (Maizels and Weiner, 1993). Irrespective of any replicatory roles, the universal occurrence of a 3′ terminal CCA on the modern adaptors for protein synthesis, tRNAs, suggests a significant current role. This study shows that the 5′ C of this universally conserved trinucleotide is not an absolute requirement for protein synthesis. However, the frameshifting and read-through results demonstrate that at least some loss of fidelity is a consequence of mutating that position.

Materials and methods

Bacterial strains and techniques

The isolation and mapping of suppressor alleles of $tRNA_1^{Val}$ (some of which are termed *hopR*) has been described (Hughes *et al.*, 1989). Each of the $tRNA_1^{Val}$ mutants described here, *valU11, valU15, valU16* and *valU530*, acts as a weak suppressor of the −1 frameshift mutation, *trpE91*. MC85 and its *recA*⁻ derivative, MC126, are derivatives of CSH41 (Miller, 1972) and contain a chromosomal copy of the *trpE91* allele. The *S.typhimurium hisR1203* mutant which is derepressed for histidine biosynthetic enzymes has been described previously (Roth *et al.*, 1966). TT10286 (Hughes and Roth, 1988) contains a M*ud-lac* (kanamycin resistant) fusion in the *hisD* gene of *Salmonella*; consequently, in this strain, the level of β-galactosidase is regulated by the same regulatory circuits that control the level of expression of the histidine operon. This *hisD–lacZ* fusion was introduced into the *hisR1203* strain by P22 mediated transduction, selecting for kanamycin resistance. TT10286 and *hisR1203 hisD9953::MudJ* were both made *recA*⁻ by transducing both strains to chloramphenicol resistance with phage prepared on TE600 (*recA*⁻ *srl::Tn10dCam*) and screening for the Rec⁻ phenotype (UV sensitivity) to give MS29 and MS28 respectively. SU1675 F′*lacI*^{q} (Weiss *et al.*, 1987a) was used as a host in routine cloning experiments. Both LB medium and the minimal (E) medium of Vogel and Bonner (1956) were used for routine cultivation of bacteria. Mutations which alleviated the growth defect of the $tRNA_1^{Val}$ GCA mutant (*moc* mutants, see text) were isolated on tryptophan-free minimal medium at 37°C. These *moc* mutations were mapped initially by a series of Hfr crosses, using the Hfr Tn*10* kit and methodology described in Wanner (1986) and using the faster-growth phenotype of the *moc* mutation to score for the presence of this marker in the progeny. Strains carrying Tn*10* insertions in the 30′ region of the *E.coli* chromosome (Bitner and Kuempel, 1981; Hazelbauer *et al.*, 1981) were obtained from Dr Barbara Bachmann. P1 transductions were carried out as described in Miller (1972).

Cloning and sequencing of $tRNA_1^{Val}$ and $tRNA^{His}$ mutants

A *valU530* clone was isolated from a *Hin*dIII *valU530* genomic library constructed in the low copy number, trimethoprim resistant vector, pUB5572 (Chopra *et al.*, 1981) by transforming MC85 with this entire library, selecting simultaneously for trimethoprim resistance and tryptophan independence. The suppressor-containing fragment of DNA was subcloned into the pSC101-based vector, pLG339 (Stoker *et al.*, 1982) on a 400 bp *Hin*cII fragment which contains only the three tandem $tRNA_1^{Val}$ genes of the *valU* operon. The other suppressor alleles, *valU11, 15* and *16*, were cloned by recombination *in vivo*. Each suppressor-containing strain was transformed with p814, a pLG339-derived plasmid containing the entire *valU* operon on a 2 kb *Xho*II fragment. Recombination between plasmid and chromosome yielded plasmids capable of transforming a *trpE91* strain to tryptophan independence. Plasmid DNA was isolated from each *valU*, p814 strain and used to transform MC85 to tryptophan independence and kanamycin resistance. Putative suppressor-containing clones were verified by a further round of transformations. For each *valU* clone, both strands of a 400 bp region encompassing the entire *valU* operon were sequenced as described previously (O'Connor *et al.*, 1989).

Cloning and sequencing of the wild-type *hisR* operon has been described previously (Bossi, 1983). The *hisR1203* mutant was cloned on a 1.5 kb *Sau*3A fragment into the *Bam*HI site of the M13 vector mWJ43. Positive clones were isolated by hybridization with ^{32}P-labelled $tRNA^{His}$ and sequenced as described by Bossi (1983). For easier manipulation of wild-type and mutant clones, a 972 bp *Eco*RI fragment containing the entire *hisR* operon was subcloned from each M13 clone into the *Eco*RI site of pACYC184 (Chang and Cohen, 1978).

Construction of further tRNA mutants

Mutants of $tRNA^{His}$ with GCA or ACA ends were constructed by site directed mutagenesis of the M13 clone carrying the wild-type *hisR* operon using the methods described in Kunkel *et al.* (1985). Each mutant was verified by sequencing and was subcloned onto pACYC184 as described above.

Variants of $tRNA_1^{Val}$ were constructed by cloning complementary oligonucleotides encoding an entire $tRNA_1^{Val}$ gene (with a mixture of all four bases at the position corresponding to base 74 of the mature tRNA) into the expression vector, pKK223-3 (Brosius and Holy, 1984).

Northern analysis of tRNAs

Bulk tRNA was isolated according to the method described in Bossi and Smith (1984). The tRNAs were separated on a 12% polyacrylamide, 7 M urea gel and transferred onto a nylon filter by electroelution (Church and Gilbert, 1984). A $tRNA_1^{Val}$-specific probe was generated by primer extension of single stranded DNA from an M13 clone containing the sense strand of the *valU* operon.

LacZ mutants

Many of the *lacZ* mutants used here were described in a previous study of $tRNA_1^{Val}$ mutants (O'Connor *et al.*, 1989). Additional mutants were constructed by ligating annealed mixtures of complementary oligonucleotides containing *Apa*I and *Hin*dIII overhangs into the *lacZ* vector, p9091 (Weiss *et al.*, 1987a). β-galactosidase was assayed as described previously (O'Connor *et al.*, 1989).

Protein purification and sequencing

Cultures were grown overnight in superbroth, spun down and resuspended in PBST (50 mM KPO_4, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 10 mM β-mercaptoethanol) and disrupted by two serial passages through a French press. Cell debris was removed by low speed centrifugation and the supernatant was further clarified by centrifugation for 4 h in a Beckman TLA100.3 rotor at 65 000 r.p.m. This post-ribosomal supernatant was then passed over an immunoaffinity column (Protosorb, Promega Biotech) and was eluted with 0.1 M Na_2CO_3. The eluate was concentrated in a Centricon spin column and loaded directly onto an Applied Biosystems model 470A gas phase sequencer, fitted with liquid pulse attachment and data analysis module.

Acknowledgements

We gratefully acknowledge Bob Weiss, Diane Dunn, Ed Meenan and Siegfried Rieble for providing oligonucleotides, plasmids and help with protein purification and sequencing. We are grateful to Drs John Roth, Barbara Bachmann, Tom Elliott, Yvonne Charteris and Millicent Masters for providing us with bacterial strains and plasmids. We thank Kate Lieberman, Steven Gregory and George Q.Pennabble for stimulating discussions on this work, Dr Albert Elvis Dahlberg for his generous support and thank the members of the Gesteland and Dahlberg labs for their continued interest in this project. This work (in Salt Lake City) was supported by the Howard Hughes Medical Institute and by a grant from the National Institutes of Health.

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Received on January 22, 1993; revised on March 2, 1993

Note added in proof

N.B.Reuven and M.P.Deutscher (1993 *Proc. Natl Acad. Sci. USA*, in press) have found that mutants of the terminal A (base 74) of $tRNA^{Tyr}$ $su3^+$ are active but dependent on repair of the mutant CCN 3′ ends, in contrast to the mutants described here.