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In vivo evidence for the prokaryotic model of extended codon–anticodon interaction in translation initiation

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Initiation codon context is an important determinant of translation initiation rates in both prokaryotes and eukaryotes. Such sequences include the Shine–Dalgarno ribosome-binding site, as well as other motifs surrounding the initiation codon. One proposed interaction is between the base immediately preceding the initiation codon (–1 position) and the nucleotide 3′ to the tRNAf^{Met} anticodon, at position 37. Adenine is conserved at position 37, and a uridine at –1 has been shown *in vitro* to favor initiation. We have tested this model *in vivo*, by manipulating the chloroplast of the green alga *Chlamydomonas reinhardtii*, where the translational machinery is prokaryotic in nature. We show that translational defects imparted by mutations at the *petA* –1 position can be suppressed by compensatory mutations at position 37 of an ectopically expressed tRNA^{fMet}. The mutant tRNAs are fully aminoacylated and do not interfere with the translation of other proteins. Although this extended base pairing is not an absolute requirement for initiation, it may convey added specificity to transcripts carrying non-standard initiation codons, and/or preserve translational fidelity under certain stress conditions.

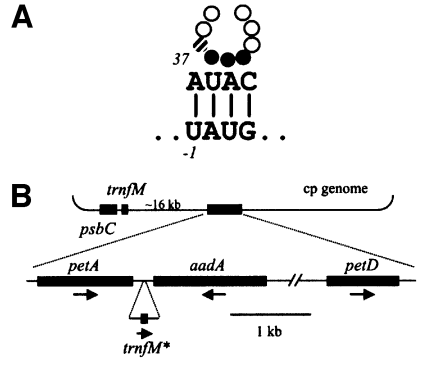
Keywords: anticodon/*Chlamydomonas*/chloroplast/initiator tRNA

Introduction

The roles of sequences surrounding the initiation codon in prokaryotic translation initiation have been extensively studied (reviewed by McCarthy and Brimacombe, 1994). The completion of the *Escherichia coli* genome has facilitated global analysis of translation initiation context, either as a gene verification/discovery tool (Delamarche *et al*., 1999; Walker *et al*., 2002) or to study motifs that potentially modulate translation initiation rates (Stenstrom *et al*., 2001). The bulk of this analysis, however, has focused on the well-known Shine–Dalgarno (SD) ribosome-binding site or on nucleotides following the initiation codon, which may (Moll *et al*., 2001) constitute a regulatory 'downstream box'. What has not been reassessed is the possibility of extended interactions between the mRNA and the initiator tRNA (tRNA^{fMet}).

The question of extended tRNA^{fMet}–mRNA base pairing was raised by the observation that residues complementary to nucleotides in the tRNA^{fMet} anticodon loop are more prevalent immediately upstream of initiation codons than predicted by chance (Ganoza *et al*., 1985). In particular, a uridine at position –1 was proposed to allow a fourth base pair with the adenine at position 37 of the tRNA^{fMet} (immediately downstream of the anticodon), a model consistent with several *in vitro* assays (see Discussion). Since many aspects of sequence context influence translation rates, and because *in vitro* experiments may not always fully reflect *in vivo* mechanisms, it is critical that the extended codon–anticodon model be tested *in vivo*. An accepted way to do so would be the demonstration that effects of –1 mutations on translation could be compensated by complementary mutations at position 37 in tRNA^{fMet}. Such a strategy was useful in confirming the interaction of the SD sequence (Shine and Dalgarno, 1974) with a complementary sequence at the 3′ end of 16S rRNA (Hui and de Boer, 1987). Unfortunately, residue A_{37} was shown to be important for the aminoacylation of tRNA^{fMet} in *E.coli* (Meinnel *et al*., 1993), rendering this approach problematic in bacteria.

Here we have chosen to test the extended base pairing model in the chloroplast, whose translational apparatus is derived from, and generally resembles that of, prokaryotes (Harris *et al*., 1994; Sugiura *et al*., 1998). In the chloroplast of the unicellular green alga *Chlamydomonas reinhardtii*, mutagenesis of the initiation codon of the *petA* mRNA, which encodes cytochrome *f* (cyt *f*), a component of the cytochrome b_6f complex in the photosynthetic electron transport chain, revealed that, as in prokaryotes, an AUG initiation codon is not required for the selection of the correct start site, but that AUU, ACG, ACC and ACU progressively reduce translation efficiency (Chen *et al*., 1995). Surprisingly, it was found in the same study that mutating the three nucleotides upstream of the initiation codon from AUU to UAA dramatically reduced cyt *f* synthesis, especially in the context of a mutant AUU initiation codon. This result raised the possibility that one or more of the –3 to –1 positions relative to the initiation codon had a functional importance. It was subsequently shown that mutation of the $_{-1}$U residue severely reduced translation when combined with an AUU initiation codon, or at elevated temperature (35°C) in the context of an AUG initiation codon (Esposito *et al*., 2001). These observations, taken together with a 75% conservation of a uridine at position –1 in the chloroplast mRNAs of *C.reinhardtii* (the genome sequence is reported in Maul *et al*., 2002), led



C

construct	petA + ectopic tRNA		base pairs with WT tRNA	base pairs with ectopic tRNA
	-1	37		
WT cell →	U - AUG	+ A	4	4
	U - AUG	+ U	4	3
	U - AUU		3	
	U - AUU	+ A	3	3
	U - AUU	+ U	3	2
	U - AUU	+ G	3	2
	A - AUU		2	
	A - AUU	+ U	2	3
	A - AUU	+ A	2	2
	C - AUU	+ G	2	3
	C - AUU	+ A	2	2
	C - AUU	+ U	2	2

Fig. 1. Expression of ectopic tRNAs in the chloroplast. (**A**) Extended interaction model between the initiator tRNA and mRNA $_{-1}$U. The three closed circles denote the tRNA anticodon; the A_{37} residue is shown by a hatched circle. (**B**) Transformation strategy to mutate the *petA* gene and, for certain constructs, to introduce an additional tRNA gene into the chloroplast genome. The top part shows the location of the endogenous *trnfM* gene downstream of *psbC*, and its distance from the *petA–petD* region. The lower line shows the altered region in transformants (the 1.8 kb *aadA/petD* intergenic region is not to scale), with gene orientations indicated by arrows. The notation *trnfM** indicates the ectopic copy. (**C**) Names of the strains created (left column), and the number of predicted base pairs that the particular version of the *petA* mRNA would form with the endogenous $tRNA^{fMet}$ (middle column) or with the ectopic $tRNA^{fMet}$ where present (right column). Strains are named by the *petA* mRNA sequences at –1 and the initiation codon (e.g. U-AUG), plus position 37 of the $tRNA^{fMet}$ (e.g. +A). Mutated residues relative to WT are in bold. In columns 2 and 3, the strongest base pairing possibility is bold and underlined.

us to consider whether the *E.coli* extended interaction model could also be proposed for plastid translation. Here we show first that in the *C.reinhardtii* chloroplast, the adenine at position 37 of $tRNA^{fMet}$ is not important for aminoacylation. Then, using a series of mutations affecting the *petA* initiation codon and –1 uridine, we show that compensatory mutations introduced at position 37 of an ectopically expressed copy of the chloroplast initiator tRNA can partially restore the cyt *f* synthesis rate.

Results

Generation of trnfM mutants and chloroplast transformation

To test the possibility of functional base pairing between the –1 uridine of a messenger RNA and A_{37} of the initiator tRNA (Figure 1A), we stably transformed the chloroplast with a plasmid that simultaneously allows the introduction of mutations in the *petA* initiation region, and adds an ectopic copy of the *trnfM* gene (Figure 1B). These altered chloroplast genomes thus combined different mutations in

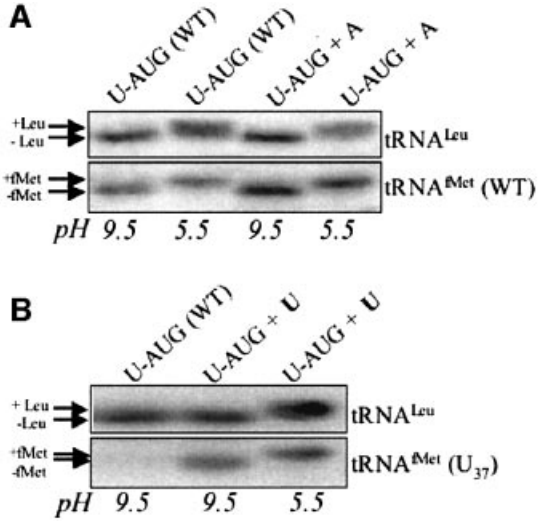


Fig. 2. Expression and aminoacylation of tRNAs. RNAs from the strains indicated above the gels were extracted at pH 5.5 or 9.5, as noted below the gels, and fractionated by electrophoresis through a 15% polyacrylamide gel buffered to pH 5.5. Gels were transferred to membranes and probed with oligonucleotides complementary to (**A**) $tRNA^{Leu}$ and WT $tRNA^{fMet}$, as indicated at the right, or (**B**) $tRNA^{Leu}$ and the ectopic $\mathbf{U}_{37}$ $tRNA^{fMet}$. The positions of charged (+Leu and +fMet) and uncharged (–Leu and –fMet) tRNAs are indicated at the left.

the *petA* RNA and the initiator tRNA. Transformants were selected by resistance to spectinomycin (conferred by the *aadA* marker; Goldschmidt-Clermont, 1991) and screened for the presence of the desired mutations and absence of wild-type (WT) genomes by PCR, DNA filter hybridization analysis and genomic sequencing (data not shown).

The different combinations of mutations in the *petA* initiation region and the ectopic *trnfM* are represented in Figure 1C. Here, as throughout the manuscript, we show WT bases in normal type and mutated bases in bold. As shown in Figure 1C, for *petA*, the WT sequence of the initiation codon ($_{-1}$U-AUG) was changed to AU**U** to reduce the translation rate, a context allowing easy analysis of the importance of the –1 residue (Chen *et al.*, 1995; Esposito *et al.*, 2001). Then, the WT –1 uridine was changed to A (**A**-AU**U**) or C (**C**-AU**U**). With these two (–1 and +3) mutations combined, only two base pairs of the four shown in Figure 1A can still occur with the initiator tRNA. When an ectopic copy of *trnfM* was introduced, we used either the WT sequence (A_{37}, denoted +A) or the compensatory $\mathbf{U}_{37}$ (noted **+U**) or $\mathbf{G}_{37}$ (**+G**) mutations. According to the model, the compensatory effect of mutations in the ectopic *trnfM* should be revealed by an improved level of cyt *f* synthesis in the following two strains: **A**-AU**U**+**U** and **C**-AU**U**+**G**, respectively. In other words, as shown in Figure 1C, these two strains should express an initiator tRNA containing a mutation that restores a third base pair with the *petA* mRNA, whereas the endogenous wild-type tRNA would form only two base pairs. Furthermore, the $\mathbf{U}_{37}$ mutation should not suppress $_{-1}$**C**, and $\mathbf{G}_{37}$ should not suppress $_{-1}$**A**.

Expression of ectopic trnfM and aminoacylation levels

To substantiate the validity of our approach, we had to demonstrate that the ectopic *trnfM* gene was expressed

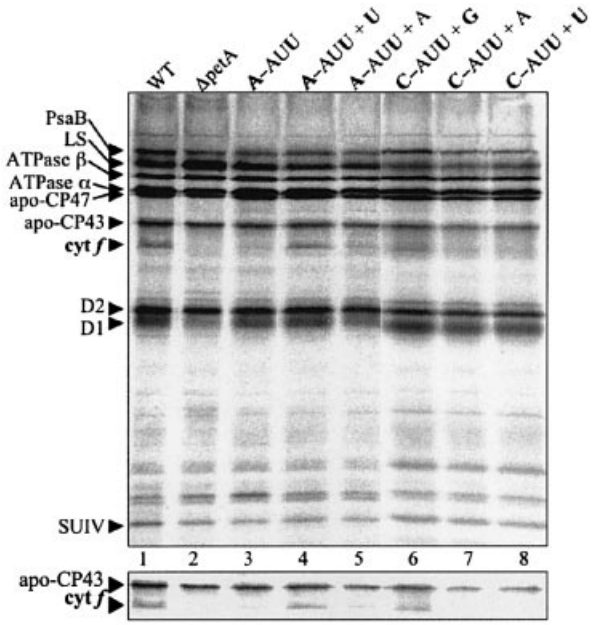


Fig. 3. Chloroplast protein pulse labeling in the presence of a cytoplasmic translation inhibitor. Strains are indicated across the top with nomenclature as described in the legend to Figure 1; ΔpetA is a strain lacking the *petA* gene. Clearly visible chloroplast proteins are indicated at the left: PsaB, core subunit B of photosystem I (PSI); LS, large subunit of Rubisco; ATPase β, β subunit of the ATP synthase; ATPase α, α subunit of the ATP synthase; apo-CP47: 47 kDa subunit of PSII; apo-CP43, 43 kDa PSII subunit; cyt*f*, cytochrome *f*; D2, PSII reaction center; D1, PSII reaction center; SUIV, subunit IV of the cytochrome b_6f complex. The lower panel shows a section of the same gel, with the exposure adjusted to emphasize cyt*f* synthesis.

(either from its own promoter or co-transcribed with *petA*, Figure 1B), and also that a mutation at position 37 of the initiator tRNA, unlike in *E.coli* (Meinnel *et al*., 1993), would not affect its aminoacylation. We used a RNA filter hybridization approach to address both questions, as shown in Figure 2. The first strains constructed for this analysis were U-AUG+A (expressing an ectopic WT *trnfM* gene) and U-AUG+**U** (expressing a $\mathbf{U}_{37}$ mutant tRNA). Comparison of lanes 1 and 3 in Figure 2A show the ~70% increased accumulation of WT $tRNA^{fMet}$ in the U-AUG +A strain, demonstrating the expression of the ectopic WT *trnfM* gene, as compared with the control $tRNA^{Leu}$. To detect expression of the $\mathbf{U}_{37}$ mutant tRNA, we developed an oligonucleotide probe that allows one mismatch-specific recognition; this probe was a 19mer complementary to residues 29–46 of the mutant tRNA (allowing 10 G/C and 9 A/U base pairs with a complete match). As shown in Figure 2B, this gives no signal with the endogenously expressed WT $tRNA^{fMet}$ present in every strain (lane 1), but identifies the $\mathbf{U}_{37}$ mutant tRNA (lane 2), which accumulated to an estimated 60–70% of the endogenous WT $tRNA^{fMet}$ level, based on comparisons with $tRNA^{Leu}$.

To analyze the aminoacylation level of the $tRNA^{fMet}$ in these strains, the RNAs were extracted either at pH 5.5 or 9.5, where the aminoacylation state is maintained or lost, respectively. Should an amino acid be present on tRNAs extracted at pH 5.5, a visible shift would occur during electrophoretic fractionation. As shown in Figure 2A, the quantitative shift in migration between lanes 1 and 2 shows that in a WT strain, $tRNA^{fMet}$ is 100% aminoacylated. Lanes 3 and 4 thus show that the expression of an ectopic *trnfM* gene does not affect the aminoacylation level, which remains at or near 100%. Similarly, lanes 2 and 3 of Figure 2B show that in the U-AUG+**U** strain, the mutant tRNA is fully aminoacylated. The chloroplast $tRNA^{Leu}$ was used in all cases as a control for successful retention or removal of the charged amino acid. Finally, to determine whether the insertion of a *trnfM* gene downstream of *petA* affected *petA* mRNA processing or stability, RNA filter hybridization analysis was performed. These experiments revealed no differences in *petA* mRNA processing and no reduction in its accumulation (data not shown).

In summary, ectopic *trnfM* genes are expressed, do not affect aminoacylation of the endogenous WT $tRNA^{fMet}$ and, importantly, mutating residue 37 does not interfere with aminoacylation. In other words, the strains represented in Figure 1C are appropriate for examining a possible extended codon–anticodon interaction.

Chloroplast protein synthesis in strains expressing ectopic trnfM genes

To visualize either specific or global effects of the ectopic tRNAs in different *petA* mRNA contexts, we analyzed chloroplast protein synthesis rates by labeling cells for 5 min with [^{14}C]acetate in the presence of cycloheximide, a cytosolic translation inhibitor. As shown in Figure 3 (lane 1), a well-defined set of proteins is labeled in WT cells, including cyt *f*, as evidenced by the specific absence of this species in strain ΔpetA, in which the *petA* gene is deleted (lane 2). Apart from these controls, the results showed that the ectopic expression of either the WT-A_{37}, $\mathbf{U}_{37}$ or $\mathbf{G}_{37}$ $tRNA^{fMet}$ (lanes 4–8) had no general effect on chloroplast translation rates, probably because of the presence of an equal or greater amount of the WT $tRNA^{fMet}$ transcribed from the endogenous gene. This important observation means that variations in cyt *f* expression should result only from a gene-specific mechanism.

Figure 3 also implies that for mRNAs which have their WT sequence in the initiation codon region (i.e. other than *petA*), mutating residue 37 of the initiator tRNA has no major consequence on translation. This result is predictable for mRNAs which, like *petA*, have the conserved U-AUG sequence, but also no change was observed for mRNAs, which have a WT A-AUG sequence, such as *rbcL* (encoding the Rubisco large subunit), *psbC* (encoding the photosystem I CP43 protein) and *psbD* (encoding the photosystem II D2 protein). Thus, at least under optimal laboratory growth conditions, a mutant $\mathbf{U}_{37}$ tRNA does not measurably stimulate translation of mRNAs with a WT A-AUG sequence by allowing the putative fourth base pair.

Finally, Figure 3 shows the effects of mutant ectopic tRNAs on expression of mutant *petA* mRNAs. Lanes 3–5 show that in **A**-AU**U**, cyt *f* synthesis is undetectable, but is substantial in the presence of mutant $\mathbf{U}_{37}$ tRNA, but not with an extra copy of the WT A_{37} tRNA. Lanes 6–8 show similarly that **C**-AU**U** can be suppressed by mutant $\mathbf{G}_{37}$ tRNA, but not by the wild-type or mutant $\mathbf{U}_{37}$ tRNAs. These data strongly suggest that allele-specific interactions can occur between the mRNA –1 base pair and position 37 of $tRNA^{fMet}$.

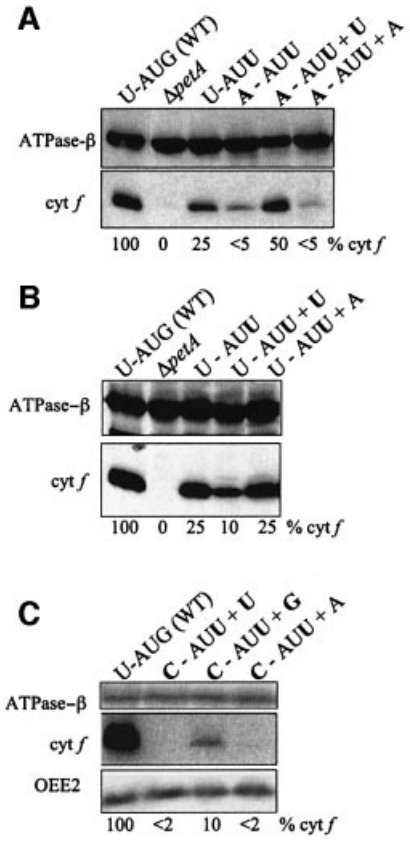


Fig. 4. Analysis of cyt *f* accumulation. Immunoblot analysis was used to measure the accumulation of cyt *f* (34 kDa) in the indicated strains, with the ATPase β subunit (55 kDa) as a loading control. The values indicated for cyt *f* accumulation, shown beneath each gel and relative to WT, were estimated from multiple blots. (**A**) Control strains compared with those with mRNA –1 mutations. (**B**) Control strains compared with those with a WT –1 sequence in *petA*, but expressing ectopic tRNAs. (**C**) A WT control compared with strains carrying $_{-1}$**C** mutations in *petA* mRNA. Here the indicated cyt *f* accumulation levels are mean values obtained for four independent clones for each strain, quantified against both the ATPase β subunit and the OEE2 protein. OEE2 is the nucleus-encoded PSII oxygen-evolving enhancer protein 2 (Mayfield *et al*., 1987).

Cytochrome f accumulation in trnfM ectopic mutants

The pulse labeling experiments of Figure 3 have limited quantitative value because cyt *f* is poorly labeled relative to other proteins. Therefore, immunoblot analysis was used, since previous work has shown that the level of cyt *f* accumulation in *Chlamydomonas* accurately reflects its synthesis rate (Kuras and Wollman, 1994; Chen *et al*., 1995). Figure 4A shows the effects of different ectopic tRNAs^{fMet} on cyt *f* translation in the **A**-AU**U** mutant. In the presence of only the WT endogenous tRNA, the context of an AU**U** initiation codon shows that the $_{-1}$U to A mutation reduces translation from 25% (in U-AU**U**, lane 3) to <5% (in **A**-AU**U**, lane 4). However, the presence of an ectopic tRNA^{fMet} harboring the $\mathbf{U}_{37}$ mutation (in **A**-AU**U+U**, lane 5) constitutes a compensatory mutation (see Figure 1C), and results in the increase of accumulation to ~50%, thus suppressing the effect of the –1 mutation. As a control, we show that an ectopic WT tRNA^{fMet} does not restore the translation level (lane 6), demonstrating that the effect observed in lane 5 is not due to the overaccumulation of an initiator tRNA, but rather to the specific presence of a uridine at position 37 of this tRNA. This result clearly shows that the –1 residues of the mRNA and position 37 of the tRNA can base pair according to the model proposed in Figure 1A.

In a similar manner, we studied the effect of an ectopic WT tRNA^{fMet} with a U-AU**U** context for the *petA* mRNA, the sequence upstream of the initiation codon being unchanged this time. The expression of an ectopic WT tRNA^{fMet} has no effect on cyt *f* synthesis (Figure 4B, lane 5), whereas the mutated $\mathbf{U}_{37}$ tRNA decreases protein accumulation from 25 to 10% (lane 4). This likely results from the mutated tRNA competing with the endogenous tRNA at an early step in translation, and ultimately decreasing the rate, since it can form only two base pairs with the U-AU**U** mRNA, instead of three for the endogenous tRNA.

To further confirm the model and our initial results, we analyzed the effect of a $_{-1}$C mutation in the mRNA and the possible compensatory effect that could be conferred by a $\mathbf{G}_{37}$ mutation in the initiator tRNA. The $_{-1}$C mutation has a more dramatic effect on translation than the $_{-1}$A mutation and, given the rarity of C residues at position –1 of chloroplast mRNAs (4 out of 64 in *C.reinhardtii*), this indicates that it is unfavorable for translation initiation. This effect could be compensated neither by an ectopic WT tRNA^{fMet} (Figure 4C, lane 4) nor by the $\mathbf{U}_{37}$ mutation (lane 2). However, the $\mathbf{G}_{37}$ mutation in the tRNA partially restored translation initiation (from <2 to ~10%), thus confirming the extended base pairing model. This compensatory effect is equal in magnitude to an A–U restored base pair (Figure 4A), i.e. about 5-fold, although the absolute levels are lower with a $_{-1}$**C**.

Discussion

We have used a compensatory mutation strategy to test the hypothesis of base pairing between the –1 nucleotide of a chloroplast mRNA and the anticodon loop of the initiator tRNA. This work was made possible in the chloroplast of *C.reinhardtii* by showing that an ectopic copy of the *trnfM* gene can be expressed without globally affecting protein synthesis rates, and that mutating position 37 does not affect its aminoacylation. The ectopic *trnfM* gene was used, in part, because we were unable to obtain strains in which the endogenous *trnfM* gene had been replaced by a mutated copy (data not shown). The ectopically expressed versions of tRNA^{fMet} carrying anticodon mutations were able to increase translation of *petA* –1 mutant mRNAs with an AUU initiation codon. In contrast, cyt *f* translation in the U-AU**U** strain was reduced in the presence of a mutant tRNA (Figure 4B). This result can be interpreted by considering that the 30S subunit can bind IF-2 and the initiator tRNA before binding the mRNA (Gualerzi and Pon, 1990; Wu *et al*., 1996). 30S subunits containing the mutant initiator tRNA would bind this mRNA relatively poorly in this scenario.

Our *in vivo* analysis supports earlier *in vitro* work, suggesting that the initiation codon–anticodon interaction may be greater than three base pairs, although the –1 position is only one determinant of translational efficiency (Hui *et al*., 1984). For example, short oligoribonucleotides

with a U upstream of the AUG initiation codon allowed 5-fold more efficient formation of an initiation complex with 70S ribosomes than those with a purine upstream of AUG (Ganoza *et al*., 1978). Moreover, 30S and 70S ribosomes bound fMet–tRNA more efficiently in response to UAUG than AAUG (Ganoza *et al*., 1982) or AUG (Eckhardt and Luhrmann, 1981) *in vitro*. Dipeptide synthesis was also 2-fold more efficient with UAUGUUU than AUGUUU or AAUGUUU (Ganoza *et al*., 1982). Sequence analysis also offers some support for the extended base pairing hypothesis. Prokaryotic initiation codons are preferentially preceded by a U compared with internal AUG codons (Ganoza *et al*., 1985) and, in *C.reinhardtii*, 75% of chloroplast genes known to be expressed possess a U at the –1 position. Moreover, a U at the –1 position is optimal for expression of foreign genes in *E.coli* (Gross *et al*., 1990; Morelle *et al*., 1991). Although we have focused here on base pairing, the –1 position may have other types of interactions with the translational machinery. Evidence for this includes the protection from chemical modification of the T4 gene 32 mRNA –1 position when studied *in vitro* in ternary complexes (Hüttenhofer and Noller, 1994).

While this work describes an extended codon–anticodon interaction in initiation, four base-pair codon–anticodon interactions have been proposed for tRNA frameshift suppressors with an expanded anticodon loop (reviewed in Culbertson *et al*., 1990). Although recent results suggest that an extended interaction may not always occur, rather that the expanded anticodon loop inhibits its use in translation forcing a near-cognate tRNA to be used (Qian *et al*., 1998), artificially created expanded anticodon loops in some cases clearly support frameshifting (e.g. Magliery *et al*., 2001). This case is not strictly analogous to our findings, however, since we have altered a base without changing normal tRNA size or structure.

Taken together, *in vitro*, sequence analysis, and now *in vivo* data strongly support a 5′ extended codon–anticodon interaction in translation initiation. Further, it can be argued that this situation, at least for some mRNAs, is likely to be widespread among bacteria and chloroplasts. In chloroplasts, the A_{37} residue is highly conserved in the initiator tRNA anticodon loop (Sprinzl *et al*., 1998), as is the $-_1$U residue upstream of mRNA initiation codons. On the other hand, position 37 of the initiator tRNA is variable in mitochondrial DNAs (Sprinzl *et al*., 1998). While ostensibly arguing against further generalization of the mechanism described here, a closer look reveals apparent co-evolution of $tRNA^{fMet}$ position 37 with mRNA –1 positions. For example, mitochondrial $tRNA^{fMet}$ of the lower fungus *Hyaloraphidium curvatum* carries a C at position 37 and, at the same time, 12 of 14 AUG initiation codons carry a $_{-1}$G (Forget *et al*., 2002). On the other hand, its close relative *Spizellomyces punctatus* (DDBJ/EMBL/GenBank accession No. AF404303) features a position 37 A in the $tRNA^{fMet}$ and 0/13 –1 positions are G, but 5/13 are U, including all three *cox* genes. Thus, this type of interaction may be evolutionarily favored both in prokaryotes and organelles, but like other initiation elements, including the SD and AUG codon itself, is undoubtedly not universal.

The advantage of an extended codon–anticodon interaction during prokaryotic-type translation initiation remains to be elucidated, and must take into account the variability of –1 residues and other regulatory motifs. One hypothesis is that the possibility of having four base pairs can improve synthesis of certain proteins under environmental stress conditions, or where mRNA or tRNA levels are limiting. Alternatively, this additional base pair may be part of the fundamental mechanism favoring the formation of the initiation complex, especially for non-AUG codons, thus conferring a more subtle evolutionary advantage.

Materials and methods

Construction of trnfM transforming plasmids

A 1280 bp *Eco*RI–*Sal*I fragment containing *trnfM* was subcloned from the plasmid p578 (*Chlamydomonas* Genetics Center, Duke University) into pBluescript-SK$^-$ (Stratagene) to create the plasmid pfmetsk-. An *Ssp*I site was introduced 18 bp downstream of the *trnfM* gene using site-directed mutagenesis (Kunkel, 1985), creating the fM-CAUAsspI mutation (CAUA is the sequence of the anticodon and first nucleotide downstream). The resulting plasmid containing the *Ssp*I site was subjected to a second round of site-directed mutagenesis yielding fM-CAUUsspI and fM-CAUGsspI. To create plasmids containing an ectopic copy of *trnfM*, a 450 bp *Hinc*II fragment from pfmetsk- containing either *trnfM* fM-CAUAsspI, fM-CAUUsspI or fM-CAUGsspI was inserted into the *Stu*I site of the plasmid pQMAD, which contains the *aadA* gene (Goldschmidt-Clermont, 1991) inserted in the *Eco*RV site between *petA* and *petD*. pQMAD had been altered to contain one of several mutations at the initiation region of *petA* (Esposito *et al*., 2001).

Chlamydomonas strains, culture conditions and chloroplast transformation

P17 (Stern *et al*., 1991), a wild-type *Chlamydomonas* strain derived from CC373 (mt+, ac-u-c-2–21; Shepherd *et al*., 1979) by bombardment with the *atpB* gene, was used as a recipient for chloroplast transformation. The ΔpetA strain was described previously (Kuras and Wollman, 1994). *Chlamydomonas* strains were grown in TAP medium (Harris, 1989) at 25°C under constant light (70 μE/m^2/s). Modified genes were introduced into the chloroplast genome by tungsten-particle bombardment (Kindle *et al*., 1991). Transformants expressing the *aadA* cassette were selected on TAP medium containing spectinomycin (100 μg/ml).

In vivo aminoacylation levels

In vivo aminoacylation levels were analyzed by RNA extraction and filter hybridization analysis according to Varshney *et al*. (1991). Total RNAs were extracted from liquid cultures of *C.reinhardtii* either with solutions buffered at pH 8.5, to obtain extracts containing deacylated tRNAs, or at pH 5.5 to obtain extracts where aminoacylation of tRNAs was preserved. Extraction at pH 8.5 was not sufficient to obtain complete deacylation of $tRNA^{fMet}$ and extracts were further treated for 1 h at room temperature in 20 mM Tris–HCl pH 9.5. Extracts were then fractionated by electrophoresis in 15% polyacrylamide gels with the running buffer and gel buffered at pH 5.5 to maintain the aminoacylated state of tRNA extracts obtained at pH 5.5. After electroblotting onto Hybond N+ membranes (Amersham), we used [^{32}P]5′-labeled oligonucleotides as probes. For $tRNA^{Leu}$, the probe was complementary to positions 18–32, with numbering according to Sprinzl *et al*. (1998). For $tRNA^{fMet}$, we used oligonucleotides that matched either the WT sequence or the $\mathbf{U}_{37}$ mutation (one mismatch with the WT sequence), complementary to positions 29–46.

Protein pulse labeling

Experiments were carried out as described previously (Drapier *et al*., 1992) with 5 μCi/ml of [^{14}C]acetate (50 mCi/mmol, Amersham) in the presence of an inhibitor of cytoplasmic translation (6.6 μg/ml cycloheximide, Sigma). Proteins of solubilized cells were then separated in 12–18% polyacrylamide/SDS–urea gels as described previously (Piccioni *et al*., 1981).

Protein preparation and immunoblotting

Total protein isolation and immunoblotting were as described previously (Drager *et al*., 1998). Blots were reacted with primary antibodies directed against the *Chlamydomonas* chloroplast-encoded cyt *f* (Chen *et al*., 1995), ATPase β subunit (a gift from F.-A.Wollman), the nuclear-encoded OEE2 protein and a secondary anti-rabbit IgG HRP-conjugated antibody

(Promega, 1:2 500 dilution), and visualized by enhanced chemiluminescence (Durant, 1990) or with ^{125}I-labeled protein A (Figure 4C only).

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