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Over Expression of a tRNA^{Leu} Isoacceptor Changes Charging Pattern of Leucine tRNAs and Reveals New Codon Reading

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During mRNA translation, synonymous codons for one amino acid are often read by different isoaccepting tRNAs. The theory of selective tRNA charging predicts greatly varying percentages of aminoacylation among isoacceptors in cells starved for their common amino acid. It also predicts major changes in tRNA charging patterns upon concentration changes of single isoacceptors, which suggests a novel type of translational control of gene expression. We therefore tested the theory by measuring with Northern blots the charging of Leu-tRNAs in *Escherichia coli* under Leu limitation in response to over expression of $tRNA^{Leu}_{GAG}$. As predicted, the charged level of $tRNA^{Leu}_{GAG}$ increased and the charged levels of four other Leu isoacceptors decreased or remained unchanged, but the charged level of $tRNA^{Leu}_{UAG}$ increased unexpectedly. To remove this apparent inconsistency between theory and experiment we postulated a previously unknown common codon for $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{UAG}$. Subsequently, we demonstrated that the $tRNA^{Leu}_{GAG}$ codon CUU is, in fact, read also by $tRNA^{Leu}_{UAG}$, due to a uridine-5-oxyacetic acid modification.

Keywords: aminoacylation; systems biology; decoding; staruation; kinetics

Introduction

Cells use different transfer RNA (tRNA) molecules aminoacylated (charged) with the same type of amino acid to decode synonymous codons in messenger RNAs (mRNAs).[40] Recently, it was suggested[1] that the charged levels of such tRNA "isoacceptors" will be selectively reduced in response to limited supply of their cognate amino acid. This prediction follows from intracellular flow balance relations, stating that the rate of aminoacylation of an isoacceptor by its cognate aminoacyl-tRNA synthetase[2] must always be equal to its rate of deacylation in protein synthesis. To a first approximation the rate of aminoacylation of an isoacceptor is proportional to the concentration of its deacylated form. Furthermore, its rate of deacylation in protein synthesis is proportional to the usage frequency of its cognate codon(s) on translating ribosomes. Therefore, the concentration of a deacylated tRNA normalized to the usage frequency of its cognate codons in translation must be equal for all tRNA isoacceptors in the same family. When amino acid limitation becomes increasingly severe, the charged level of the tRNA isoacceptor with the smallest ratio between its total concentration and the usage frequency of its cognate codons in translation will therefore approach zero, while the charged levels of the other tRNAs in the family will stabilize at intermediate values.[1] From this follows also the prediction that the charged fraction of a tRNA isoacceptor, originally at a low level during starvation for its cognate amino acid, will increase significantly when it is over expressed. Accordingly, one way that cells could regulate gene expression during amino acid limitation would be by tuning the relative concentrations of tRNAs in families of isoacceptors.

The theory successfully identified the choices of synonymous control codons in attenuation leader sequences in *Escherichia coli*,[3] assuming that these should be read by isoacceptors for which the charged levels respond most sensitively to amino

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Abbreviations used: mRNA, messenger RNA; tRNA; transfer RNA.

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acid limitation. It also explained codon usage patterns in genes, like those in amino acid biosynthetic operons, for which expression under amino acid limitation is vital.[1] Here, the assumption was that synonymous codons, read by isoacceptors for which the charged levels were insensitive to amino acid starvation, should be statistically over-represented.

The theory of selective charging of tRNA isoacceptors[1] has been directly verified for *E. coli* in the Leu, Arg and Thr cases by tRNA micro array and Northern hybridization experiments by Dittmar *et al.*[4] It was, for example, shown that under Leu limitation, the isoacceptors $tRNA^{Leu}_{CmAA}$ and $tRNA^{Leu}_{cmnm^5UmAA}$ (see Table 1 for annotation of $tRNA^{Leu}$ isoacceptors and their codons) retain comparatively large charged fractions, while the charged fractions of the other three $tRNA^{Leu}$ isoacceptors are close to zero. These results[4] are in qualitative agreement with theory,[1] but the predicted charged levels of isoacceptors $tRNA^{Leu}_{CmAA}$ and $tRNA^{Leu}_{cmnm^5UmAA}$ are higher than the measured ones (see below).

The frequency of ribosomal bypassing of short mRNA stretches downstream from the ribosomal P site depends on the rate of reading of the A-site codon.[5] Selective charging of two members of an isoacceptor family that read different codons, should therefore map to selective bypassing frequencies for ribosomes with one or the other of these codons in the A site. Lindsley *et al.*[6] demonstrated strongly diverging frequencies of bypassing at two different serine codons for increasingly severe inhibition of the serinyl-tRNA synthetase, in full quantitative agreement with theory.[1]

Here, we address another class of theoretical predictions from the theory, which concerns greatly changed charging patterns of a family of tRNA isoacceptors in response to varying concentration of one of its members. If true, this prediction would immediately suggest a novel type of translational control of gene expression under conditions of amino acid limitation. We over expressed one member of the leucine tRNA isoacceptor family, and found its charged fraction to increase, perfectly in line with theory. However, the charged fraction of also a second member of the same isoacceptor family increased significantly, in apparent contradiction to theory, but the inconsistency was eventually removed by the discovery of a previously unknown codon reading by the second leucine tRNA isoacceptor and the identification of the tRNA modification that allowed for it.

Results

Responses in the charged levels of leucine isoacceptors to over expression of $tRNA^{Leu}_{GAG}$

In the *E. coli* study by Dittmar *et al.*,[4] it was shown experimentally that the charged fraction of $tRNA^{Leu}_{GAG}$ approaches zero upon leucine starvation as predicted by theory.[1] If, however, $tRNA^{Leu}_{GAG}$ is over expressed, it will compete better with the other leucine isoacceptors in aminoacylation although it will still be deacylated at the same rate in protein synthesis. Therefore, the charged fraction of the over expressed $tRNA^{Leu}_{GAG}$ should remain high during leucine limitation.[1] Furthermore, since $tRNA^{Leu}_{GAG}$ has been reported not to share cognate codons with any other tRNA in the leucine family,[40] theory also predicted that its over expression should result in reduced or unchanged charged fractions of all other $tRNA^{Leu}$ isoacceptors.

For over expression of $tRNA^{Leu}_{GAG}$, we inserted the coding portion of its structural gene *leuU*, controlled by the inducible p*tac* promoter, into the medium copy number plasmid pACtac (Materials and Methods). The plasmids pACtac and p*leuU* were introduced into a *recA1 lacI*^{q1} derivative of the strain CP78,[7] which is an auxotroph for the amino acid residues arginine, threonine, histidine and leucine.

In the experiments, performed to measure the charged fractions of the $tRNA^{Leu}$ isoacceptors during over expression of *leuU*, we grew the two plasmid-containing strains in minimal glycerol medium and induced full expression of $tRNA^{Leu}_{GAG}$ one generation time before the onset of starvation. Leucine starvation was introduced by filtration of the cultures, followed by washing and resuspension of the cells in leucine free medium. Samples for purification of tRNA were made from the cultures 1 min before, and 10 min and 30 min after the onset of leucine starvation. Northern blot analysis was

Table 1. Nomenclature of the leucyl isoacceptor tRNAs

$tRNA_{anti\text{-}codon}$	Coding genes	Older nomenclature	Cognate codons
$tRNA^{Leu}_{CAG}$	*leuPQVT*	tRNALeu1	CUG
$tRNA^{Leu}_{GAG}$	*leuU*	tRNALeu2	CUC,CUU
$tRNA^{Leu}_{cmo^5UAG}$ [a]	*leuW*	tRNALeu3	CUG,CUA,CUU[b]
$tRNA^{Leu}_{CmAA}$ [c]	*leuX*	tRNALeu5 (or 6 or 4)	UUG
$tRNA^{Leu}_{cmnm^5UmAA}$ [d]	*leuZ*	tRNALeu4 (or 5)	UUG,UUA

[a] cmo^5U, uridine-5-oxyacetic acid modification is established here.
[b] Decoding capacity for codon CUU found in this work.
[c] Cm, 2′-*O*-methylcytidine.
[d] $cmnm^5Um$, 5-carboxymethylaminomethyl-2′-*O*-methyluridine.

used to directly measure the charged fractions of the $tRNA^{Leu}$ isoacceptors.[8]

Under these conditions, $tRNA^{Leu}_{GAG}$ was sixfold over expressed in the strain containing the plasmid p*leuU* compared to the expression in the pACtac control strain (see Materials and Methods). This over expression did not change the charged fraction of any $tRNA^{Leu}$ isoacceptors when the cultures were grown with leucine in the medium (Figure 1(a) and data not shown). However, when growth was limited by the supply of leucine, the sixfold over expression of $tRNA^{Leu}_{GAG}$ led to a tenfold increase in the charged fraction of $tRNA^{Leu}_{GAG}$, an unchanged charged fraction of $tRNA^{Leu}_{CAG}$ and a threefold decrease in the charged fractions of $tRNA^{Leu}_{CmAA}$ and $tRNA^{Leu}_{cmnm^5UmAA}$ (Figure 1). These

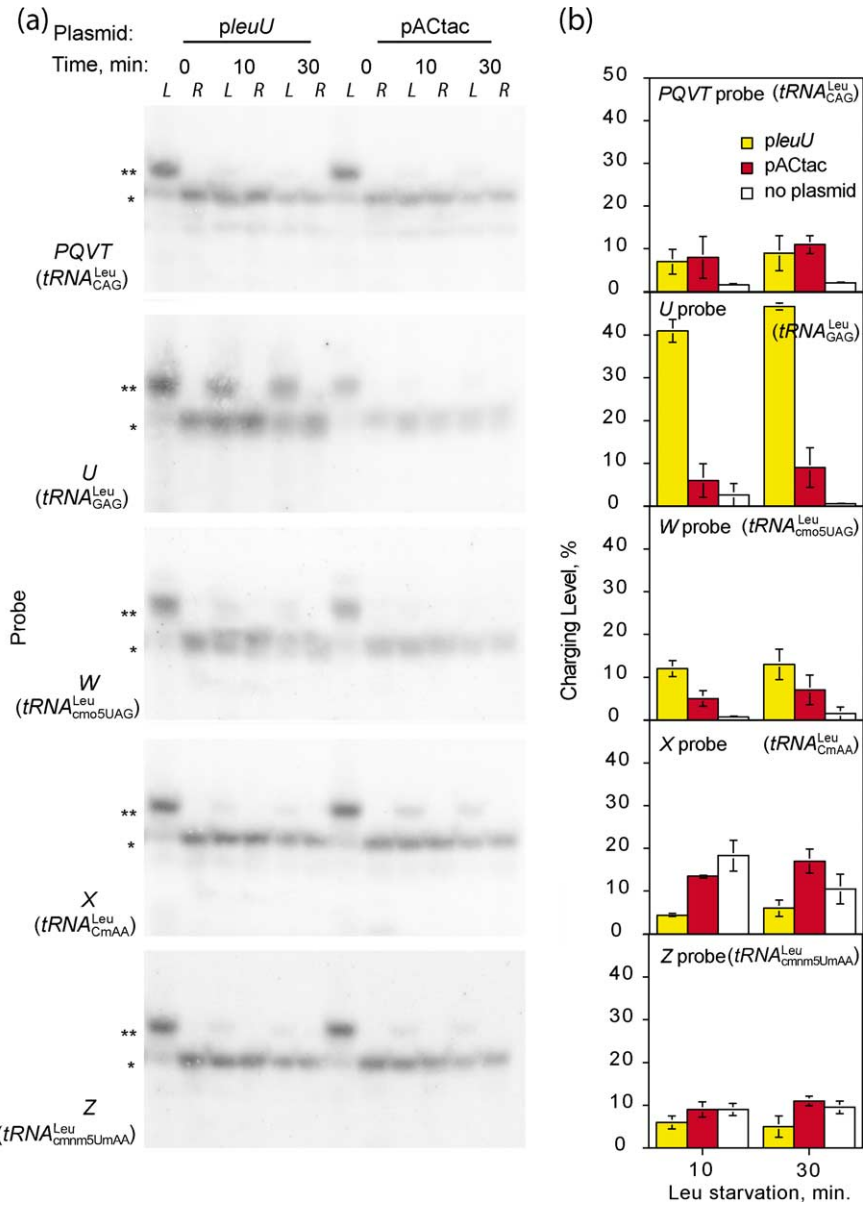

Figure 1. Charged fractions of the leucine isoacceptor tRNAs after over expression of $tRNA^{Leu}_{GAG}$. (a) PhosphoImager pictures of a Northern blot containing tRNA harvested from the two plasmid-containing strains before and after removal of Leu from the medium, as indicated above the lanes. Each panel represents the radioactivity found after hybridization with the individual ^{32}P labeled DNA probes specific for the tRNAs indicated at the left. One and two stars indicate the position of the deacylated and aminoacylated $tRNA^{Leu}$, respectively. Equal amounts of the same tRNA preparation were loaded in neighboring lanes (*L* and *R*) and the tRNA in the rightmost lane (*R*) were deacylated by incubation at pH 9.0 for 2 h at 37 °C before loading onto the gel. This deacylated tRNA was used for background subtraction. (b) Bar diagram showing the charged fractions of the individual Leu isoacceptor tRNAs, 10 min and 30 min after removal of Leu from the medium. Yellow and red bars represent the p*leuU* and the pACtac containing strain, respectively, and error bars symbolize ± SEM. The white bars show the charged fraction found in the parental Rec^+ Lac^+ strain without any plasmid (reproduced from Dittmar *et al.*[4]). Here, error bars symbolize ± SD. The white bars at 10 min represent an average of measurements made at 5 min and 15 min after removal of Leu.

results were in line with the theoretical predictions, but the further observation of a twofold increase in the charged fraction of the $tRNA^{Leu}_{UAG}$ isoacceptor (Figure 1) could not be accounted for by the theory.

The most straight-forward way to remove the apparent inconsistency between theory and experiment due to the increased charged level of $tRNA^{Leu}_{UAG}$ by over expression of $tRNA^{Leu}_{GAG}$, was to revise the existing codon reading rules[40] by postulating that $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{UAG}$ have at least one cognate codon in common. If this were the case, over expression of $tRNA^{Leu}_{GAG}$ and the resulting large increase in its charged level during starvation (Figure 1) would lead to rapid reading of a codon that were cognate also for $tRNA^{Leu}_{UAG}$, which would reduce the demand for the latter isoacceptor in protein synthesis and thereby lead to the observed increase in its charged level during starvation.

The *recA1* derivative of the "wild-type" strain was used here to avoid recombination between the chromosome and plasmid *leuU* sequences. The *recA1* allele reduces growth rate by 25%, and the $tRNA^{Leu}$ isoacceptor concentrations vary significantly and differently with growth rate.[9] This led to residual charging level differences between the *recA1* derivative and the previously studied "wild-type" strain (Figure 1(b), see Dittmar *et al.*[4]) also without over expression of *leuU*. However, regardless of this the effect of over expressing *leuU* was clearly demonstrated in the comparison with the isogenic pACtac control strain (Figure 1(b)). Over expression of individual tRNAs may disturb the cellular physiology by saturating aminoacyl-tRNA synthetases and tRNA modification enzymes.[10] However, the sixfold over expression of *leuU* did not affect the growth rate. Since, furthermore, no extra tRNA bands appeared in the Northern blots upon *leuU* over expression (Figure 1), the integrity as well as the modification patterns of all leucine tRNA isoacceptors remained unchanged.

Identification of codon recognition patterns by *in vitro* translation

To decide the identity of the codons that are read by $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{UAG}$, we took advantage of an *E. coli* system for protein synthesis with components of high purity.[11] Initiated 70 S ribosome complexes were prepared with fMet-$tRNA^{fMet}$ in the P site and varying codons in the A site. The extent of fMet-Leu formation was monitored as a function of time after rapid mixing in a quench flow instrument of an initiated ribosome complex with a $tRNA^{Leu}_{GAG}$, $tRNA^{Leu}_{CAG}$ or $tRNA^{Leu}_{UAG}$ isoacceptor in ternary complex with EF1-Tu and GTP (see Materials and Methods). The experiments demonstrated that, indeed, $tRNA^{Leu}_{UAG}$ reads the CUU codon with the same rate constant (k_{cat}/K_m) as it reads its previously identified cognate codons CUG and CUA, but is unable to read CUC (Figure 2). It can be noted that $tRNA^{Leu}_{GAG}$ translates its cognate codons (CUC, CUU) between three and one and a half times as fast as $tRNA^{Leu}_{UAG}$ and $tRNA^{Leu}_{CAG}$ translate their cognate codons (Table 2).

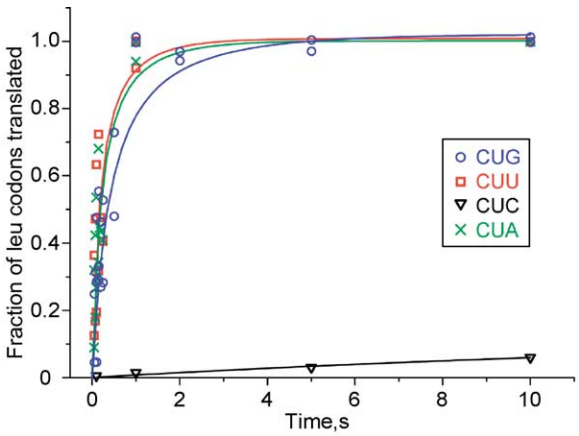

Figure 2. Rate of reading of four Leu codons by $tRNA^{Leu}_{cmo^5UAG}$. *Y*-axis: Fraction of active ribosomes containing fMet-Leu. *X*-axis: Time. The experiment shows that $tRNA^{Leu}_{cmo^5UAG}$ reads CUG, CUA and from this study CUU as cognate codons, but not CUC.

Analysis of $tRNA^{Leu}_{UAG}$ modifications

According to the DNA sequence of the structural gene *leuW*, $tRNA^{Leu}_{UAG}$ has a U as wobble nucleoside. If this U nucleoside were unmodified, it should according to Crick[12] base-pair only with A or G. The reading of CUU as cognate codon by $tRNA^{Leu}_{UAG}$ (Figure 2, Table 2) therefore suggested this tRNA to have special features allowing for U:U wobble base-pairing. An extended version of Crick's wobble hypothesis includes the effects of various modifications of U that either extend or restrict its coding capacity.[13] It postulates, in particular, that U modified to uridine-5-oxyacetic acid (cmo^5U) extends its coding capacity to base-pairing not only with A and G but also with U, as now confirmed by *in vitro* experiments.[40] Accordingly, the capacity of $tRNA^{Leu}_{UAG}$ to read CUU codons (Figure 2, Table 2) could be due to the presence of cmo^5U in the wobble position. We therefore analyzed the modification pattern of purified $tRNA^{Leu}_{UAG}$ and $tRNA^{Leu}_{CAG}$ and found that, indeed, cmo^5U was present in $tRNA^{Leu}_{UAG}$ but, as expected,

Table 2. Codon association rates

Codon	tRNA k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)		
	$tRNA^{Leu}_{CAG}$	$tRNA^{Leu}_{cmo^5UAG}$	$tRNA^{Leu}_{GAG}$
CUG	18.2±1.0	9.6±1.6	<0.01
CUC	<0.01	≈0.01	30.3±3.6
CUU	<0.01	11.4±1.3	32.8±1.4
CUA	<0.01	11.6±1.1	<0.01

The Table displays the association rate constant measured for each $tRNA^{Leu}$ isoacceptor on the four different codons. Each number is an average (±SEM) of at least three independent measurements.

not in $tRNA^{Leu}_{CAG}$ (Figure 3). Thus, the anticodon of $tRNA^{Leu}_{UAG}$ is most likely cmo^5UAG, which would explain the extended codon reading found here (Figure 2, Table 2) and motivate the name $tRNA^{Leu}_{cmo^5UAG}$ for this isoacceptor.

Discussion

We have demonstrated experimentally that a cell can change its selective charging pattern in a predictable way simply by altering the concentration of a tRNA isoacceptor. In *E. coli*, the relative abundances of different tRNA isoacceptors change with the quality of the growth medium.[14] Different patterns of selective charging of tRNA isoacceptors during amino acid limitation are therefore expected for growth in different media. Translation of different sets of mRNAs could therefore be shut down or greatly stimulated in a medium-dependent way during amino acid limitation depending on choices of synonymous codons. In eukaryotes, where the rate of protein synthesis is thought to be amino acid limited in many cases,[15–17] coordinated regulation of amino acid supply and tRNA isoacceptor concentrations may by the same principle direct the translational activity to a subset of an intracellular mRNA pool.

Initially, the theoretical prediction of how the charging pattern of members of the $tRNA^{Leu}$ isoacceptor family will respond to over expression of $tRNA^{Leu}_{GAG}$ under conditions of leucine limitation (Figure 4) appeared to be contradicted by results from the Northern hybridization experiments (Figure 1). However, instead of refuting the theory of selective charging of tRNA isoacceptors,[1] the contradiction was eventually removed by the unexpected discovery that $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{UAG}$ have a common codon.

We found that $tRNA^{Leu}_{UAG}$ reads the CUU codon (Figure 2), previously assigned only to $tRNA^{Leu}_{GAG}$,[40] with the help of a previously unknown uridine-5-oxyacetic acid modification of its wobble nucleoside (Figure 3), so that this isoacceptor may now be annotated $tRNA^{Leu}_{cmo^5UAG}$. Our observation that $tRNA^{Leu}_{cmo^5UAG}$ reads CUG, CUA as well as CUU but not CUC is perfectly in line with the Nishimura codon reading rule[18] and a stereochemical model for cmo^5U base-pairing.[19] This seems to be valid for $tRNA^{Ser}_{cmo^5UGA}$[20] and $tRNA^{Val}_{cmo^5UAC}$[21] but neither for $tRNA^{Pro}_{cmo^5UGG}$[22] nor for $tRNA^{Ala}_{cmo^5UGC}$,[23] since these latter tRNAs appear to read also the C-ending codon. Our result that $tRNA^{Leu}_{cmo^5UAG}$ cannot read CUC is also in line with the recent observation[24] that $tRNA^{Leu}_{GAG}$ is essential for *E. coli*, since it is the sole reader of CUC (Table 1). Our clear-cut observations that $tRNA^{Leu}_{CAG}$ reads only CUG and

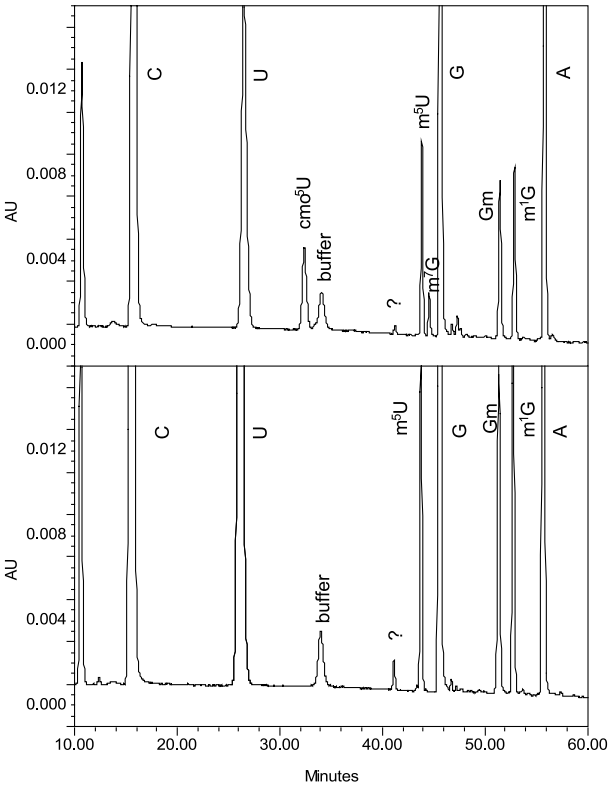

Figure 3. Analysis of modified nucleosides of $tRNA^{Leu}_{cmo^5UAG}$ (upper panel) and $tRNA^{Leu}_{CAG}$ (lower panel). The nucleosides were monitored at 280 nm, which is the λ_{max} of cmo^5U. The positions of cmo^5U (uridine-5-oxy acetic acid), m^5U (5-methyluridine), m^7G (7-methylguanosine), Gm (2′-*O*-guanisone) and m^1G (1-methylguansine) are indicated close to the respective compound. The peak labeled "buffer" is a compound originating from the polymix buffer, in which the tRNAs were dissolved. The peak labeled "?" is an unknown G derivative (see Materials and Methods for details).

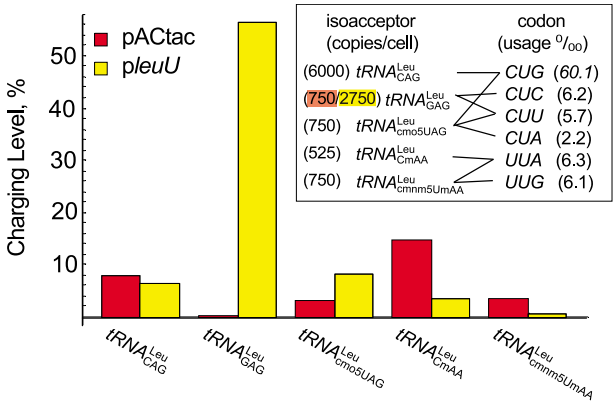

Figure 4. Modeling of the charged fractions of the members of the Leu isoacceptor family with or without over expression of $tRNA^{Leu}_{GAG}$. *Y*-axis: charged fractions of Leu isoacceptors without (red) or with (yellow) over expression of $tRNA^{Leu}_{GAG}$. *X*-axis: type of isoacceptor. Inset: Isoacceptor copy number per cell and codon frequencies on translating ribosomes used to calculate the charged fractions in the Figure from the theory of selective charging (Ibba & Söll[2] and Supplementary Data). k_{cat}/K_m-values were taken from Table 2 and the total rate of protein synthesis was assumed to be reduced to 60% of its maximal value by Leu limitation (see Supplementary Data).

that $tRNA^{Leu}_{GAG}$ reads CUC and CUU are consistent with early low-resolution estimates of specific tRNA isoacceptor binding to programmed ribosomes.[25] They are, however, at variance with a report, based on protein synthesis experiments,[26] claiming that $tRNA^{Leu}_{CAG}$ reads CUC, CUU, CUA (CUG not tested) and that $tRNA^{Leu}_{GAG}$ reads CUA in addition to CUC and CUU. It is likely that these apparent discrepancies are caused by poor isoacceptor separation in the experiments by Goldman *et al*. (E.B., unpublished results).

With the present addition to the previous rules of codon reading by Leu isoacceptors,[40] the theory of selective charging accounts for the leucine starved pattern of $tRNA^{Leu}$ isoacceptor charging before as well as after induction of over expression of $tRNA^{Leu}_{GAG}$ (Figures 1 and 4). That is, when the total concentration of an isoacceptor increases, the charged percentage at which its rate of aminoacylation matches the rate of consumption of its aminoacylated form in protein synthesis must always increase (Supplementary Data).[1] It is therefore easy to understand why the charged fraction of $tRNA^{Leu}_{GAG}$ increased when it was over expressed (Figure 1). The concomitant increase in the charged level of $tRNA^{Leu}_{cmo^5UAG}$ can be explained by taking into account that an increasing intracellular concentration of Leu – $tRNA^{Leu}_{GAG}$ causes an increasing fraction of CUU codons on translating ribosomes to be read by this isoacceptor, rather than by $tRNA^{Leu}_{cmo^5UAG}$. This, in turn, will reduce the demand for $tRNA^{Leu}_{cmo^5UAG}$, which will allow its charged fraction to increase (Figure 4). The charged fractions of the other leucine isoacceptors decreased upon $tRNA^{Leu}_{GAG}$ over expression (Figure 1). This follows from the fact that the total rate of supply of leucine is always equal to the total rate of leucine incorporation into nascent peptides. Therefore, when the rates of translation of codons cognate to $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{cmo^5UAG}$ increase, the rates of translation of other Leu codons must decrease so that the total rate of leucine consumption remains unaltered. The mathematical details behind this reasoning are found in Supplementary Data. Full quantitative agreement between theory and experiment (Figure 4 and Supplementary Data) requires adjustment of the intracellular concentrations of $tRNA^{Leu}_{CmAA}$ and $tRNA^{Leu}_{cmnm^5UmAA}$ to lower values than those reported by Dong *et al.*[14] for K12-*E. coli*. The notion that these two isoacceptor concentrations could have been over estimated by Dong *et al*.[14] was pointed out by Dittmar *et al*.,[4] and is supported by earlier observations by Ikemura.[27]

The way we found a novel codon assignment for $tRNA^{Leu}_{UAG}$, depended on properties of the theory of selective isoacceptor charging which are robust to variation of unknown parameters but sensitive to some underlying qualitative assumptions, like the codon reading rules for an organism. This robustness, in combination with the universal character of the theory,[1] suggests that it will be useful for future clarifications of codon reading rules, control of gene expression and codon usage patterns in cells from different species.

Materials and Methods

Charged levels of leucine isoacceptors in *E. coli* cells

Plasmids

The pACtac vector was constructed by replacing the HindIII-BamHI restriction fragment of plasmid pACYC184[28] with a synthetic DNA fragment (aagcttttga caatt aatcggctcgtataa tgtgtggaattgtga gcggataacaattgg atcc) cut with HindIII and BamHI. This introduces the hybrid *trp-tac*, p*tac*[29] promoter into the plasmid.

The genomic sequence encoding *leuU*, PCR amplified with primers ccgcggccgcatgacggcgctgctgg and gggcccgttgacacaataaagtgcc was cloned into pGEM-T Easy vector (Promega). From this plasmid, the SalI-NaeI restriction fragment was ligated into the SalI-NruI restriction sites of pACtac vector resulting in plasmid p*leuU*. This vector has a modest copy number in *E. coli* and the expression of the cloned sequences can be modulated by the level of the *lac* inducer IPTG in the medium. The inserts in the pGEM-easy and final plasmids were confirmed by sequencing.

Strains

The two plasmids, p*leuU* and the empty vector, pACtac, were introduced into the *E. coli* strain MAS397 (*thr leu his argH thi mtl relA*$^+$ *spoT*$^+$ *lacI*^{q1}*lacZ*::Tn*5 recA1srl*::Tn*10*) that is a *recA1*, *lacI*^{q1} and λ^- derivative of CP78.[7] The auxotrophy of MAS397, together with the long history of starvation experiments with the parent strain, CP78, made it well suited for this study.

Expression level of $tRNA^{Leu}_{GAG}$ from p*leuU*

The relative amounts of tRNA isoacceptors in the different lanes of a Northern blot are expected to reflect their relative amounts in the cell population from which they were harvested. Accordingly, the relative amounts of $tRNA^{Leu}_{GAG}$ in the p*leuU* and in the pACtac containing strains were estimated from the radioactivity of the probe for $tRNA^{Leu}_{GAG}$, summed from the two bands representing its acylated and deacylated forms, normalized to the radioactivity of the probe for each of the other four leucine isoacceptor tRNAs. In each case, the ratio was six times higher in the p*leuU* containing strain compared to the control (data not shown). The ratio was the same independent of the reference tRNA used, thus taken as evidence for the invariance of the concentration of the other $tRNA^{Leu}$ isoacceptors.

Starvation and Northern blots

The strains were grown at 37 °C in Mops medium[30] supplemented with 15 μg/ml chloramphenicol, 0.4% glycerol, 5 μg/ml thiamin, 100 μg/ml each of arginine, histidine, isoleucine and leucine. The strains grew exponentially for at least ten generations before the onset of the starvation experiments. The expression from the plasmids was induced by the addition of 1.0 mM IPTG (final concentration) for one generation time (95 min) before starvation was initiated. Leucine

starvation was introduced by filtration of the cultures, followed by washes and re-suspension in a leucine-lacking, but otherwise identical medium.[8] The tRNA was harvested, and the acylated and deacylated forms were separated on sequencing gels, which were then used for the Northern blots. After probing for the individual tRNAs, the charged level was in each case estimated as the radioactivity of the probe hybridized to the acylated form of the tRNA isaocceptor, normalized to the sum of the radioactivities of the probes hybridized to its acylated and deacylated forms as described.[4,8]

Codon reading *in vitro*

Chemicals and buffers

Nucleoside triphosphates (ATP, UTP, and GTP), radioactive amino acid residues and unlabelled nucleotides were from Amersham (USA). Phosphoenolpyruvate (PEP), myokinase (MK), pyruvate kinase (PK), putrescine, spermidine and non-radioactive amino acid residues were from Sigma (USA). All other chemicals were of analytical grade from Merck (Germany). BD-Sepharose was a kind gift from Professor J. C. Janson, Uppsala. All experiments were carried out in polymix buffer[31] which has the following final composition: 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg(OAc)_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate (KP) (pH 7.5) and 1 mM DTE.

Preparation of mRNAs

Synthetic mRNAs, all with a strong Shine-Dalgarno sequence encoding the tri-peptide Met-Leu-Ile, with different leucine codons, were prepared[11] by *in vitro* phage T7 RNA polymerase transcription of the PCR amplified sequence. The sequence of the forward primer used for PCR was designed to introduce a strong phage T7 promoter into the PCR product. The product mRNAs contained a poly(A) tail for purification on a poly(dT) column (Amersham Biosciences).The full sequence of one mRNA was:

GUA CCG AAA UUA AUA CGA CUC ACU AUA GGG AAU UCG GGC CCU UGU UAA CAA UUA AGG AGG UAU ACU AUG **CUG** AUC UAA UUG CAG $(A)_{21}$

In the other variants of mRNA the second codon for leucine (bold letters) in the open reading frame (underlined) was replaced with either one of the following codons: CUC, CUU or CUA. The codon sequences were checked through translation by their established cognate tRNA isoacceptors.

Purification of tRNAs

$tRNA^{bulk}$ was purified from crude tRNA (*E. coli*) as described.[32] [^{3}H]fMet-$tRNA^{fMet}$ was prepared from $tRNA^{bulk}$ as described.[31] $tRNA^{Leu}$ isoacceptors were purified from $tRNA^{bulk}$ by BD-Sepharose chromatography with minor modifications as described.[33] $tRNA^{Leu}_{GAG}$ was used without further purification, while $tRNA^{Leu}_{CAG}$ and $tRNA^{Leu}_{cmo^5UAG}$ were separated from each other on 15% polyacrylamide gels containing 7 M urea. The purity of the tRNA isoacceptors was checked by Northern blot analysis with specific DNA oligos (data not shown). Pure $tRNA^{Leu}$ isoacceptors prepared by the solid-phase DNA probe method[34] were used as a marker and in control experiments. At each purification step, the fractions containing $tRNA^{Leu}$ isoacceptors were identified by aminoacylation test in the presence of *E. coli* LeuRS.[35]

Other components of the in vitro *translation system*

70 S ribosomes were prepared from the *E. coli* strain MRE 600, using sucrose gradient zonal ultracentrifugation as described.[36] Initiation factors were purified from overproducing strains as described.[31] Elongation factors EF-Tu and EF-Ts were purified as described.[35]

Dipeptide formation assay

The rate constants (k_{cat}/K_m) by which aminoacylated $tRNA^{Leu}$ isoacceptors (Leu-$tRNA^{Leu}$) read different codons were estimated from di-peptide formation experiments with pure components from an *E. coli* system for protein synthesis. Initiated ribosomes, containing [^{3}H]fMet-$tRNA^{fMet}$ in the P site and either one of four Leu codons in the A site, were prepared in an initiation mix, containing 70 S ribosomes (0.4 μM active), [^{3}H]fMet-$tRNA^{fMet}$ (1.2 μM), mRNA (1.0 μM), IF1, IF2, IF3 (0.8 μM each) and GTP (1 mM). Ternary complexes were formed in an aminoacylation mix, containing $tRNA^{Leu}$ (0.5 μM), EF-Tu (4 μM), EF-Ts (0.4 μM), LeuRS (0.2 U/μl), leucine (400 μM), ATP (2 mM), PEP (20 mM), GTP (1 mM), PK (100 μg/ml) and MK (4 μg/ml). Both mixes were pre-incubated for 10 min at 37 °C and loaded onto a quench-flow instrument (Chemical-Quench-Flow ModelQF-3, KinTek Corp.). They were rapidly mixed in equal volumes, incubated for varying times and quenched by formic acid to final concentration 17% (w/w). Dipeptide formation was quantified by HPLC with an on line radioactivity detector (βRAM3; INUS Inc.) from the ratio of the area of the [^{3}H]fMet-Leu dipeptide peak and the sum of the areas of the [^{3}H]fMet-Leu and [^{3}H]fMet peaks. Samples for the HPLC were prepared and processed as described.[37] The rates of peptide bond formation were determined by the rates of association of ternary complex in this concentration range (k_{cat}/K_m range; E.B., unpublished results), and k_{cat}/K_m values were estimated with Origin by fitting the model expression:

$$\mathrm{dip}(t) = \frac{r_0 t_0 (1 - e^{-tk(t_0 - r_0)})}{(t_0 - r_0 e^{-tk(t_0 - r_0)})} + b$$

to the time traces in Figure 2. In the model, r_0 is the total, active ribosome concentration, t_0 is the initial, active ternary complex concentration, t is the incubation time, k is the k_{cat}/K_m value to be estimated, b is the background and dip(t) is the concentration of dipeptide at incubation time t.

Analysis of modified nucleosides in purified $tRNA^{Leu}_{cmo^5UAG}$ and $tRNA^{Leu}_{CAG}$

Purified $tRNA^{Leu}_{cmo^5UAG}$ and $tRNA^{Leu}_{CAG}$ were digested to nucleosides by nuclease P_1 and bacterial alkaline phosphatase. The hydrolysate was applied to a Develosil 5 μ RP-AQEUOSE C-30 column (Phenomenax) and the gradient was optimized to separate cmo^5U from other nucleosides.[22] The identification of the compound denoted cmo^5U in $tRNA^{Leu}_{cmo^5UAG}$ was based on similarity of its UV spectrum and its retention time to that of synthetic cmo^5U. The RNA sequence of $tRNA^{Leu}_{CAG}$ is known, and we expected it to contain pseudouridine (Ψ), 4 thiouridine (s^4U), 5-methyluridine (m^5U), 2′-*O*-methylguanosine (Gm), and an uncharacterized guanosine derivative.[38,39] We monitored the eluate at 280 nm, at

which wavelength *D* (dihydrouridine) does not absorb and is not well separated using this chromatographic method. When monitored at 314 nm, s^4U was observed (data not shown). m^7G present in the hydrolysate of $tRNA^{Leu}_{cmo^5UAG}$ might be originating from a small contamination of another tRNA but may also be present at submolar level in $tRNA^{Leu}_{cmo^5UAG}$, since its structural gene *leuW* has a G in the position where m^7G normally is present in the tRNA. In addition we found in both tRNAs an unknown compound ("?" in Figure 3), which has a spectrum as a G-derivative but migrates at a position where no known G-derivative migrates. The modified nucleoside at position 37 was earlier identified as a guanosine derivative[39] or as m^1G or m^2G[38] and we know now that it must be a derivative of m^1G.[40] Our results show that both these tRNAs contain m^1G but *in vivo* it might be further modified and this modification may be removed during its purification or analysis. The compound "?" may be such a derivative. The relative response factor of cmo^5U to m^5U was determined from the content of cmo^5U in purified $tRNA^{Val}$ and this response factor was used to estimate the molar level of cmo^5U in $tRNA^{Leu}_{cmo^5UAG}$ to 0.7–0.8 mol cmo^5U/mol of tRNA. Although the HPLC analysis does not show the position in the tRNA of the modified nucleosides, cmo^5U is likely to be present in the wobble position, where it has been seen in all previous cases.[40]

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2005.08.076

The Supplementary Data describes the mathematical details on the theoretical predictions.

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