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Cysteine tRNAs of plant origin as novel UGA suppressors

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ABSTRACT

We have isolated and sequenced chloroplast (chl) and cytoplasmic (cyt) cysteine tRNAs from *Nicotiana rustica*. Both tRNAs carry a GCA anticodon but beyond that differ considerably in their nucleotide sequences. One obvious distinction resides in the presence of N^6-isopentenyladenosine (i^6A) and 1-methylguanosine (m^1G) at position 37 in chl and cyt $tRNA^{Cys}$ respectively. In order to study the potential suppressor activity of $tRNAs^{Cys}$ we used *in vitro* synthesized zein mRNA transcripts in which an internal UGA stop codon had been placed in either the tobacco rattle virus (TRV)- or tobacco mosaic virus (TMV)-specific codon context. *In vitro* translation was carried out in a messenger- and tRNA-dependent wheat germ extract. Both $tRNA^{Cys}$ isoacceptors stimulate read-through over the UGA stop codon, however, chl $tRNA^{Cys}_{GCA}$ is more efficient than the cytoplasmic counterpart. The UGA in the two viral codon contexts is suppressed to about the same extent by either of the two $tRNAs^{Cys}$, whereas UGA in the β-globin context is not recognized at all. The interaction of $tRNA^{Cys}_{GCA}$ with UGA requires an unconventional G:A base pair in the wobble position, as postulated earlier for plant $tRNA^{Tyr}_{G\Psi A}$ misreading the UAA stop codon. This is the first case that a cysteine-accepting tRNA has been characterized as a natural UGA suppressor.

INTRODUCTION

Suppressor tRNAs can be classified into two groups according to their decoding capacities. The first group includes tRNAs carrying an anticodon that permits classical base pairings in the interaction with any of the three stop codons. All tRNAs derived from mutagenesis of tRNA genes leading to 'induced suppression' belong to this group. The second group comprises tRNAs which misread stop codons by means of unconventional base interactions. Virtually all known natural suppressor tRNAs, with the exception of $tRNA^{[Ser]Sec}_{U*CA}$, are members of this group.

The first natural UGA suppressor tRNA to be characterized was normal $tRNA^{Trp}$ with a CCA anticodon from *Escherichi coli*. This tRNA stimulates read-through over the UGA stop codon at the end of the coat protein cistron in phage Qβ RNA *in vivo* and *in vitro* (1,2). Translation of the UGA nonsense codon by $tRNA^{Trp}_{CCA}$ necessitates a C:A mispairing at the first anticodon position. We have recently isolated two natural UGA suppressor tRNAs from tobacco plants on the basis of their ability to promote read-through over the leaky UGA stop codon in RNA-1 of tobacco rattle virus (TRV) in a wheat germ extract (3). Amino acid acceptance and nucleotide sequence identified the two UGA suppressors as chloroplast (chl) and cytoplasmic (cyt) $tRNA^{Trp}$ with a CmCA anticodon, i.e. translation of the UGA stop codon by the two plant $tRNA^{Trp}$ isoacceptors involves an unorthodox Cm:A interaction comparable with the C:A mispair of the *E.coli* suppressor tRNA mentioned above. Possibly as a consequence of this unconventional codon reading, natural suppressor tRNAs generally require a special codon context for their action (4–6).

A second well-known UGA suppressor tRNA in prokaryotic and animal cells is $tRNA^{[Ser]Sec}$ with a U*CA anticodon which inserts selenocysteine at defined in-frame UGA codons in mRNAs coding for selenoproteins (7). A third potential natural UGA suppressor might be cysteine tRNA with a GCA anticodon. Two indirect lines of evidence support this assumption. A G:A mispairing in the first anticodon position would be a prerequisite for reading of the UGA codon by $tRNA^{Cys}_{GCA}$. Such an unconventional interaction has been shown to exist in another example of stop codon suppression: we have shown that $tRNA^{Tyr}$ with a GΨA anticodon is capable of reading *in vitro* not only the UAG but also the UAA stop codon in a tobacco mosaic virus (TMV)-specific context (8). The latter interaction involves a G:A mismatch. Furthermore, Feng *et al.* (9) have demonstrated that mutation of the leaky UAG codon at the *gag–pol* junction in murine leukemia virus (MuLV) RNA to a UGA codon resulted in the incorporation of tryptophan, cysteine and arginine at the corresponding position of the read-through product upon translation in a reticulocyte lysate, indicating that UGA can possibly be read as cysteine.

In order to examine the presumable suppressor activity of $tRNA^{Cys}$ we have first isolated chl and cyt $tRNAs^{Cys}$ from tobacco leaves and have then studied the ability of these two $tRNA^{Cys}$ isoacceptors to suppress the UGA stop codon in a wheat germ extract. We show here that $tRNA^{Cys}_{GCA}$ is indeed capable of promoting read-through over a UGA stop codon as long as it is located in a favourable codon context.

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MATERIALS AND METHODS

Enzymes and reagents

SP6 RNA polymerase, T4 polynucleotide kinase, RNase A, RNase inhibitor from human placenta and BD-cellulose were from Boehringer (Mannheim, Germany). T4 RNA ligase was purchased from Pharmacia Biotech (Freiburg, Germany). [^{14}C]Methylated proteins used as molecular weight markers, L-[^{35}S]methionine and L-[^{35}S]cysteine with specific activities of 37 and 48 TBq/mmol respectively were obtained from Amersham Buchler (Braunschweig, Germany). Untreated wheat germ was a gift from Synpharma GmbH (Augsburg, Germany).

Plasmids

The recombinant plasmid pSP65-ML1 carries a zein gene from maize seedlings (10) cloned into the *Bam*HI and *Pst*I sites of pSP65 vector DNA. The construction of the expression vectors pSP65-TRV, pSP65-TMV$_3$ and pSP65-globin is described elsewhere (3,11).

Transcription *in vitro*

In vitro transcription of pSP65 plasmid DNAs by SP6 RNA polymerase was essentially carried out as described by Zerfaß and Beier (3). RNase inhibitor from human placenta was added to the reaction mixture at a concentration of 0.6 U/µl.

Fractionation and isolation of tRNAs^{Cys} from total tobacco tRNA

Preparation of unfractionated tRNA from leaves of *Nicotiana rustica* was performed as described by Beier *et al.* (12). Total tobacco tRNA was loaded onto a BD-cellulose column and fractionated by elution with a linear gradient of 0.35–1 M NaCl (in 0.01 M $MgCl_2$, 0.02 M NaOAc, pH 4.5). The hydrophobic tRNAs were eluted with 2 M NaCl, 15% ethanol. The tRNA^{Cys}-containing fractions were further purified by electrophoresis in a 10% non-denaturing polyacrylamide gel, pH 8.3, followed by a 10% denaturing polyacrylamide gel, pH 3.5.

Preparation of yeast UGA suppressor tRNA

Total tRNA was prepared from the *Schizosaccharomyces pombe* strain sup3-5h$^-$, which contains a serine-incorporating UGA suppressor tRNA with a U*CA anticodon. U* is predominantly 5-(methoxycarbonyl methyl)uridine, mcm^{5}U (13). The suppressor tRNA was partially purified by BD-cellulose chromatography.

Sequencing of tRNA by post-labelling techniques

Nucleotide sequences were determined according to Stanley and Vassilenko (14), with some modifications as described earlier (3,12). tRNA 5′- and 3′-labelling, controlled digestion with alkali and two-dimensional mobility shift analyses were carried out as described by Beier and Gross (15).

RNA dot blot hybridization

tRNA samples (100–500 ng) derived from BD-cellulose or gel fractions were suspended in 50 µl TE buffer, pH 7.6 (10 mM Tris–HCl, 1 mM Na_2EDTA), denatured by adding 30 µl 20× SSC, pH 7.0 (3 M NaCl, 0.3 M sodium citrate) and 20 µl 37% formaldehyde solution and incubated at 60°C for 15 min. One sheet of Whatman 3 MM paper and a nylon membrane (Biodyne B, Pall, Dreieich) soaked in 10× SSC prior to use were placed in a multiwell filtration manifold (Schleicher & Schüll, Minifold™). The denatured tRNA probes were applied into individual wells under low vacuum and washed with 500 µl 10× SSC. After removal and drying of the membrane the tRNAs were immobilized by ultraviolet cross-linking according to Heitzler *et al.* (16). The dried membrane was first prehybridized for 1 h at 40°C in 6× SSC, 0.1% SDS, 10× Denhardt's solution and 50 µg/ml sonicated salmon sperm DNA. Hybridization was performed overnight at 40°C in the same solution containing in addition ~10^5 c.p.m./ml 5′-labelled oligonucleotide. After hybridization the membrane was washed twice in 6× SSC, 0.1% SDS for 10 min at room temperature and once at the appropriate T_H (= T_D – 5°C) for 7 min.

Synthesis and labelling of oligodeoxyribonucleotides

Oligonucleotides were synthesized with a Gene Assembler Plus from Pharmacia LKB. They were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified in a 15% polyacrylamide–8 M urea gel. The oligonucleotides 5′-GGATTTGCAGTCCTCTGCC-3′, 5′-GATCTGCAGTCAAATGCTCT-3′ and 5′-GTTTTGGAG ACCCACGTTCT-3′ were used for identification of tobacco chl tRNA^{Cys}, cyt tRNA^{Cys} and chl tRNA^{Trp} respectively.

Aminoacylation of tRNAs

Assay of cysteine acceptance by tobacco tRNAs was performed in a reaction mixture containing 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 5 mM ATP, 3 mM dithiothreitol, 0.5 µM CTP, 30 mM cysteine, 45 Bq/µl ^{35}S-labelled cysteine, 2.6% (v/v) crude aminoacyl-tRNA synthetase from wheat germ (15 mg protein/ml) and appropriate amounts of tRNA.

In vitro translation in wheat germ extract

A wheat germ cell-free extract depleted of endogenous mRNAs and tRNAs was prepared according to Pfitzinger *et al.* (17). *In vitro* translation was carried out in a total volume of 10 µl containing 25% (v/v) wheat germ extract, 10% (v/v) wheat germ initiation factor solution, 20 mM HEPES–KOH, pH 7.5, 2 mM dithiothreitol, 1 mM ATP, 20 µM GTP, 8 mM creatine phosphate, 60 µg/ml creatine phosphokinase, 50 µM spermine, 2.5 mM $Mg(OAc)_2$, 120 mM KOAc, 1 U/µl RNase inhibitor, 70 µM each of 19 L-amino acids (without methionine), 15 kBq/µl [^{35}S]methionine and 50 µg/ml template RNA. The reaction mixture was incubated for 60 min at 30°C and was followed by an incubation with RNase A (0.05 mg/ml) for 15 min at 30°C in order to remove labelled methionyl-tRNA obscuring the protein analysis.

Analysis of translation products

Proteins were analysed by electrophoresis in 15% polyacrylamide slab gels containing 0.1% SDS. Gels were fixed overnight, fluorographed and exposed to Fuji RX X-ray film at –80°C. The ratio of the protein products derived from termination and read-through events respectively was determined by densitometric quantification of incorporated radioactive methionine using an LKB UltraScan XL® laser densitometer. Appropriate corrections

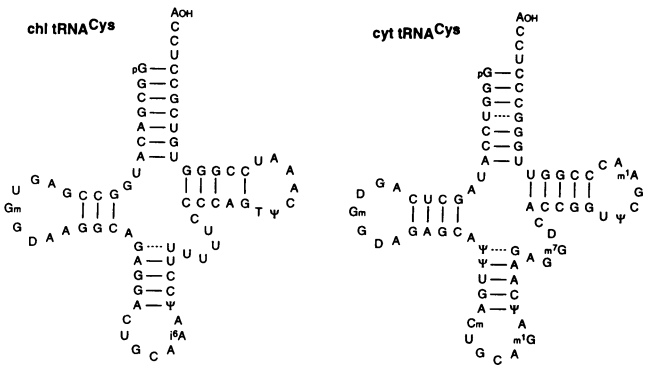

Figure 1. Nucleotide sequences of chloroplast (chl) and cytoplasmic (cyt) $tRNA^{Cys}$ from *N.rustica* in the cloverleaf arrangement. i^6A, N^6-isopentenyladenosine; m^1A, 1-methyladenosine; Cm, 2′-*O*-methylcytidine; D, dihydrouridine; Gm, 2′-*O*-methylguanosine; m^1G, 1-methylguanosine; m^7G, 7-methylguanosine; Ψ, pseudouridine; T, ribothymidine.

were made for the two additional methionine residues present in the read-through products.

RESULTS

Purification of chloroplast (chl) and cytoplasmic $tRNA^{Cys}$ from tobacco leaves

Total tRNA from *Nicotiana rustica* was fractionated by BD-cellulose chromatography as described previously (3,12). Two major $tRNA^{Cys}$-containing fractions (i.e. I and II) eluted from the column as shown by aminoacylation assays with [^{35}S]cysteine in the presence of a crude wheat germ aminoacyl-tRNA synthetase preparation. The $tRNAs^{Cys}$ were further purified by electrophoresis in two successive polyacrylamide gel electrophoreses and subsequently sequenced. The $tRNA^{Cys}$ in the late eluting fraction II turned out to be chloroplast (chl) $tRNA^{Cys}$ with a GCA anticodon (Fig. 1). The tRNA nucleotide sequence is co-linear with the corresponding gene sequence of chl $tRNA^{Cys}$ from *N.tabacum* var. Bright Yellow 4 (18). As with most organelle tRNAs, very few modifications are present in chl $tRNA^{Cys}$, the most important being i^6A at position 37.

The $tRNA^{Cys}$ in fraction I was identified as cytoplasmic (cyt) $tRNA^{Cys}$ with a GCA anticodon. In contrast to chl $tRNA^{Cys}$, it carries m^1G at position 37. A further difference in the anticodon loop region of the two $tRNAs^{Cys}$ lies in the presence of Cm at position 32 in cyt $tRNA^{Cys}$ (Fig. 1). Although tobacco chl and cyt $tRNA^{Cys}$ show only an overall sequence homology of 65%, they are both charged with cysteine to the same level by a wheat germ aminoacyl-tRNA synthetase preparation (not shown). This was not the case for tobacco chl and cyt $tRNA^{Trp}$, the former being only poorly charged with [^{3}H]tryptophan by wheat germ aminoacyl-tRNA synthetase, but a good substrate for *Thermus thermophilus* or *Bacillus subtilis* synthetase (3).

In order to study the suppressor activity of tobacco $tRNAs^{Cys}$ we needed larger quantities of these tRNAs. After having established the nucleotide sequences of chl and cyt $tRNA^{Cys}$ we applied dot blot hybridization analyses with specific probes instead of aminoacylation assays for further purifications, which facilitated the unambigous identification of chl and cyt $tRNA^{Cys}$ in column and gel fractions. At the very end the purity and identity of the isolated $tRNAs^{Cys}$ were always confirmed by mobility shift analyses of 5′-end-labelled tRNA.

Chl $tRNA^{Cys}$ promotes read-through over leaky UGA stop codons

We have recently established an SP6 RNA polymerase-specific expression vector containing a zein gene which naturally harbours an internal in-frame TAG stop codon (i.e. pSP65-ML1; Fig. 2). The *in vitro* run-off transcript generated by SP6 RNA polymerase was shown to be efficiently translated in a messenger- and tRNA-dependent wheat germ extract (3,8). In the present study we have used zein mRNA transcripts in which the internal UAG had been exchanged for the UGA stop codon and in which three codons on each side of this stop codon had been replaced by the corresponding codons flanking the leaky UAG and UGA codons in tobacco mosaic virus (TMV) and tobacco rattle virus (TRV) RNA (i.e. pSP65-TMV_3 and pSP65-TRV, Fig. 2) respectively. The two original viral read-through regions, consisting of a total of only seven codons, have been shown to efficiently support suppression by plant $tRNA^{Tyr}_{G\Psi A}$ and $tRNA^{Trp}_{CmCA}$ respectively (3,8).

Translation *in vitro* of the two transcripts pSP65-TRV and pSP65-TMV_3 in the presence of purified chl $tRNA^{Cys}_{GCA}$ resulted in synthesis of the 26 kDa read-through protein (Fig. 3, lanes c and d), indicating that the UGA stop codon in either the TRV- or TMV-specific context is suppressed. We repeated these experiments several times with chl $tRNA^{Cys}$ derived from different tRNA preparations and obtained reproducible read-through efficiencies ranging from 4 to 7% (Table 1). The yeast UGA suppressor $tRNA^{Ser}_{U*CA}$ derived from chemical mutagenesis stimulates read-through over the UGA codon in the TMV or TRV context up to 60 and 70% respectively (Fig. 3, lanes e and f).

Table 1. Read-through (%) over the UGA stop codon by *Nicotiana* $tRNAs^{Trp}$ and $tRNAs^{Cys}$ as a function of codon context[a]

tRNA	TRV context	TMV context	β Globin context
chl $tRNA^{Cys}_{GCA}$	5.3	5	0
chl $tRNA^{Trp}_{CmCA}$	25	19.5	0
cyt $tRNA^{Cys}_{GCA}$	1.2	3	0
cyt $tRNA^{Trp}_{CmCA}$	12.5	11.3	0

[a]The values given in the table are average numbers from three single experiments.

Comparison of UGA suppressor activity of tobacco chl $tRNA^{Cys}$ and chl $tRNA^{Trp}$

In a recent study we identified tobacco chl $tRNA^{Trp}_{CmCA}$ as a strong UGA suppressor which stimulates read-through over the internal UGA stop codon in RNA-1 of TRV with an efficiency ranging from 25 to 30% (3,11). To directly compare the UGA suppressor activity of chl $tRNA^{Cys}_{GCA}$ and chl $tRNA^{Trp}_{CmCA}$ we examined the read-through activity of the two UGA suppressors in relation to the nucleotides surrounding the UGA codon, choosing the TRV-, TMV- and β-globin-specific codon contexts. As seen in Figure 4 (lanes b and e), chl $tRNA^{Trp}_{CmCA}$ reads the UGA codon in the TRV and TMV contexts with efficiencies of 20–28 and 15–22% respectively (Table 1), whereas chl $tRNA^{Cys}_{GCA}$ stimulates read-

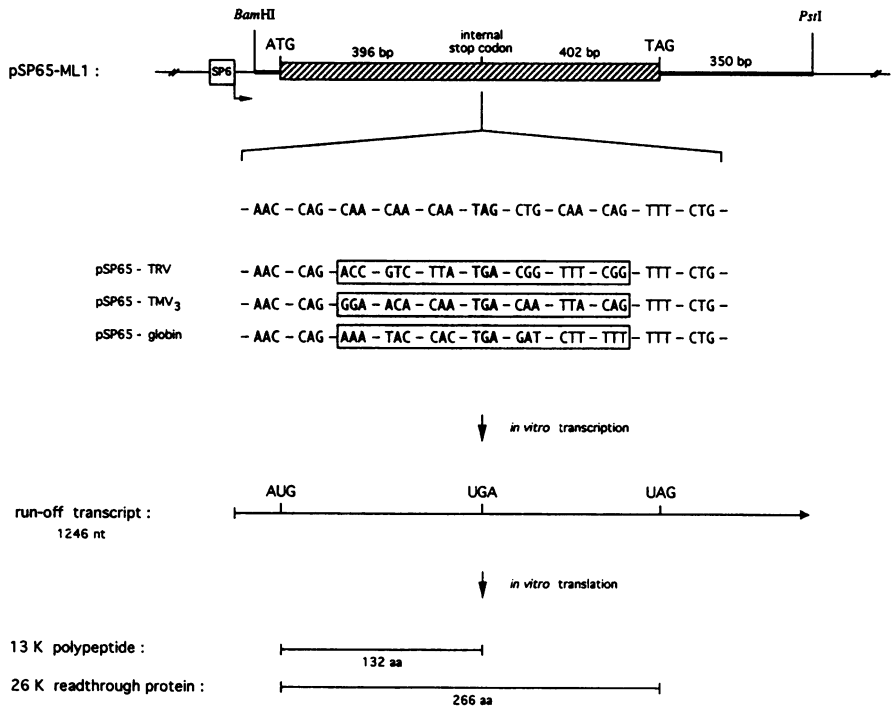

Figure 2. Structures of expression vector pSP65-ML1 and its derivatives. A 1.2 kb fragment from *Zea mays* DNA, harbouring a zein gene, was cloned into the *Bam*HI and *Pst*I sites of the SP6 RNA polymerase-specific vector pSP65 (3). The coding region of the zein gene is indicated by a hatched box. The transcripts generated by constructs pSP65-TRV, pSP65-TMV_3 and pSP65-globin contain a UGA instead of the original internal in-frame UAG stop codon in the zein mRNA and a total of six codons flanking the leaky UGA in RNA-1 of TRV (36), the leaky UAG codon in TMV RNA (37) and the UGA stop codon at the end of the β-globin mRNA (38) respectively. *In vitro* translation of the run-off transcript results in the synthesis of a 13 kDa termination protein and, in the presence of an appropriate UGA suppressor tRNA, in the production of a 26 kDa read-through protein.

through over the UGA codon in the two contexts with an efficiency ranging from 4 to 7% (Fig. 4, lanes c and f and Table 1). Neither chl $tRNA^{Trp}_{CmCA}$ nor chl $tRNA^{Cys}_{GCA}$ are capable of interacting with the UGA stop codon at the end of the β-globin mRNA (Fig. 4, lanes h and i).

Comparison of UGA suppressor activity of tobacco cyt $tRNA^{Cys}$ and cyt $tRNA^{Trp}$

In addition to chl $tRNA^{Cys}_{GCA}$ we have also examined cyt $tRNA^{Cys}_{GCA}$ for its UGA suppressor activity. *In vitro* translation of the transcript pSP65-TMV_3 in the presence of purified cyt $tRNA^{Cys}_{GCA}$ resulted in the synthesis of low amounts (up to 3%) of the 26 kDa read-through product (Fig. 5, lane d) and translation of the pSP65-TRV transcript yielded even lower amounts (~1%) of the 26 kDa protein (Fig. 5, lane c). cyt $tRNA^{Trp}_{CmCA}$ was more effective as a UGA suppressor, yielding 10–15% of the read-through protein in the TRV- as well as in the TMV-specific contexts (Fig. 5, lanes e and f and Table 1). As shown with the two types of chl UGA suppressor tRNAs, cyt $tRNA^{Cys}_{GCA}$ and cyt $tRNA^{Trp}_{CmCA}$ do not support read-through over the UGA stop codon in the β-globin context (not shown).

DISCUSSION

We have identified $tRNA^{Cys}$ with a GCA anticodon as a novel UGA suppressor. Although some recent observations have made the existence of cysteine-accepting UGA suppressors very likely,

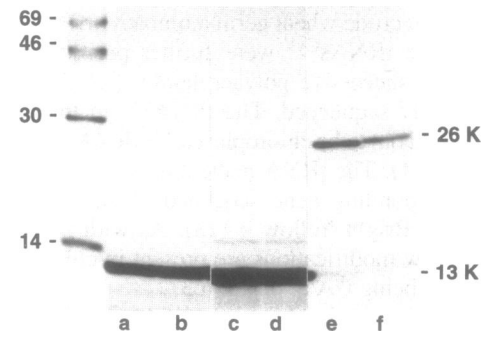

Figure 3. Suppression of leaky UGA stop codons by chl $tRNA^{Cys}_{GCA}$ from *N.rustica*. *In vitro* transcripts derived from pSP65-TRV (lanes a, c and e) and pSP65-TMV_3 (lanes b, d and f) respectively were translated in wheat germ extract in the absence of tRNA (lanes a and b) or in the presence of 30 μg/ml purified *Nicotiana* chl $tRNA^{Cys}_{GCA}$ (lanes c and d). The effect of added yeast UGA suppressor $tRNA^{Ser}_{U*CA}$ (50 μg/ml) on protein synthesis is shown in lanes e and f. [^{35}S]Methionine-labelled proteins were separated in a 15% denaturing gel. [^{14}C]Methylated protein standards ranging in size from 14 to 69 kDa are shown at the left side of the gel. The positions of the 13 kDa and 26 kDa termination and read-through proteins respectively are indicated at the right.

$tRNA^{Cys}_{GCA}$ has not been studied for its suppressor activity *in vitro* or *in vivo* until now. One reason for this neglect in eukaryotic systems might simply be the fact that only very few $tRNA^{Cys}$ or $tDNA^{Cys}$ sequences of nuclear origin are known (19). Besides the $tRNA^{Cys}$ sequence from *Saccharomyces cerevisiae* there is one report about the isolation of a mouse genomic clone containing

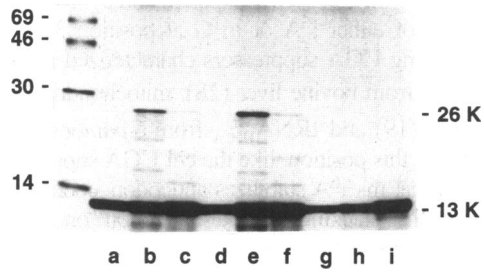

Figure 4. Comparison of UGA read-through activities of chl $tRNA^{Cys}_{GCA}$ and chl $tRNA^{Trp}_{CmCA}$. *In vitro* transcripts derived from pSP65-TRV (lanes a–c), pSP65-TMV_3 (lanes d–f) and pSP65-globin (lanes g–i) were translated in wheat germ extract in the absence of added tRNA (lanes a, d and g) or in the presence of ~30 µg/ml purified *Nicotiana* chl $tRNA^{Trp}_{CmCA}$ (lanes b, e and h) and chl $tRNA^{Cys}_{GCA}$ (lanes c, f and i) respectively.

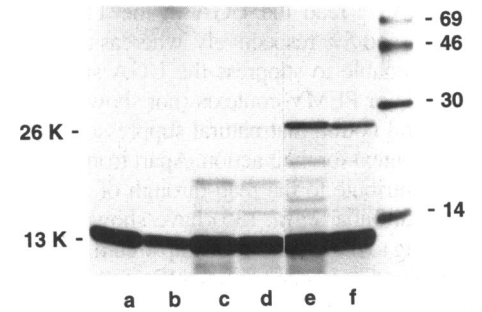

Figure 5. Comparison of UGA read-through activities of cyt $tRNA^{Cys}_{GCA}$ and cyt $tRNA^{Trp}_{CmCA}$. *In vitro* transcripts derived from pSP65-TRV (lanes a, c and e) and pSP65-TMV_3 (lanes b, d and f) were translated in wheat germ extract in the absence of added tRNA (lanes a and b) or in the presence of ~30 µg/ml purified *Nicotiana* cyt $tRNA^{Cys}_{GCA}$ (lanes c and d) and cyt $tRNA^{Trp}_{CmCA}$ (lanes e and f) respectively.

four $tRNA^{Cys}$-encoding genes (20). The cyt $tRNA^{Cys}$ from *N.rustica* described here (Fig. 1) shows 85% sequence homology with the coding region of these $tRNA^{Cys}$ genes and only 61% homology with the corresponding yeast gene.

As mentioned earlier, Feng *et al.* (9) have demonstrated that replacement of the leaky UAG stop codon in MuLV RNA by a UGA codon directs incorporation of cysteine in addition to tryptophan and arginine upon translation in a reticulocyte lysate. Furthermore, Meyer *et al.* (21) have shown that three in-frame UGA codons in pheromone 3 of *Euplotes octocarinatus* are translated as cysteine. In both studies the tRNAs responsible for the observed decoding of UGA as cysteine were not characterized. Another interesting report by Baron *et al.* (22) supports our finding that a tRNA with the anticodon GCA can in principle interact with the UGA codon. These authors found that changing the U*CA anticodon of *E.coli* $tRNA^{[Ser]Sec}$ to UGA (specific for a serine-inserting tRNA) abolished the capacity to decode UGA in formate dehydrogenase mRNA, whereas alteration to a GCA anticodon (specific for a cysteine-inserting tRNA) permitted 30% read-through *in vivo*.

For the detection and quantification of UGA suppressor activity displayed by plant $tRNA^{Cys}_{GCA}$ we have employed two zein mRNA *in vitro* transcripts which contain an internal in-frame UGA codon and six codons surrounding the leaky stop codon in TRV RNA-1 and TMV RNA respectively (Fig. 2). Both read-through regions have been proven to efficiently stimulate stop codon suppression *in vitro* and *in vivo*. Thus we have shown that the UAG codon in the TMV-specific codon context is read by plant $tRNA^{Tyr}_{G\Psi A}$ with an efficiency of 25–30% in a wheat germ extract (8,11). Valle *et al.* (23) also found that the UAG codon in a TMV-like context is efficiently suppressed in reticulocyte lysate. Moreover, if UAG in the same context was replaced by the UGA stop codon, an increased suppressor activity was measured, indicating an intrinsic leakiness of this read-through region which is not associated with a specific stop codon. The UAG codon in the TMV context is also suppressed *in vivo*, with an efficiency of 4–5%, as shown by Skuzeski *et al.* (24), who have studied stop codon read-through in tobacco protoplasts by means of a β-glucoronidase reporter gene. Recently we have shown that the UGA codon in TRV RNA-1 is as leaky as the UAG in TMV RNA if tobacco chl $tRNA^{Trp}_{CmCA}$ was added to a wheat germ extract, i.e. up to 25% read-through protein was synthesized (3).

The first identification of natural suppressor tRNAs was based on the utilization of the two read-through regions described above and only those tRNAs which yielded the highest suppressor activity in all purification steps were characterized in more detail (3,12). Consequently we had selected the relatively strong suppressors $tRNA^{Tyr}_{G\Psi A}$ and $tRNA^{Trp}_{CmCA}$. We had further stated that these are the potential UAG and UGA suppressors used by TMV and TRV *in vivo* for the synthesis of their corresponding read-through products. However, in the meantime further data has accumulated indicating that a given stop codon in a given codon context can be recognized by more than one specific tRNA. For instance, UAG in the TMV context can be recognized by $tRNA^{Tyr}_{G\Psi A}$ and, in addition, albeit less efficiently, by *Tetrahymena* cyt $tRNA^{Gln}_{UmUG}$ (11) and by tobacco cyt $tRNA^{Gln}_{CUG}$ (M.Grimm and H.Beier, unpublished). Furthermore, Valle *et al.* (25) have recently identified two $tRNA^{Leu}$ isoacceptors from calf liver as suppressors which are capable of misreading the leaky UAG stop codon in TMV RNA *in vitro*. As shown here, the UGA codon in the TRV context not only supports read-through by $tRNA^{Trp}_{CmCA}$, but also by $tRNA^{Cys}_{GCA}$ (Fig. 3). The finding that leaky read-through regions are active with all three stop codons and with different suppressor tRNAs argues against a specific interaction of the nucleotides surrounding the stop codon with the tRNA.

The suppressor activity of chl or cyt $tRNA^{Cys}$ in the two viral codon contexts is clearly lower than that of the two $tRNA^{Trp}$ isoacceptors (Figs 4 and 5 and Table 1). As observed for most natural suppressor tRNAs, an unconventional base pair is involved in interaction of $tRNA^{Cys}_{GCA}$ with the UGA stop codon (Fig. 6c). Thus one reason for the weaker suppressor activity displayed by $tRNA^{Cys}_{GCA}$ as compared with $tRNA^{Trp}_{CmCA}$ could possibly be that a G:A base pair is less often formed or less tolerated by the ribosome than a Cm:A interaction. In this context it should be noted that in the case of $tRNA^{Tyr}_{G\Psi A}$ reading the two stop codons UAG and UAA the interaction with UAA is weaker (8), indicating that a G:G base pair is favoured over a G:A pair by this tRNA.

Apart from anticodon–codon affinity, structural elements of the $tRNA^{Cys}$ itself could further contribute to the observed lower UGA suppressor activity of $tRNA^{Cys}$ as compared with $tRNA^{Trp}$. For instance, it has been shown by Smith and Yarus (26) that a G→A mutation at position 24 in *E.coli* $tRNA^{Trp}_{CCA}$ reduces the rate

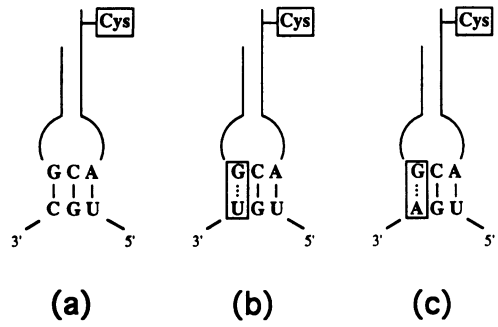

Figure 6. Schematic presentation of $tRNA^{Cys}_{GCA}$ interacting with the two cysteine codons UGC (**a**), UGU (**b**) and the stop codon UGA (**c**). The reading of UGU involves a G:U wobble base pair (b), whereas the decoding of UGA necessitates an unconventional base pair (c). We have recently presented two alternative models for the putative G:A interaction and dicussed their relevance with respect to known data (8).

at which the ribosome rejects non-cognate tRNAs. As a consequence this mutated $tRNA^{Trp}$ is a stronger UGA suppressor than the wild-type $tRNA^{Trp}$. Vacher *et al.* (27) have examined the UGA suppressor activity of a yeast $tRNA^{Cys}$ with a UCA anticodon injected into *Xenopus laevis* oocytes together with rabbit β-globin mRNA. They found that yeast $tRNA^{Cys}_{UCA}$ was a much less efficient suppressor than yeast $tRNA^{Ser}_{U*CA}$ or yeast mitochondrial $tRNA^{Trp}_{U*CA}$, in accordance with the general observation that different suppressor tRNAs differ in their abilities to read a given stop codon.

We have identified cyt $tRNA^{Trp}_{CmCA}$ and cyt $tRNA^{Cys}_{GCA}$, as well as the corresponding chl tRNAs, as natural UGA suppressors in plants. Our results clearly indicate that in both cases the chl tRNAs are the stronger UGA suppressors as compared with their cytoplasmic counterparts (Figs 4 and 5; 8). Since the overall sequence homology is not very high we can only speculate about the reason for this marked difference in UGA suppressor activity.

One obvious distinction between the chl and cyt isoacceptors lies in the presence of either i^6A or m^1G at position 37 (Fig. 1; 8). Actually, all strong UGA suppressors characterized in eukaryotes, i.e. $tRNA^{[Ser]Sec}_{U*CA}$ from bovine liver (28), mitochondrial $tRNA^{Trp}_{U*CA}$ from mammals (19) and $tRNA^{Ser}_{U*CA}$ from *S.pombe* (13) carry an i^6A or ms^2i^6A at this position, like the chl UGA suppressors. It has been proposed that ms^2i^6A stabilizes anticodon–codon interactions by increasing the stacking of the anticodon on neighbouring nucleotides, thus modulating the efficiency of suppressor tRNAs for reading stop codons (29,30), whereas m^1G is thought to prevent translational frame-shifting (31).

Read-through of stop codons has been documented for a number of viral genes (32–34). Plant viral RNAs harbouring a suppressible UGA stop codon include members of the tobravirus, furovirus, machlomovirus and enamovirus groups (Table 2). Of these RNAs all but MCMV RNA and PEMV RNA-1 contain a UGA in a codon context very similar to that of TRV RNA-1. As shown in Table 1, cyt and chl $tRNA^{Cys}$ read the UGA in the TRV context with efficiencies of ~1 and 5% respectively, whereas the two $tRNA^{Cys}$ isoacceptors are unable to suppress the UGA stop codon in the β-globin (Table 1) or PEMV contexts (not shown). These results support the general notion that natural suppressor tRNAs require a special codon context for their action. Apart from a leaky context, other features contribute to the read-through of stop codons. For instance, Mottagui-Tabar *et al.* (35) have shown that the amino acid corresponding to the second codon upstream of the stop codon seems to be a major determinant for the efficiency of read-through.

While the biological significance of stop codon read-through in cellular mRNAs is unknown, there is ample evidence that suppression of stop codons in viral RNAs results in the synthesis of read-through products that are essential for the virus (32–34). Although we do not know what role $tRNA^{Cys}$ may play as a potential UGA suppressor it should be emphasized that even apparent weak suppressors producing only small amounts of read-through proteins might have an essential function in cellular metabolism, proliferation or differentiation.

Table 2. Natural UGA read-through sites in different plant viruses

Virus	Genus	Sequence of the read-through region									Reference
TRV	Tobravirus	E	T	V	L	*	R	F	R	S	
(RNA-1)		GAG	ACC	GUC	UUA	UGA	CGG	UUU	CGG	UCU	(36)
PEBV	Tobravirus	D	A	M	K	*	R	C	R	S	
(RNA-1)		GAU	GCU	AUG	AAA	UGA	CGG	UGU	CGG	UCA	(39)
SBWMV	Furovirus	E	L	T	K	*	R	F	G	S	
(RNA-1)		GAG	CUU	ACU	AAA	UGA	CGG	UUU	GGG	UCG	(40)
PCV	Furovirus	E	Q	T	K	*	R	F	G	S	
(RNA-1)		GAA	CAG	ACC	AAA	UGA	CGG	UUU	GGG	UCA	(41)
SBWMV	Furovirus	E	G	S	S	*	R	D	G	V	
(RNA-2)		GAA	GGU	UCG	AGU	UGA	CGG	GAC	GGC	GUC	(40)
MCMV	Machlomovirus	F	N	F	N	*	A	G	V	C	
		UUC	AAU	UUC	AAC	UGA	GCU	GGA	GUG	UGU	(42)
PEMV	Enamovirus	N	A	S	L	*	G	D	D	A	
(RNA-1)		AAU	GCC	UCC	CUC	UGA	GGG	GAC	GAC	GCU	(43)

TRV, tobacco rattle virus; PEBV, pea early browning virus; SBWMV, soil-borne wheat mosaic virus; PCV, peanut clump virus; MCMV, maize chlorotic mottle virus; PEMV, pea enation mosaic virus.

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