The EMBO Journal vol.6 no.10 pp.3049-3055, 1987

Novel amber suppressor tRNAs of mammalian origin

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Communicated by A.-L.Haenni

Two amber suppressor tRNAs have been isolated from calf liver. They are different from previously identified naturally occurring amber suppressors of eukaryotes in so far as they are neither $tRNA^{Tyr}$ nor $tRNA^{Gln}$. They are leucine isoacceptors and their nucleotide sequence indicates that they harbour a CAA and a CAG anticodon respectively. Both species are functional as amber suppressors as demonstrated by readthrough of the amber codon which terminates the 126 kd protein gene of tobacco mosaic virus RNA. The results bring new information in the discussion of codon-anticodon recognition and regulation of termination in eukaryotic protein synthesis.

Key words: codon-anticodon recognition/eukaryotic amber suppressor tRNAs/natural suppression/termination of protein synthesis/tobacco mosaic virus

Introduction

Amber (UAG), ochre (UAA) and opal (UGA) codons specify termination in the 'universal' genetic code and as such are recognized by release factors during protein synthesis. In 'divergent' codes, such as those used in mitochondria and in the prokaryote *Mycoplasma capricolum*, UGA is used to specify tryptophan (for a review see Fox, 1985), and in ciliates UAG and UAA code for glutamine (Hanju *et al.*, 1986).

However in the 'universal' code, nonsense codons may also at times be recognized by tRNAs called 'suppressor' tRNAs, that allow the synthesis with various efficiencies of readthrough proteins. Recognition of termination codons might also serve to insert modified amino acids into proteins (Hatfield, 1985; Chambers *et al.*, 1986), although this still has to be supported by experimental evidence.

Nonsense suppressor tRNAs were initially found to be mutated tRNA species isolated from mutagenized cells and generally modified in their anticodon. These so-called 'genetic' suppressor tRNAs have been extensively studied in prokaryotes and yeast (for a review, see Celis and Smith, 1979) and only recently characterized in another eukaryote namely the worm *Caenorhabditis elegans* (Wills *et al.*, 1983). In contrast, 'natural' suppressor tRNAs are, as defined here, normal non mutated tRNA species that can suppress termination codons and are isolated from wild-type cells. They were discovered a few years ago when examining translation of certain viral mRNAs. A well-documented example is that of TMV (tobacco mosaic virus). The genomic RNA of TMV has a '+' polarity and encodes two high molecular weight nonstructural proteins: the 183 kd and 126 kd proteins. These proteins are initiated at the same site on the mRNA and the 183 kd protein results from suppression of the amber codon at the end of the open reading frame of the 126 kd protein gene (Pelham, 1978; Goelet *et al.*, 1982). Both proteins are observed *in vitro* (Pelham, 1978) and *in vivo* (Beier *et al.*, 1980). Since the first report of readthrough in TMV, suppression of termination codons has been proposed as a mechanism of regulating gene expression for an ever growing number of plant and animal RNA viruses (for reviews, see Kozak, 1986; Morch *et al.*, 1987).

In vitro translation of TMV RNA has been used as a convenient system for assaying 'natural' amber suppressor tRNAs. These tRNAs are expected to stimulate the synthesis of the 183 kd readthrough product at the expense of the 126 kd protein. 'Natural' suppressor tRNAs were identified in *Drosophila melanogaster* (Bienz and Kübli, 1981), in tobacco where they are thought to be responsible for the *in vivo* suppression of TMV RNA (Beier *et al.*, 1984a), in wheat (Beier *et al.*, 1984b) and in lupin (Barciszewski *et al.*, 1985). They all correspond to major cytoplasmic $tRNA^{Tyr}$ species with a GψA anticodon. Only very recently a minor $tRNA^{Gln}_{UmUG}$ was isolated from mouse liver that is also capable of reading the UAG codon of TMV RNA (Kuchino *et al.*, 1987).

We have observed that the addition of calf liver tRNA to a reticulocyte lysate translation system programmed by TMV RNA stimulates the synthesis of the 183 kd readthrough protein. This prompted us to search for a naturally occurring amber suppressor tRNA in calf liver. In this paper we report the isolation and sequencing of two major cytoplasmic tRNA species from calf liver that act as amber suppressors *in vitro*.

Results

Calf liver amber suppressor activity is not related to a $tRNA^{Tyr}$

Total calf liver tRNA was partially purified by BD-cellulose column chromatography. The elution profile is shown in Figure 1A. Individual fractions were assayed for suppressor activity by *in vitro* translation using a reticulocyte lysate programmed by TMV RNA: fractions 77-143 were found to stimulate the synthesis of the 183 kd readthrough product (data not shown). Since all reports available at the time these experiments were performed showed that naturally occurring amber suppressors of eukaryotic origin are $tRNA^{Tyr}$ with a GψA anticodon, the BD-cellulose fractions were also assayed for their ability to aminoacylate [^{3}H]tyrosine (Figure 1A). Fractions were pooled on the basis of these two assays and the three pooled samples tested again for these same two activities (Figure 1B and Table I). Pool P_3 (fractions 45-69) contains tyrosine-accepting activity (Figure 1A and Table I) and is inactive in amber suppression (Figure 1B, lane 3). Pool P_1 (fractions 70-115) contains tyrosine-accepting activity of lower specific activity than P_3 (Figure 1A and Table I); in addition, it displays amber suppressor activity (Figure 1B, lane 4). Pool P_2 (fractions 130-150) lacks tyrosine-accepting activity (Figure 1A and Table I) but shows amber suppressor activity (Figure 1B, lane 5). These results suggest that calf liver tRNA contains at least two major $tRNA^{Tyr}$ species and that part of the amber suppressor activity coelutes with one of them under the conditions used.

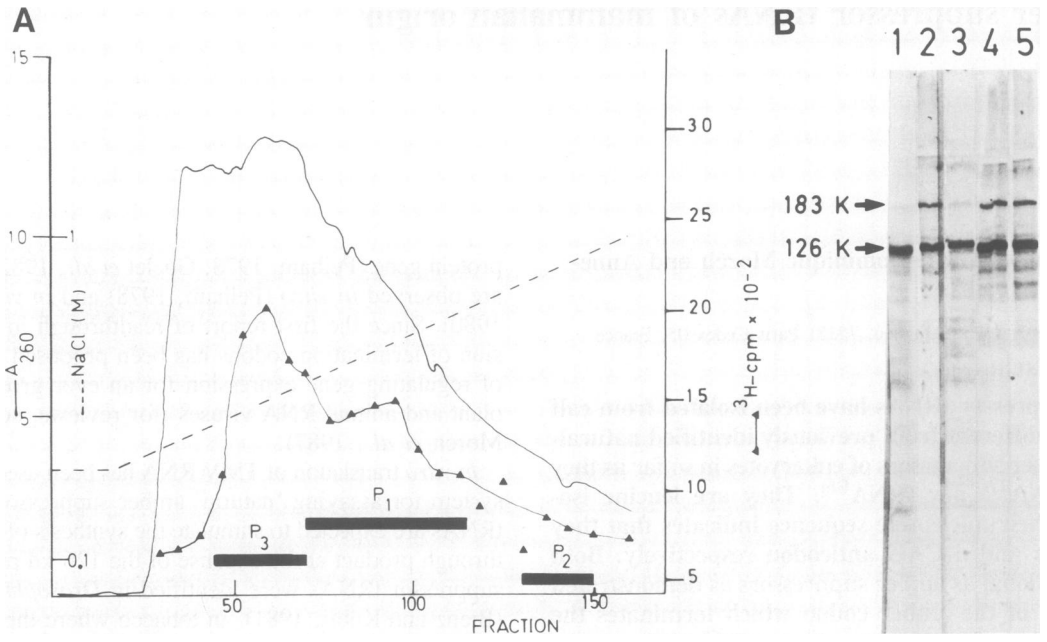

Fig. 1. (**A**). BD-cellulose column chromatography of unfractionated calf liver tRNA. (—): A_{260} profile; (▲—▲): [^{3}H]tyrosine-accepting activity; (---): linear NaCl gradient. Conditions were as described under Materials and methods except that in the tyrosylation assay 5 μg of tRNA from the corresponding column fractions were aminoacylated in a 15 μl reaction volume by the yeast extract (Beier *et al.*, 1984a). Bold bars represent the pooled fractions P_3, P_1 and P_2. (**B**). The [^{35}S]methionine-labeled proteins synthesized in a reticulocyte lysate programmed by TMV RNA, analyzed by SDS—PAGE and autoradiographed. tRNAs were added at the final concentration of 50 μg/ml. All lanes come from the same gel; 250 000 c.p.m. were loaded per slot. The two high mol. wt non-structural proteins (183 kd and 126 kd) of TMV are indicated. **Lane 1**, no tRNA; **lane 2**, unfractionated calf liver tRNA; **lane 3**, P_3; **lane 4**, P_1; **lane 5**, P_2.

P_3 and P_1 were further purified by one-dimensional urea-PAGE (polyacrylamide gel electrophoresis). Seven tRNA bands, designated A, D, E, G, G′, H and I, from the slowest to the faster migrating species could be separated from P_3 and 10 bands designated A, B, C, D, E, F, G, G′, H and I from P_1; the results of a typical aminoacylation assay performed on the tRNAs eluted from each band are shown in Table II. In both pools the major $tRNA^{Tyr}$ isoacceptor species are distinctly localized in band G′. The tRNAs eluted from the bands obtained by one dimensional urea—PAGE of P_1 were also tested for their amber suppressor activity by the *in vitro* translation assay. As shown in Figure 2, in the absence of exogenous tRNA only the 126 kd protein of TMV is synthesized in the reticulocyte lysate used (lane 1). Addition of calf liver tRNA at a final concentration of 50 μg/ml stimulates the synthesis of the 183 kd protein (lane 2) as does also addition of P_1 at 5 or 50 μg/ml (lanes 3 and 4). The readthrough product is also synthesized when tRNAs eluted from bands A and B are added to the translation system at 2.5 or 5 μg/ml (lanes 5—8). On the contrary, no 183 kd protein is observed when the tRNAs eluted from the other bands are used (lanes 9—18), including those eluted from band G′ (lanes 15 and 16) that contains a $tRNA^{Tyr}$ isoacceptor species. This result demonstrates that calf liver amber suppressor activity is displayed by a tRNA other than a $tRNA^{Tyr}$.

Identification of the amber suppressor tRNAs

The tRNAs eluted from the band with the highest suppressor activity (P_1A) were finally purified by two-dimensional urea—PAGE according to the two procedures described in Materials and methods. In each case two spots contained amber suppressor activity and the major one was selected: spot Ac and spot A4, respectively. The tRNAs eluted from spot Ac and spot A4 stimulate the synthesis of the TMV readthrough protein when

Table I. [^{3}H]Tyrosine-accepting activity of pooled fractions derived from the BD-cellulose column (Figure 1A)[a]

tRNA	[^{3}H]Tyr bound (pmol/A_{260})
Total	16
P_3	48
P_1	24
P_2	6

[a]The assay was performed in the conditions described under Materials and methods with 0.5 μg of tRNA. The background value obtained in the absence of tRNA has been substracted.

Table II. [^{3}H]Tyrosine-accepting activity of tRNAs derived from the one-dimensional urea—PAGE purification of P_1 and P_3[a]

tRNA	[^{3}H]Tyr bound (pmol/A_{260})	tRNA	[^{3}H]Tyr bound (pmol/A_{260})
P_3	34	P_1	16
A	<1	A	<1
D	<1	B	<1
E	<1	C	<1
G	7	D	<1
G′	107	E	<1
H	16	F	<1
I	2	G	27
		G′	133
		H	6
		I	<1

[a]The assay was performed with 0.5 μg of tRNA in the conditions described under Materials and methods.

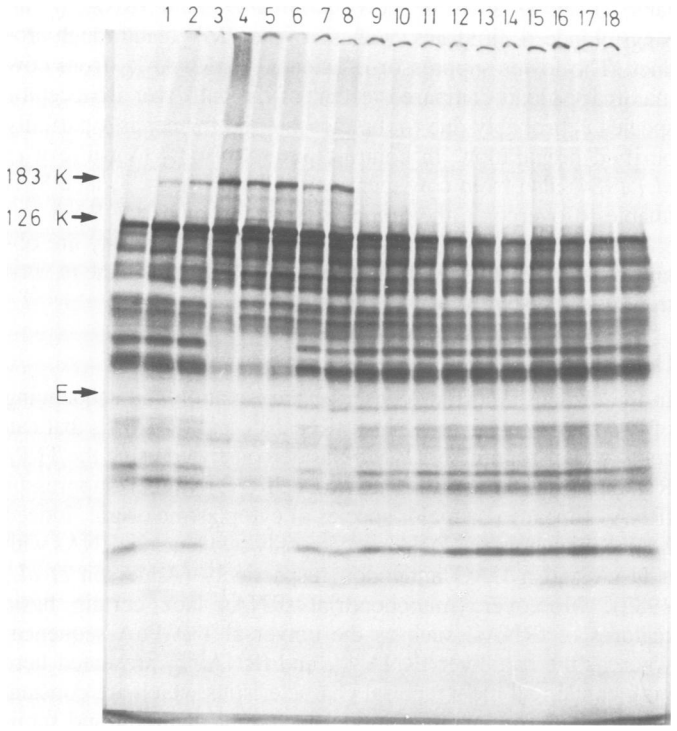

Fig. 2. Test of suppression activity of the P_1 pool and of the tRNA bands derived from the one-dimensional urea−PAGE purification of P_1. The tRNAs were added at various concentrations to a reticulocyte lysate programmed by TMV RNA. The [^{35}S]methionine-labeled proteins were analyzed by SDS−PAGE and autoradiographed. Each slot of the gel was loaded with 250 000 c.p.m. The position of the major TMV translation product (126 kd), of the readthrough product (183 kd) and of an endogenous reticulocyte protein E are indicated. **Lane 1**, no tRNA; **lane 2**, 50 μg/ml of unfractionated calf liver tRNA; **lanes 3** and **4**, 5 and 50 μg/ml of P_1; **lanes 5** and **6**, 2.5 and 5 μg/ml of A; **lanes 7** and **8**, 2.5 and 5 μg/ml of B; **lanes 9** and **10**, 2.5 and 5 μg/ml of C; **lane 11**, 5 μg/ml of D; **lane 12**, 5 μg/ml of E; **lane 13**, 5 μg/ml of F; **lane 14**, 5 μg/ml of G; **lanes 15** and **16**, 2.5 and 5 μg/ml of G′; **lane 17**, 5 μg/ml of H; **lane 18**, 5 μg/ml of I.

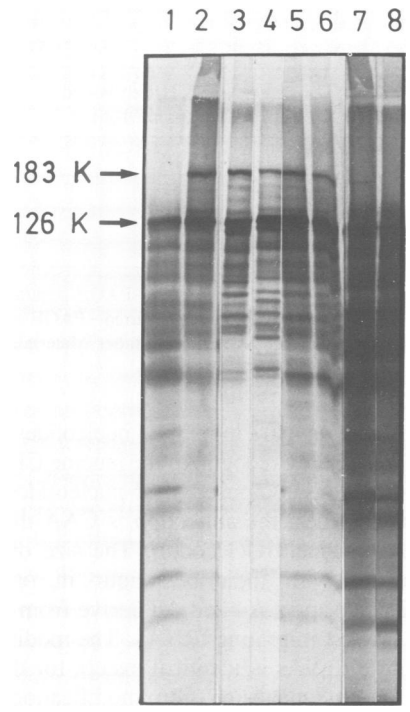

Fig. 3. *In vitro* translation assay of TMV RNA in the presence of purified amber suppressor tRNAs from calf liver. The [^{35}S]methionine-labeled proteins were analyzed by SDS−PAGE followed by autoradiography. **Lane 1**, no tRNA; **lane 2**, 50 μg/ml of unfractionated calf liver tRNA; **lanes 3** and **4**, 2.5 and 0.25 μg/ml of spot Ac ($tRNA^{Leu}_{CAA}$); **lanes 5** and **6**, 2.5 and 0.25 μg/ml of spot A4 ($tRNA^{Leu}_{CAG}$); **lane 7**, 5 μg/ml of cow mammary gland $tRNA^{Leu}_{CAG}$; **lane 8**, 5 μg/ml of cow mammary gland $tRNA^{Leu}_{IAG}$. All lanes were loaded with 500 000 c.p.m. and are from the same gel except lanes 3 and 4; lanes 7 and 8 are overexposed to allow better detection of the translation products.

tested at 2.5 or 0.25 μg/ml (Figure 3, lanes 3−6). The ability of these tRNAs to recognize the amber termination codon *in vitro* was further investigated using another mRNA harbouring a UAG codon in an open reading frame. RNA2 of BNYVV (beet necrotic yellow vein virus), a multipartite plant RNA virus, encodes the p22 coat protein that terminates with an amber codon (Bouzoubaa *et al.*, 1986). This protein can be elongated *in vitro* yielding a p81 readthrough protein upon addition of tobacco amber suppressor $tRNA^{Tyr}_{G\psi A}$ (Ziegler *et al.*, 1985). Similarly the tRNAs from spots Ac and A4 stimulate the synthesis of p81 in the *in vitro* translation system used here and programmed by BNYVV RNA (data not shown).

The tRNAs from spots Ac and A4 were sequenced. The overall sequence was obtained by post-labeling techniques. The modified nucleosides were analyzed by t.l.c. in one dimension using the solvents of Nishimura (1979) in parallel runs, or in two dimensions as stated under Materials and methods. In addition, the sequence of the 3′ end and of the 5′ end was determined both by mobility shift analyses and enzymatic sequencing. In particular the latter method resolved the ambiguities in the D arm resulting from band compression that appears in the post-labeling technique.

The sequences of the tRNAs from spot A4 and spot Ac are presented in Figure 4. The tRNA from spot A4 is composed of

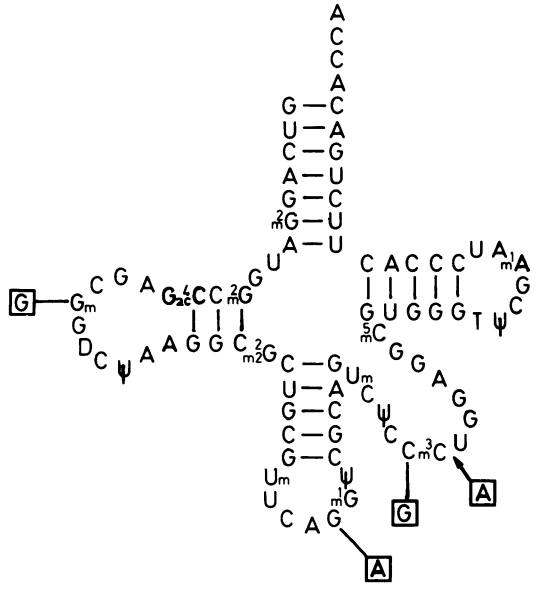

Fig. 4. Nucleotide sequences of calf liver amber suppressor tRNAs. The clover leaf model shows the nucleotide sequence of $tRNA^{Leu}_{CAG}$ (spot A4). Bases in boxes are those found in $tRNA^{Leu}_{CAA}$ (spot Ac); arrow indicates the insertion of an A residue in $tRNA^{Leu}_{CAA}$. Mobility shift analyses and nuclease P_1 digestions revealed that the 5′-terminal nucleotide G was partially replaced by an A residue in both species. No corresponding heterogeneity was detected at the C residue in position 72. The 2′-*O*-methylguanosine (Gm) modification in position 18 in $tRNA^{Leu}_{CAG}$ is partial.

Table III. [^{3}H]Leucine-accepting activity of partially purified and purified tRNAs[a]

tRNA	[^{3}H]Leu bound (pmol/A_{260})
P_1	18
P_1A	84
Spot Ac	126
Spot Aa	12

[a]P_1A derives from the one-dimensional urea−PAGE separation of P_1. Spots Ac and Aa derive from the two-dimensional urea−PAGE separation of P_1A. The assay was performed as indicated under Materials and methods with 0.2 μg of tRNA in each case.

85 nucleotides with an extra loop of 13 nucleotides; the anticodon is CAG that presumably recognizes the leucine CUG codon. The tRNA from spot Ac is composed of 86 nucleotides with an extra loop of 14 nucleotides; the anticodon is CAA that presumably recognizes the leucine UUG codon. The size of these tRNAs is in agreement with their behaviour in one-dimensional urea−PAGE since spots A4 and Ac derive from band A which contains the slowest migrating tRNAs. The modified base composition of these tRNAs is identical except for the presence of a partial 2′-*O*-methylguanosine (Gm) modification in position 18 of $tRNA_{CAG}$ (numbering of nucleotides is according to the compilation of tRNA sequences (Sprinzl *et al.*, 1985)). Apart from the anticodon, the two isoacceptors sequenced here differ in the extra loop: $tRNA_{CAA}$ has a G in position 47:A instead of the C found in $tRNA_{CAG}$, and an additional A residue following m^3C. On the basis of sequence data it appears that calf liver $tRNA_{CAG}$ is also related to a $tRNA^{Leu}_{CAG}$ isolated from cow mammary gland (Tukalo *et al.*, 1980; see also Sprinzl *et al.*, 1985). The latter has an unmodified G_{18} and differs at position 20:A, 20:B, 38 and 47:B where a D, a C, an unidentified nucleotide and a C respectively in the cow mammary gland $tRNA^{Leu}_{CAG}$ are replaced by C, ψ, ψ and m^3C respectively in the calf liver $tRNA_{CAG}$.

Leucine aminoacylation activity was checked on the tRNA eluted from spot Ac and compared to that of the partially purified tRNAs, P_1 and P_1A, from which it derives. The results are presented in Table III. A significant increase in leucine aminoacylation activity is observed as purification proceeds from pool P_1 to the pure tRNA in spot Ac. This confirms that the tRNA in spot Ac which has amber suppressor activity is a leucine isoacceptor. The tRNA eluted from another spot (spot Aa) that derives from a two-dimensional separation of band P_1A and displays no amber suppressor activity, is virtually not aminoacylated with leucine.

The material in spots Ac and A4, identified as calf liver $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ respectively, appears to be pure as judged not only by sequencing but also by dilution experiments. Indeed, $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ are still fully active in amber suppression when added to the *in vitro* system at concentrations as low as 0.25 μg/ml (Figure 3, lanes 4 and 6). A comparable level of stimulation of the 183 kd protein synthesis is obtained with 5 μg/ml of P_1 (Figure 2, lane 3). These results indicate that the successive purification steps of P_1 have increased the purity of the suppressor species by a factor of 20 and that the possibility of the presence of a contaminating species in spots Ac or A4 is very remote.

The establishment of amber suppressor activity associated with calf liver $tRNA^{Leu}_{CAG}$ prompted us to verify whether the $tRNA^{Leu}_{CAG}$ from cow mammary gland is also able to recognize the amber codon of TMV RNA in an *in vitro* translation assay. Figure 3 (lane 7) shows that the cow mammary gland $tRNA^{Leu}_{CAG}$ at 5 μg/ml indeed stimulates synthesis of the TMV readthrough product. The lower suppressor efficiency of $tRNA^{Leu}_{CAG}$ from cow mammary gland compared to that of the calf liver isoacceptor species is probably due to the fact that the former is a partially purified preparation. In contrast pure $tRNA^{Leu}_{IAG}$ (Vasil'ieva *et al.*, 1984) also from cow mammary gland (Figure 3, lane 8) is unable to overcome the amber stop codon of TMV RNA.

The same results with respect to readthrough activity are obtained using BNYVV RNA in place of TMV RNA in the *in vitro* translation system (data not shown).

Discussion

In the present report, we describe the isolation and sequencing of two major calf liver tRNAs: $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ that can act as amber suppressors *in vitro* as demonstrated using the TMV RNA translation assay. The two calf liver amber suppressor tRNAs appear to be major species of cytoplasmic origin. Indeed bovine mitochondrial DNA encodes only two leucine tRNAs with a UAA and a UAG anticodon respectively (Anderson *et al.*, 1982). Moreover, mitochondrial tRNAs lack certain basic features of tRNAs, such as the universal TψCPuA sequence, whereas the calf liver $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ presented here both contain all the structural characteristics which are constant in the standard cloverleaf model including invariant and semi-invariant residues (Dirheimer *et al.*, 1979).

The novel feature of the amber suppressor tRNAs described here is their nature: they are leucine isoacceptors. Indeed they are neither $tRNA^{Tyr}_{G\psi A}$ (Figures 2 and 4) like the 'natural' amber suppressors previously identified in *D. melanogaster* (Bienz and Kübli, 1981) and plants (Beier *et al.*, 1984a,b; Barciszewski *et al.*, 1985) nor are they $tRNA^{Gln}_{UmUG}$ or $tRNA^{Gln}_{CUG}$ as very recently described in mouse liver (Kuchino *et al.*, 1987) and in yeast (Weiss and Friedberg, 1987) respectively.

The two major calf liver $tRNA^{Tyr}$ have also been purified from P_3 and P_1 (Figure 1) using a procedure similar to the one described here to understand why they display no amber suppressor activity. Sequence data reveal (manuscript in preparation) that both $tRNA^{Tyr}$ species have a Q*ψA anticodon where Q* is probably an as yet unidentified modification of Q. This finding is in agreement with the previously proposed hypothesis (Bienz and Kübli, 1981) that the Q modification in the wobble position of the anticodon restricts recognition of the tRNA to its cognate codon and precludes reading of the amber codon. However, the molecular mechanism by which the unmodified G in the GψA anticodon of the amber suppressor $tRNAs^{Tyr}_{G\psi A}$ interacts with the G in the UAG codon remains unclear. A hypothesis concerning G:G interaction in which the G base of the anticodon would adopt a *syn* conformation has been proposed (Beier *et al.*, 1984b).

The amber suppressor activity of calf liver $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ is supported by two *in vitro* suppression assays, the standard TMV RNA and BNYVV RNA2. Sequencing of the tRNAs and *in vitro* translation assays using very low concentrations of tRNAs (Figure 3) confirm that the two tRNAs are pure species; thus contaminating species cannot be responsible for the observed suppression activity. Taken together, these data suggest that calf liver $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{CAG}$ can act as amber suppressor tRNAs. Moreover, the finding that partially purified $tRNA^{Leu}_{CAG}$ from cow mammary gland obtained through a different procedure (Tukalo *et al.*, 1980) can also overcome the amber codon of TMV RNA (Figure 3) and BNYVV RNA2 (not shown) strongly supports the observation that recognition of the UAG codon by a CAG anticodon indeed occurs *in vitro*.

This raises the question of how recognition between the CAA or CAG anticodons and the UAG codon takes place. This recognition involves in the case of $tRNA^{Leu}_{CAA}$, the proper C:G and A:U interactions at the first and third positions of the anticodon respectively and an unusual A:A base pair in the middle position. In the case of $tRNA^{Leu}_{CAG}$, recognition involves proper C:G interaction at the first position, an unusual A:A pairing in the second position and also a G:U wobble-type pairing in the third position of the anticodon. Such unorthodox interactions cannot be explained in the light of the wobble hypothesis (Crick, 1966), or of the 'two out of three' reading mechanism (Lagerkvist, 1978). Codon–anticodon interactions inconsistent with these two hypotheses have already been reported and mainly deal with unconventional pairings at the first or even third position of the anticodon (Hirsch, 1971; Weissenbach and Dirheimer, 1977; Reeves *et al.*, 1968; Holmes *et al.*, 1977; Jank *et al.*, 1977; Pure *et al.*, 1985; Weiss and Friedberg, 1986). Fewer are the cases in which unconventional pairing has been reported in the middle position of the anticodon. Strigini and Brickman (1973) have shown that the *Escherichia coli* opal suppressor tRNA which bears a CCA anticodon (Hirsh, 1971) reads UGA *in vivo* via C:A pairing in the first position of the anticodon, and misreads UAA via another C:A pairing in the second position of the anticodon. An *E. coli* $tRNA^{Gly}$ derived from the wild-type *glyT* tRNA gene can read both the GGA glycine and GAA glutamic acid codons. This unexpected result amounts to second-position 'wiggle' (Murgola *et al.*, 1984; Murgola, 1985).

From a structural standpoint, it has been postulated on theoretical grounds (Topal and Fresco, 1976) that a wider set of complementary base pairs than the two originally proposed by Watson and Crick (1953), namely A:T and G:C, can be formed that are compatible with the steric constraints of a regular B-DNA helix, provided the bases assume minor tautomeric forms (enol, imino) and/or minor conformations (*syn*) as mentioned above. Recently in view of understanding DNA mismatches and their implications for mismatch repair, the molecular structure of the G:A base pair (Brown *et al.*, 1986) and the C:A base pair (Hunter *et al.*, 1986) was reported based on X-ray crystallographic analyses. In the former study, the adenine is in the *syn* conformation whereas the guanine adopts the usual *anti* conformation. In the latter study, both the cytosine and the adenine are in their major tautomeric form and the adenine is protonated; this encouraging result indicates that minor base conformations do not always need to be invoked to explain base interactions. Thus, it appears that although a comprehensive knowledge of the structure of base pairs is still lacking, there is growing evidence that non Watson–Crick base pairs occur in mismatched DNA as well as during the process of codon–anticodon recognition when it involves suppressor tRNAs as also suggested here.

It is noteworthy that suppressor $tRNA^{Leu}_{CAG}$ contains a C at the first or wobble position of its anticodon whereas the non suppressor $tRNA^{Leu}_{IAG}$ contains an I in this position. The possibility of a strong C:G interaction in this position could favour recognition of the UAG codon by $tRNA^{Leu}_{CAG}$. In contrast I:G interaction has as yet never been reported. Thus when compared to $tRNA^{Leu}_{CAG}$, $tRNA^{Leu}_{IAG}$ is incapable of forming a single correct base pair with the UAG codon that would compensate for the two other unorthodox base pairs.

Recognition of termination codons by suppressor tRNAs and efficiency of suppression are known to be influenced by codon context effects (Bossi and Roth, 1980; Bossi, 1983; Miller and Albertini, 1983). In this respect, it is striking to note that the leaky amber codon of TMV RNA and BNYVV RNA2, that is bypassed *in vitro* by the two calf liver suppressor tRNAs and by the cow mammary gland $tRNA^{Leu}_{CAG}$ (Figure 3 and data not shown), are flanked on either side by a CAA glutamine triplet (Goelet *et al.*, 1982; Bouzoubaa *et al.*, 1986). Work is in progress in our laboratory to set up an *in vitro* system that could be used to compare the suppression efficiency of various suppressor tRNAs with regards to termination codons in various codon contexts. Such a system would be of interest in establishing the significance of codon context in natural suppression.

The identification of an increasing number of 'natural' suppressor tRNAs in higher eukaryotes has several consequences. It raises the question of the biological role of such molecules in the cell. The 'natural' amber suppressor tRNAs isolated to date, including the calf liver amber suppressors reported here, have been identified on the basis of their ability to recognize amber codons in viral messenger RNAs. Whether or not they can also read amber codons in cellular mRNAs is still unknown. Experiments using mutagenized human tRNA genes introduced into mammalian cell lines, indicate that the expression of amber or ochre suppressor tRNAs does not affect cell viability (Capone *et al.*, 1985). This implies that cellular amber and ochre termination codons are somehow protected from recognition by suppressor tRNAs. In this respect, codon context could play a role in discriminating suppressible from non-suppressible termination codons. On the contrary, attempts to date to obtain cell lines expressing opal suppressor tRNA genes have failed, thus suggesting that efficient expression of opal suppressor tRNAs is detrimental to the cell (Capone *et al.*, 1985). Interestingly, natural opal suppressor tRNAs do exist in mammals. This suggests that recognition of UGA codons could specify additional biological functions besides termination of protein synthesis. One such suppressor tRNA is a minor species that is aminoacylated by serine which in turn can be phosphorylated to form phosphoseryl-tRNA (Hatfield *et al.*, 1982). It harbours an NCA anticodon (N is probably an unknown modification of U) complementary to the UGA termination codon (Kato *et al.*, 1983). The nature of the anticodon has been confirmed by nucleotide sequencing of the corresponding gene (O'Neill *et al.*, 1985). However, it remains to be established whether this tRNA is functional in deciphering UGA codons during protein synthesis. Another consequence of the identification of naturally occurring suppressor tRNAs that are major species is that they may become tools for the study of codon–anticodon recognition, fidelity of translation and regulation of protein synthesis. Finally, the existence of major and minor tRNA species able to read termination codons, together with the recent findings on 'divergent' genetic codes (for a review, see Fox, 1985) should ultimately lead us to envisage the termination step of eukaryotic protein synthesis on the basis of a much more flexible mechanism than was previously assumed.

Materials and methods

Materials

[γ-^{32}P]ATP (~111 TBq/mmol), L-[^{35}S]methionine (>29.6 TBq/mmol) and L-[^{3}H]leucine (2.59 TBq/mmol) were purchased from Amersham, and [^{3}H]-tyrosine (2.2 TBq/mmol) from the C.E.A. (France). T4 polynucleotide kinase was from NEN; calf intestine alkaline phosphatase for molecular biology, nuclease P1, RNase U2, nuclease S7 and RNase CL3 were from Boehringer Mannheim. RNase T1 was a kind gift of S.Nishimura (National Cancer Research Center, Tokyo). Tyrosyl-tRNA synthetase was either from a crude yeast extract kindly provided by H.Beier (Institut für Biochemie, Würzburg) or from a partially purified preparation from sheep liver provided by J.P.Waller (C.N.R.S., Gif-sur-Yvette) and R.L.Joshi. Rabbit liver leucyl-tRNA synthetase was supplied in the form of a purified multienzyme complex by J.P.Waller. TMV RNA (*Vulgare* strain) and BNYVV RNA (F13 isolate) were kindly provided by A.Schön (Institut für Biochemie, Würzburg) and G.Jonard (I.B.M.C., Strasbourg) respectively. Yeast

soluble RNA, BD-cellulose and t.l.c. plates were from Boehringer Mannheim. Purified $tRNA^{Leu}_{IAG}$ and partially purified $tRNA^{Leu}_{CAG}$ from cow mammary gland were generously provided by M.Tukalo (Institute of Molecular Biology and Genetics, Kiev).

tRNA purification

tRNA (4500 A_{260} units; 1 A_{260} ~40 μg) isolated from calf liver (Roe, 1975) in 10 mM $MgCl_2$, 0.35 M NaCl and 12 mM NaOAc pH 4.5 was loaded onto a 36 × 1.2 cm BD-cellulose column and eluted with 400 ml of a linear gradient of 0.35 M to 1 M NaCl in the same buffer. Fractions were tested both for tyrosine-accepting and suppressor activities and pooled accordingly. The tRNA pools were separated by one-dimensional electrophoresis on a 15% polyacrylamide/7 M urea gel at pH 8.3 (Silberklang *et al.*, 1979). tRNA bands were stained with 0.4% toluidine blue, 50% methanol and 1% acetic acid, excised from the gel and eluted according to Krupp and Gross (1983). The tRNAs were ethanol precipitated and redissolved in water to a final concentration of 250 μg/ml.

Further purification was accomplished by two-dimensional gel electrophoresis. Two procedures were used. Either a 10% polyacrylamide/7 M urea gel (pH 3.5) in the first dimension and a 15% polyacrylamide/7 M urea gel (pH 8.3) in the second dimension (Krupp and Gross, 1983), or a 10% polyacrylamide/8 M urea gel (pH 8.3) in the first and a 20% polyacrylamide/4 M urea gel (pH 8.3) in the second dimension using the same buffer and electrophoretic conditions as in the second dimension of the first procedure. Gel spots were stained, excised and eluted as described above and the purified tRNAs were brought to a final concentration of 25 μg/ml in water.

tRNA sequencing

The post-labeling procedure was that of Stanley and Vassilenko (1978) as modified by Krupp and Gross (1983). Pure tRNA (1 μg in 1 μl of water) was subjected to limited hydrolysis in a sealed capillary for 30 s at 90°C. In these conditions an average of one nick per molecule occurs. The RNA fragments were 5′-terminally labeled in 10 μl containing 100 mM Tris–HCl pH 8, 20 mM $MgCl_2$, 3.4 mM spermine, 10 mM DTT with 9.25 MBq of [γ-^{32}P]ATP and 7 U of T4 polynucleotide kinase for 30 min at 37°C, and separated on a 10% or 12.5% polyacrylamide/8 M urea gel (90 cm × 24 cm × 0.35 mm) at pH 8.3. Electrophoresis was carried out at 2400 V until the bromophenol blue dye reached 5 cm from the bottom of the gel. After autoradiography, the individual bands of the ladder were excised, eluted as described above in the presence of 5 μg of yeast soluble RNA as carrier and ethanol precipitated. Complete digestion of each fragment with 100 ng of nuclease P1 in 10 μl containing 50 mM NH_4OAc pH 5.3 was performed for 2 h at 50°C. The 5′-labeled nucleotide of each eluted fragment was identified by t.l.c. in the presence of 20 μg each of the four major nucleotides as markers using the two-solvent system of Nishimura (1979) in parallel runs. For further identification of modified nucleotides (m^2G, m^2_2G, m^3C and m^3C) labeled spots were eluted from the thin-layer plate and rechromatographed in two dimensions using different combinations of three solvents as described by Nishimura and Kuchino (1983). Uncleaved dinucleotides containing a 2′-*O*-methyl nucleotide such as UmU and UmC were eluted from the thin-layer plate and further digested with a 100-fold higher concentration of nuclease P1. The 5′-terminal nucleotide and the sequence at the 5′ end of the tRNA were determined by nuclease P1 digestions, enzymatic sequencing and mobility shift analyses of 5′-labeled full-length tRNA (Krupp and Gross, 1983). Sequence at the 3′ end was determined by enzymatic sequencing and mobility shift analyses of the 5′-labeled tRNA fragments eluted from the ladder obtained by the post-labeling procedure. Controlled enzymatic digestion of the 5′-labeled material was performed in 8 M urea for 15 min at 50°C in the presence of RNase T1, RNase U2, nuclease S7 or RNase CL3 (Krupp and Gross, 1983). The resulting labeled fragments were separated on a 20% polyacrylamide/8 M urea gel (pH 8.3) together with a ladder derived from a partial acid hydrolysis of the 5′-labeled material (Krupp and Gross, 1983). The sequence was directly read off the autoradiogram. Short and long runs were used as well as gels with a thickness gradient from top (0.35 mm) to bottom (0.5 mm) to ensure reading of a maximum number of nucleotides. For mobility shift analysis 5′-labeled material was hydrolyzed for 10 min in boiling water in 0.5 mM EDTA and 50 mM $NaHCO_3$ pH 9.1. Electrophoresis, blotting and homochromatography in 20 mM KOH homomix were performed according to Krupp and Gross (1983).

Translation assay in vitro

The reticulocyte lysate was prepared according to Jackson and Hunt (1983). Incubations were performed in 10 μl as described previously (Morch *et al.*, 1982) except that the tRNA samples to be tested were the only exogenous tRNAs added. After 90 min at 30°C, aliquots (1 μl) were removed to determine the hot TCA (trichloroacetic acid)-precipitable radioactivity. The translation products were analyzed on a 12.5% polyacrylamide–0.1% SDS slab gel (Laemmli, 1970).

Aminoacylation assay

Partially purified or pure tRNA (0.5 μg or 0.2 μg as indicated in the Tables) was incubated for 30 min at 37°C in 10 μl of 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, with 50 μg of partially purified sheep liver tyrosyl-tRNA synthetase and 3 μM (74 kBq) of [^{3}H]tyrosine or with 130 ng of rabbit liver leucyl-tRNA synthetase and 14.3 μM (370 kBq) of [^{3}H]leucine. The incubation was stopped with 10% cold TCA and the samples filtered through Whatmann GF-C filters. The filters were dried and counted, and the amino acid-accepting activity was determined.

Acknowledgements

We are very grateful to Dr H.J.Gross for his continued interest in this work and for having opened his laboratory to one of us (M.-D.M.) during an EMBO Long-Term Fellowship. We are indebted to Dr H.Beier for her precious help in introducing us to tRNA sequencing and to H.-D.Sickinger for technical assistance on this occasion. We are grateful to Dr M.Tukalo for having supplied us with tRNAs from cow mammary gland,, to Dr A.Schön and Dr G.Jonard for a gift of TMV RNA and of BNYVV RNA respectively and to Dr J.P.Waller for the aminoacyl-tRNA synthetases. R.V. is grateful to the 'Ministère de la Recherche et de l'Enseignement Supérieur' for a fellowship. The Institut Jacques Monod is an 'Institut Mixte, C.N.R.S.-Université Paris 7'.

References

Anderson,S., de Bruijn,M.H.L., Coulson,A.R., Eperon,I.C., Sanger,F. and Young,I.G. (1982) *J. Mol. Biol.*, **156**, 683–717.

Barciszewski,J., Barciszewska,M., Suter,B. and Kübli,E. (1985) *Plant Sci.*, **40**, 193–196.

Beier,H., Mundry,K.W. and Issinger,O.-G. (1980) *Intervirology*, **14**, 292–299.

Beier,H., Barciszewska,M., Krupp,G., Mitnacht,R. and Gross,H.J. (1984a) *EMBO J.*, **3**, 351–356.

Beier,H., Barciszewska,M. and Sickinger,H.-D. (1984b) *EMBO J.*, **3**, 1091–1096.

Bienz,M. and Kübli,E. (1981) *Nature*, **294**, 188–190.

Bossi,L. (1983) *J. Mol. Biol.*, **164**, 73–87.

Bossi,L. and Roth,J.R. (1980) *Nature*, **286**, 123–127.

Bouzoubaa,S., Ziegler,V., Beck,D., Guilley,H., Richards,K. and Jonard,G. (1986) *J. Gen. Virol.*, **67**, 1689–1700.

Brown,T., Hunter,W.N., Kneale,G. and Kennard,O. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2402–2406.

Capone,J.P., Sharp,P.A. and RajBhandary,U.L. (1985) *EMBO J.*, **4**, 213–221.

Celis,J.E. and Smith,J.D. (eds) (1979) *Nonsense Mutations and tRNA Suppressors.* Academic Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Chambers,I., Frampton,J., Goldfarb,P., Affara,N., McBain,W. and Harrison,P.R. (1986) *EMBO J.*, **5**, 1221–1227.

Crick,F.H.C. (1966) *J. Mol. Biol.*, **19**, 548–555.

Dirheimer,G., Keith,G., Sibler,A.P. and Martin,R.P. (1979) In Schimmel,P.R., Söll,D.G. and Abelson,J.N. (eds), *Transfer RNA: Structure, Properties and Recognition.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 19–41.

Fox,T.D. (1985) *Nature*, **314**, 132–133.

Goelet,P., Lomonossoff,G.P., Butler,P.J.G., Akam,M.E., Gait,M.J. and Karn,J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5818–5822.

Hanju,N., Kuchino,Y., Nishimura,S. and Beier,H. (1986) *EMBO J.*, **5**, 1307–1311.

Hatfield,D.L. (1985) *Trends Biochem. Sci.*, **10**, 201–204.

Hatfield,D.L., Diamond,A. and Dudock,B.S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6215–6219.

Hirsh,D. (1971) *J. Mol. Biol.*, **58**, 439–458.

Holmes,W.M., Goldman,E., Miner,T.A. and Hatfield,D.L. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1393–1397.

Hunter,W.N., Brown,T. Anand,N.N. and Kennard,O. (1986) *Nature*, **320**, 552–555.

Jackson,R.J. and Hunt,T. (1983) *Methods Enzymol.*, **96**, 50–71.

Jank,P., Shindo-Okada,N., Nishimura,S. and Gross,H.J. (1977) *Nucleic Acids Res.*, **4**, 1999–2008.

Kato,N., Hoshino,H. and Harada,F. (1983) *Biochem. Int.*, **7**, 635–645.

Kozak,M. (1986) *Adv. Virus Res.*, **31**, 229–290.

Krupp,G. and Gross,H.J. (1983) In Agris,P.F. and Kopper,R.A. (eds), *The Modified Nucleosides in Transfer RNA, II: A Laboratory Manual of Genetic Analysis, Identification and Sequence Determination.* Alan R.Liss, New York, pp. 11–58.

Kuchino,Y., Beier,H., Akita,N. and Nishimura,S. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2668–2672.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lagerkvist,U. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1759–1762.

Miller,J.H. and Albertini,A.M. (1983) *J. Mol. Biol.*, **164**, 59–71.

Morch,M.-D., Zagorski,W. and Haenni,A.-L. (1982) *Eur. J. Biochem.*, **127**, 259–265.

Morch,M.-D., Valle,R.P.C. and Haenni,A.-L. (1987) In Perez Bercoff,R. (ed.), *The Molecular Basis of Viral Replication.* Plenum Press, New York, 113–151.
Murgola,E.J. (1985) *Annu. Rev. Genet.*, **19**, 57–80.
Murgola,E.J., Pagel,F.T. and Hijazy,K.A. (1984) *J. Mol. Biol.*, **175**, 19–27.
Nishimura,S. (1979) In Schimmel,P.R., Söll,D.G. and Abelson,J.N. (eds), *Transfer RNA: Structure, Properties and Recognition.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 59–79.
Nishimura,S. and Kuchino,Y. (1983) In Weissman,S.M. (ed.), *Methods of DNA and RNA Sequencing.* Praeger, New York, pp. 235–260.
O'Neill,V.A., Eden,F.C., Pratt,K. and Hatfield,D.L. (1985) *J. Biol. Chem.*, **260**, 2501–2508.
Pelham,H.R.B. (1978) *Nature*, **272**, 469–471.
Pure,G.A., Robinson,G.W., Naumovski,L. and Friedberg,E.C. (1985) *J. Mol. Biol.*, **183**, 31–42.
Reeves,R.H., Imura,N., Schwan,H., Weiss,G.B., Schulman,L.H. and Chambers,R.W. (1968) *Proc. Natl. Acad. Sci. USA*, **60**, 1450–1457.
Roe,B.A. (1975) *Nucleic Acids Res.*, **2**, 21–41.
Silberklang,M., Gillum,A.M. and RajBhandary,U.L. (1979) *Methods Enzymol.*, **59**, 58–109.
Sprinzl,M., Moll,J., Meissner,F. and Hartmann,T. (1985) *Nucleic Acids Res.*, **13**, r1–r49.
Stanley,J. and Vassilenko,S. (1978) *Nature*, **274**, 87–89.
Strigini,P. and Brickman,E. (1973) *J. Mol. Biol.*, **75**, 659–672.
Topal,M.D. and Fresco,J.R. (1976) *Nature*, **263**, 289–293.
Tukalo,M.A., Vlasov,V., Vasil'chenko,I., Matsuka,G. and Knorre,D. (1980) *Dokl. Akad. Nauk. SSSR*, **253**, 253–256.
Vasil'ieva,I.G., Tukalo,M.A., Kriklivi y,I.A. and Matsuka,G.Ch. (1984) *Mol. Biol. Akad. Nauk. SSSR*, **18**, 1321–1325.
Watson,J.D. and Crick,F.H.C. (1953) *Nature*, **171**, 737–738.
Weiss,W.A. and Friedberg,E.C. (1986) *J. Mol. Biol.*, **192**, 725–735.
Weissenbach,J. and Dirheimer,G. (1977) *FEBS Lett.*, **82**, 71–76.
Wills,N., Gesteland,R.F., Karn,J., Barnett,L., Bolten,S. and Waterston,R.H. (1983) *Cell*, **33**, 575–583.
Ziegler,V., Richards,K., Guilley,H., Jonard,G. and Putz,C. (1985) *J. Gen. Virol.*, **66**, 2079–2087.

Received on February 4, 1987; revised on July 2, 1987