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UAG readthrough during TMV RNA translation: isolation and sequence of two tRNAs^{Tyr} with suppressor activity from tobacco plants

Hildburg Beier*, Miroslawa Barciszewska[1], Guido Krupp, Rita Mitnacht and Hans J.Gross

Institut für Biochemie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, FRG

[1]Present address: Institute of Organic Chemistry, Department of Stereochemistry and Natural Products, 61-704 Poznan, Poland
*To whom reprint requests should be sent
Communicated by H.J.Gross

The hypothetical replicase or replicase subunit cistron in the 5′-proximal part of tobacco mosaic virus (TMV) RNA yields a major 126-K protein and a minor 183-K 'readthrough' protein *in vivo* and *in vitro*. Two natural suppressor tRNAs were purified from uninfected tobacco plants on the basis of their ability to promote readthrough over the corresponding UAG termination codon *in vitro*. In a reticulocyte lysate the yield of 183-K readthrough protein increases from ~10% in the absence of added tobacco plant tRNA up to ~35% in the case of pure tRNA^{Tyr} added. Their amino acid acceptance and anticodon sequence (GΨA) identifies the two natural suppressor tRNAs as the two normal major cytoplasmic tyrosine-specific tRNAs. tRNA$_1^{Tyr}$ has an A:U pair at the base of the TΨC stem and an unmodified G_{10}, whereas tRNA$_2^{Tyr}$ contains a G:C pair in the corresponding location and m^2G in position 10. This is the first case that, in a higher eukaryote, the complete structure is known of both the natural suppressor tRNAs and the corresponding viral RNA on which they exert their function. The corresponding codon-anticodon interaction, which is not in accordance with the wobble hypothesis, and the possible biological significance of the readthrough phenomenon is discussed.
Key words: readthrough/tobacco mosaic virus (TMV)/tobacco plant tRNAs^{Tyr}/translation/UAG suppression

Introduction

Mutagenesis of tRNA genes, mainly in the anticodon, leads to 'induced suppression', i.e., misreading of termination codons, and has preferentially been studied in prokaryotes and lower eukaryotes. In contrast, 'natural suppression' is the readthrough over termination codons stimulated by any tRNA, i.e., 'natural' suppressor tRNA, in wild-type cells. In higher eukaryotes, only a few cases of natural suppression have been observed. In murine leukemia virus, the viral RNA-dependent DNA polymerase appears to be a product of an amber codon misreading (Philipson *et al.*, 1978; Murphy *et al.*, 1980). Here, the biological significance of the readthrough process is obvious, whereas the corresponding suppressor tRNA is as yet unknown. A tRNA^{Trp} has been identified in rabbit reticulocytes which promotes readthrough over the UGA termination codon of rabbit β-globin mRNA in reticulocyte extracts as well as in reticulocyte cells (Geller and Rich, 1980). Furthermore, a tRNA^{Tyr} has been identified in *Drosophila* which stimulates UAG readthrough in *Xenopus* oocytes (Bienz and Kubli, 1981). Another UGA suppressor tRNA was purified from bovine liver and sequenced. It has unusual structural and functional features, in that it has a tryptophan anticodon, which binds exclusively to the UGA codon, and it is charged with serine, which in turn can be phosphorylated to form phosphoseryl-tRNA (Diamond *et al.*, 1981; Hatfield *et al.*, 1982). In these three latter cases, natural suppressor tRNAs have been identified and sequenced, respectively, however the biological function of the readthrough process is as yet completely obscure.

Readthrough also occurs in several plant virus systems. One example is the suppression of a UAG termination codon in tobacco mosaic virus (TMV) RNA. This RNA can be translated in various cell-free systems to yield a 'normal' 126-K protein, and a 183-K readthrough protein (Bruening *et al.*, 1976; Beachy and Zaitlin, 1977; Pelham, 1978). Viral proteins of the same size as the two polypeptides synthesized *in vitro* were detected in TMV-infected cells (Paterson and Knight, 1975; Siegel *et al.*, 1978; Beier *et al.*, 1980).

Here we show that two normal tRNAs^{Tyr} from tobacco plants, the natural host of TMV, promote the UAG readthrough during TMV translation *in vitro* and we propose that they have the same function in the infected plant *in vivo*.

Results

TMV-specific proteins synthesized in tobacco protoplasts

Virus-induced protein synthesis in tobacco infected with TMV has been investigated in intact plants (Zaitlin and Hariharasubramanian, 1972) and in isolated tobacco protoplasts (Sakai and Takebe, 1974; Paterson and Knight, 1975; Siegel *et al.*, 1978; Beier *et al.*, 1980). A polypeptide of ~120 000 mol. wt. has been reported in all cases to be the major non-coat protein synthesized in tobacco cells after infection with TMV. A second virus-specific protein with mol. wt. of ~180 000 has been identified by some workers (Paterson and Knight, 1975; Siegel *et al.*, 1978) but its nature as a virus-induced protein has been doubted by others (Huber, 1979).

We re-investigated the presence of the 180-K protein in tobacco protoplasts and compared the virus-specific proteins synthesized *in vivo* with the products translated *in vitro* from TMV RNA (Figure 1). Furthermore, we wanted to confirm that the pattern of TMV protein synthesis is the same, independent of the nature of the tobacco variety chosen as a source for protoplasts. All experiments up till now were done with different cultivars of *Nicotiana tabacum,* whereas we preferred *N. rustica* as a host because this species yields very stable protoplasts independent from growth conditions of the intact plant and since the tRNAs^{Tyr} described here were mainly isolated from this source. As can be seen in Figure 1, we obtained essentially the same pattern of virus-induced proteins as previously described for *N. tabacum*. The same two high mol. wt. proteins (126 K and 183 K) could be detected in infected protoplasts and *in vitro* (Figure 1). The TMV coat protein (17.5 K) is synthesized late during infection *in vivo*, but not at all *in vitro*.

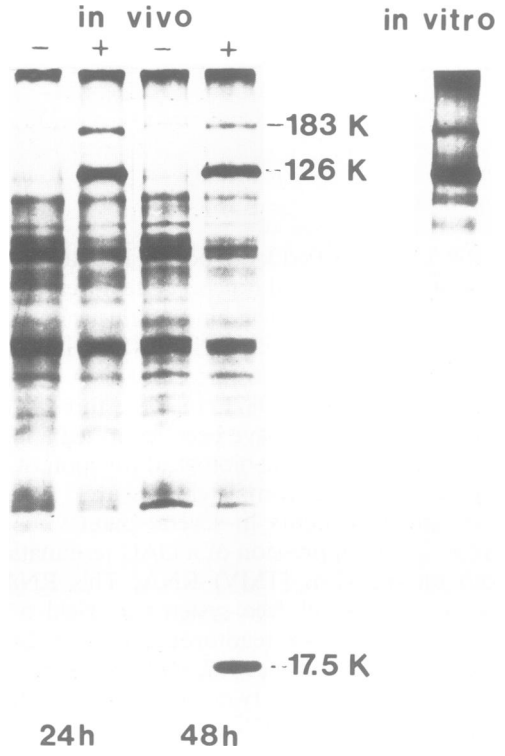

Fig. 1. Comparison of virus-specific proteins from TMV vulgare-infected tobacco protoplasts with the corresponding *in vitro* translation products. The left panel shows the gel electrophoretic analysis of ^{35}S-labeled proteins synthesized in tobacco protoplasts. Protoplasts were inoculated with 4 μg/ml TMV vulgare (+) or with buffer only (–) and incubated for the times indicated below. [^{35}S]Methionine (3 μCi/ml) was added 16 h after inoculation. The analysis of polypeptides translated *in vitro* from TMV RNA is shown in the right panel. The translation mixture was supplemented with 50 μg/ml calf liver tRNA. Numbers indicate mol. wts. of the products.

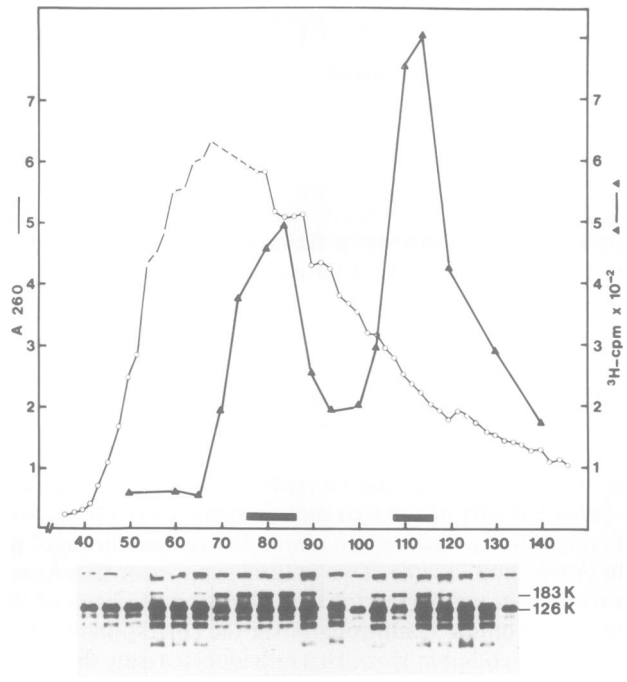

Fig. 2. BD-cellulose chromatography of unfractionated tobacco tRNA. Approximately 1000 A_{260} units of total tRNA were loaded onto a BD-cellulose column and fractionated by elution with a linear gradient of 0.35 – 1 M NaCl. The fraction volume was 2 ml. A_{260} absorbance is indicated by open circles (○——○). One A_{260} unit of each of the appropriate column fractions was removed and the tRNA was precipitated with 2 volumes of ethanol. Following centrifugation the tRNA was redissolved in water to a final concentration of 5 mg/ml. These solutions were used for aminoacylation and *in vitro* translation assays. The tRNA^{Tyr} content of fractions 50 – 140 was determined by aminoacylation with [^{3}H]tyrosine (△——△). The reaction volume was 15 μl, contained 5 μg of tRNA collected from the corresponding column fractions and aminoacyl-tRNA synthetases from a yeast extract. The lower panel shows a fluorogram of a 10% SDS-polyacrylamide gel of [^{35}S]methionine-labeled proteins after *in vitro* translation of TMV RNA in the presence of 100 μg/ml tRNA from the corresponding column fractions. The mol. wts. of the major translation product and the readthrough protein are shown. The bars indicate the fractions with maximal suppressor activity which were pooled for further purification of tRNA$_1^{Tyr}$ and tRNA$_2^{Tyr}$. The ratio of 183 K: (126 K + 183 K) was 0.1 to 0.12 in fractions without suppressor tRNA, and it was practically the same if the lysate was supplemented with calf liver tRNA. This ratio increased up to 0.22 in the fractions enriched in tRNAs^{Tyr}.

Purification of tobacco tRNA$_1^{Tyr}$ and tRNA$_2^{Tyr}$ and detection of suppressor activity

Tobacco tRNAs^{Tyr} were isolated from unfractionated tRNA by a two-step chromatographic procedure consisting of BD-cellulose followed by Sepharose 4B chromatography. Two major tyrosine tRNAs eluted from BD-cellulose as shown by aminoacylation assays with [^{3}H]tyrosine (Figure 2). Essentially the same elution pattern was obtained for the fractionation of tRNA derived from different tobacco varieties; however, the relative yield of the two isoacceptors varied in some preparations. The two tRNAs^{Tyr} are likely to be of cytoplasmic origin since they are the prominent tyrosine-accepting species in the total tobacco tRNA fraction, and since chloroplast tRNA^{Tyr} is not or only very slowly aminoacylated by cytoplasmic synthetases (A.Steinmetz, personal communication) as used here. Mitochondrial tRNA^{Tyr} would be expected in trace amounts only.

Suppressor activity was detected in a messenger-dependent reticulocyte lysate to which TMV RNA and tRNA of the corresponding column fractions was added. Both fractions containing tyrosine tRNAs stimulated readthrough of the UAG codon in TMV RNA, e.g., enhanced the synthesis of the 183-K protein (Figure 2, lower panel). The two fractions from the BD-cellulose column with highest suppressor activity (e.g., tRNA$_1^{Tyr}$ and tRNA$_2^{Tyr}$) were pooled and used for further fractionation on a Sepharose 4B column in parallel chromatographic runs. As can be seen in Figure 3, suppressor activity was again correlated with a tyrosine tRNA-rich fraction. Further aminoacylation assays indicated that this fraction contained, in addition to tRNA^{Tyr}, a tRNA^{Ser} and no tRNA^{Trp}.

The final purification of the tobacco tyrosine tRNAs consisted of a two-dimensional gel electrophoresis step. This procedure resulted in the separation of three tRNA species, only one of which (No. 3) upon elution, could be charged with tyrosine and showed suppressor activity (Figure 4, panel B). This material appeared to be quite pure as judged by the absence of extra bands in the RNA 'ladder' obtained during fragment analysis according to Stanley and Vassilenko (1978) and in the 'ladder' produced after alkaline digestion of the corresponding 3′-^{32}P- or 5′-^{32}P-labeled material isolated as shown in Figure 4, panel C.

Successive purification of tRNAs^{Tyr} led to an increase of their relative suppressor activity as measured by the percentage of 183-K protein synthesized *in vitro* in relation to the synthesis of both, the 126-K and 183-K protein. In our reticulocyte lysate used for the *in vitro* translation of TMV RNA,

~10% of the 183-K product is synthesized in the absence of added tRNA. Addition of tRNA^{Tyr} to the lysate results in an increase of readthrough protein synthesis, from 25% in tyrosine-rich fractions collected after BD-cellulose chromatography up to ~35% with pure tRNA^{Tyr}.

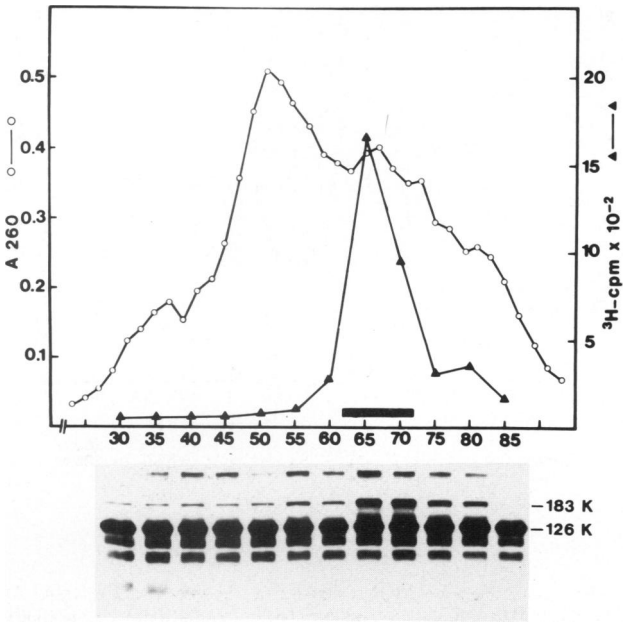

Fig. 3. Sepharose 4B chromatography of partially purified tRNA$_1^{Tyr}$. Approximately 20 A_{260} units of tRNA$_1^{Tyr}$ partially purified by BD-cellulose chromatography (Figure 2, fractions 75–86) were loaded onto a Sepharose 4B column and fractionated by elution with a reverse $(NH_4)_2SO_4$ gradient ranging from 2 M to 1 M. The line with open circles indicates A_{260} absorbance (○——○). An aliquot of each appropriate fraction was removed, dialysed against distilled water and the tRNA was then precipitated with 2.5 volumes of ethanol and 0.1 volume of 2 M KOAc, pH 6.0. Following centrifugation the tRNA was redissolved in water to a final concentration of 1 mg/ml. These solutions were used for aminoacylation with [^{3}H]-tyrosine (△——△) and as probes for readthrough activity as described in Figure 2. The ratio of 183 K: (126 K + 183 K) was 0.12 in fractions not containing tRNA^{Tyr}, and increased up to 0.28 in the tRNA^{Tyr} fractions.

Two-dimensional gel electrophoretic separation of total tRNA and of partially purified tRNA^{Tyr} yields a variety of tRNA species which were also tested for their tyrosine acceptance ability and for suppressor activity (Figure 4, panel A). The arrow indicates the position of suppressor tRNA^{Tyr}. It is obvious that even in unfractionated tobacco tRNA the suppressor tRNA^{Tyr} migrates as the fastest tRNA species in the second dimension and is clearly separated from the bulk of the tRNAs. The two tRNA^{Tyr} species eluting from the BD-cellulose column at different salt concentrations are not separated by the two-dimensional gel electrophoresis.

Nucleotide sequence of the two tRNAs^{Tyr} isoacceptors from tobacco plants

The most informative procedure in determining the nucleotide sequence of tRNAs^{Tyr} was the fragment analysis of partially digested tRNA according to Stanley and Vassilenko (1978), which was used with some modifications.

The 5′ nucleotide of each eluted fragment was analyzed by cellulose t.l.c. in two different solvents as shown in Figure 5. Modified nucleotides were identified according to their relative position in the two-dimensional system of Nishimura (1979). In some cases they were further examined by re-chromatography with authentic standards. The dinucleotide pGmpG was eluted and digested with a 100-fold concentration of nuclease P1 and re-chromatographed with authentic pGm as a marker. Additional confirmation for the sequences of tRNAs^{Tyr} was obtained from RNA sequence gels (Donis-Keller *et al.*, 1977; Peattie, 1979; Krupp and Gross, 1983) and by mobility shift analysis.

The complete nucleotide sequence of the tobacco tRNAs^{Tyr} is shown in Figure 6. Ribothymidine which was not detectable in the TΨC loop of the tRNAs^{Tyr} is replaced by unmodified U. The modified nucleoside U* next to m^{7}G is most likely 3-(3-amino-3-carboxypropyl)uridine (apc^{3}U) for the following reasons: (a) chemical sequence analysis (Peattie, 1979) showed a strong U-specific reaction, whereas there was no cleavage in base-specific enzymatic digestions; (b) the chromatographic behaviour was that of apc^{3}U, although we

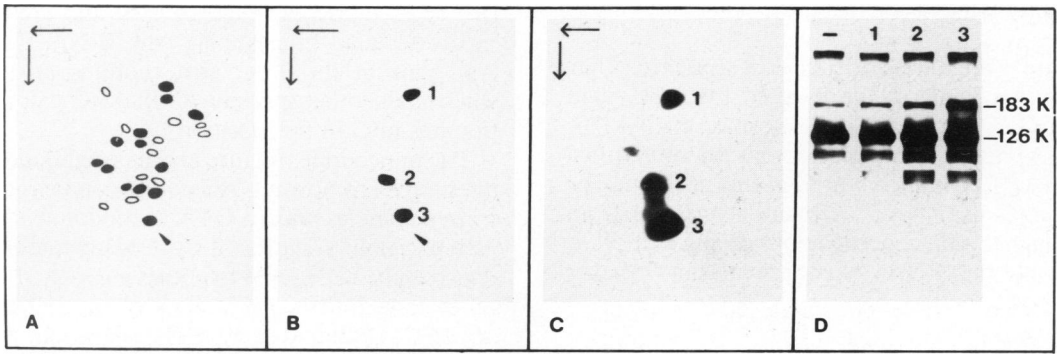

Fig. 4. Two-dimensional gel electrophoretic separation of unfractionated tobacco tRNA and partially purified tRNA$_1^{Tyr}$. About 40 μg of total tRNA **(A)** and 5 μg of tRNA$_1^{Tyr}$ partially purified by successive chromatographic runs on BD-cellulose and Sepharose 4B columns **(B)** were first loaded on a 10% polyacrylamide gel, pH 3.5 (from right to left), followed by a second dimension (12.5% polyacrylamide, pH 8.3, from top to bottom). Gels were stained with toluidine blue and destained as described in Materials and methods. Under these conditions ~0.5 μg of tRNA per spot can be detected. The stained spots are presented schematically. The total number of spots (closed and open circles) in **panel A** represent the tRNA pattern of unfractionated tRNA whereas the closed circles alone indicate the tRNA pattern of partially purified tRNA$_1^{Tyr}$ after BD-cellulose chromatography. All spots visible in **panels A** and **B** were excised from the gel, the tRNA was eluted in the absence of carrier RNA and precipitated with ethanol. The dried material eluted from each spot (~2 μg) was directly used for aminoacylation with [^{3}H]tyrosine and *in vitro* translation experiments in parallel assays. An example for the latter is given in **panel D** which shows a fluorogram of a 10% SDS-polyacrylamide gel of tRNA species No. 1–3 eluted from **B**. The arrow in **panels A** and **B** indicates the tRNA species which showed highest readthrough activity and could be charged with [^{3}H]tyrosine. The non-supplemented system (−) showed a 183 K: (126 K + 183 K) ratio of 0.12, whereas the tRNA fractions 1, 2 and 3 **(panel B)** produced a ratio of 0.11, 0.15 and 0.31, respectively **(panel D)**. **Panel C** shows the two-dimensional gel electrophoretic pattern of 3′-^{32}P-labeled tRNAs of the same origin as the material analyzed in **panel B**. The labeled tRNA from spot No. 3 (tRNA$_1^{Tyr}$) was eluted for enzymatic and chemical sequence analysis. Spot No. 3 from **B** was used for sequence determination according to Stanley and Vassilenko (1978).

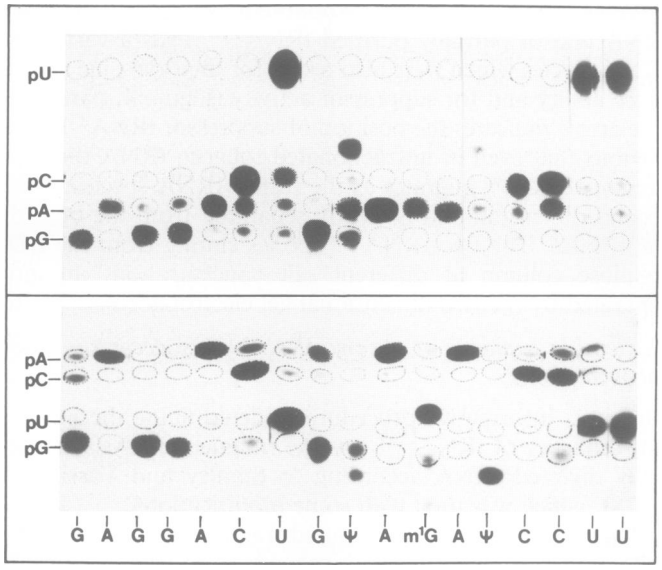

Fig. 5. Analysis of [5′-^{32}P]terminal nucleotides of fragments of $tRNA_1^{Tyr}$ by cellulose t.l.c. Purified tobacco $tRNA_1^{Tyr}$ was subjected to limited hydrolysis with water, the fragments were 5′-^{32}P-labeled and then separated on a 15% polyacrylamide gel as described in Materials and methods. Each fragment was subjected to nuclease P1 digestion and the 5′ nucleotide identified by t.l.c. in two different solvents (Nishimura, 1979). The lower panel shows the autoradiogram of such an analysis in isobutyric acid/concentrated ammonia/H_2O = 580:38:385 (by vol.); the upper panel shows the analysis of the same fragments in isopropanol/concentrated HCl/water = 700:150:150 (by vol.). The dotted circles indicate the location of unlabeled pN markers as visualized under u.v. light. As an example, the nucleotide sequence analysis of the anticodon stem and loop is shown.

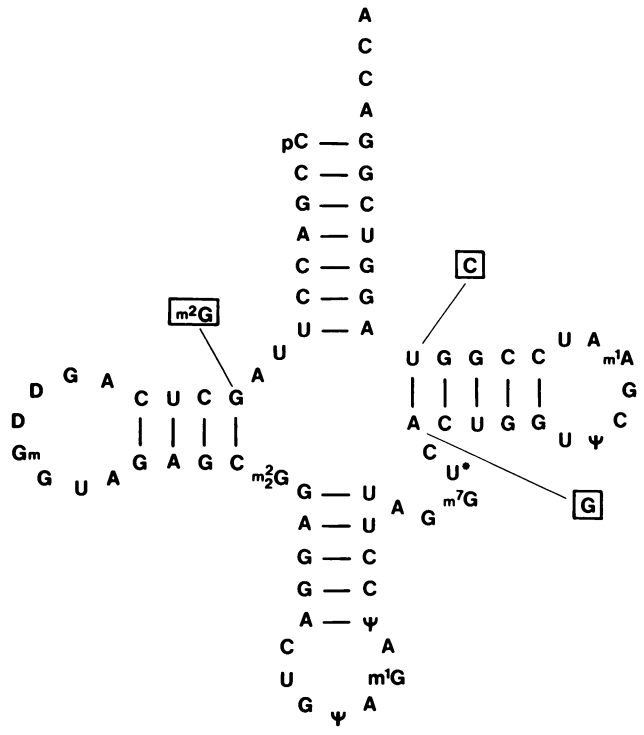

Fig. 6. Nucleotide sequences of tobacco $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$. The clover leaf model shows the nucleotide sequence of $tRNA_1^{Tyr}$. Bases in boxes are those found in $tRNA_2^{Tyr}$. There are indications that U* in position 47 is 3-(3-amino-3-carboxypropyl)uridine, acp^{3}U.

had no authentic standard; (c) apc^{3}U has rather frequently been found in this position.

The nucleoside at the 3′ side of the anticodon was identified as m^{1}G (Figure 5) by comparison with authentic [^{32}P]pm^{1}G, which was obtained from pure yeast $tRNA^{Trp}$ and *Salmonella typhimurium* $tRNA_3^{Pro}$, respectively, by parallel sequence and nucleotide analyses according to Stanley and Vassilenko (1978). A corresponding analysis of pure *Escherichia coli* $tRNA^{Ile}$ excluded the possibility of t^{6}A in this position.

The two $tRNAs^{Tyr}$ are almost identical in sequence. They differ only in an A:U pair at the base of the TΨC stem ($tRNA_1^{Tyr}$) and a G:C pair in the same position ($tRNA_2^{Tyr}$), respectively. $tRNA_1^{Tyr}$ was only isolated with an unmodified G_{10}, whereas $tRNA_2^{Tyr}$ contained a m^{2}G in this position. The presence of this m^{2}G in $tRNA_2^{Tyr}$ explains its elution from BD-cellulose at higher salt concentrations (Figure 2). It is remarkable that only the $tRNA^{Tyr}$ species with the G:C pair contains m^{2}G in position 10.

Discussion

Natural suppression, the readthrough over termination codons by tRNAs from wild-type cells, may be of certain general importance because it would allow an organism or a virus to produce two proteins from one cistron (Picard-Bennoun, 1982; Ryoji *et al.*, 1983). A classic example for natural readthrough are two major viral proteins of the TMV-infected plant cell, which, on the basis of the known TMV sequence (Goelet *et al.*, 1982) are now designated as the 126-K protein and the 183-K readthrough protein. These proteins differ by 499 amino acids at the C terminus. Using the readthrough assay (Pelham, 1978) and established rapid RNA sequencing procedures, we identify here the two major cytoplasmic $tRNAs^{Tyr}$ of the tobacco plant as the UAG suppressors which promote the TMV RNA-dependent 183-K protein synthesis *in vitro*. This finding may indicate that the host plant does not use UAG termination, or that an unknown structural feature of TMV RNA favors the interaction with this UAG codon and normal tyrosine tRNAs. These tRNAs differ in two nucleotides, indicating that at least two types of $tRNA^{Tyr}$ genes exist and are expressed in the tobacco plant. Interestingly, the $tRNA^{Tyr}$ species with an A:U pair in the TΨC arm contains an unmodified G_{10}, whereas the other species with the G:C pair in the same location has m^{2}G in this position.

The anticodon structure and its neighbourhood lead to the question as to how a UAG codon can be recognized by, and form a complex with, a GΨA anticodon, with an A:U and an A:Ψ pair only. Clearly, this type of interaction is not in accordance with the wobble hypothesis (Crick, 1966), as in a few other cases (Hirsh, 1971; Jank *et al.*, 1977; Weissenbach *et al.*, 1977; Diamond *et al.*, 1981). Also, the 'two out of three' reading (Lagerquist, 1978) does not apply here, since it excludes codons of the UA family. We can also preclude suppression mediated by frameshift, since there is no tyrosine codon next to the UAG codon in the other two reading frames. Furthermore, on the basis of the known TMV sequence (Goelet *et al.*, 1982) we can also exclude a context effect, i.e., a 'swollen' codon (Bossi, 1983) which would involve bases outside the codon and anticodon, respectively, to form a stable interaction. Also, there is no experimental basis for the suggestion that the Ψ in the second position of the anticodon, which is a unique feature of eukaryotic $tRNAs^{Tyr}$, may have a favourable influence on the codon-anticodon

interaction. In summary, the nature of the recognition between UAG termination codon and GΨA anticodon of wild-type tRNA^{Tyr} remains obscure.

We have not detected any Q nucleoside in place of G in the first position of the GΨA anticodon. This is in some contrast to the situation in *Drosophila*, where two tRNAs^{Tyr} have been detected, one of which is supposed to contain a GNN anticodon and to act as a UAG suppressor, whereas the other does not read UAG codons and may have a QNN anticodon (Bienz and Kubli, 1981). However, these tRNAs have not been well characterized, and they exert their function only in a non-homologous assay, using TMV RNA as a messenger. It also remains unknown whether the readthrough over the UAG codon has any biological significance for the plant cell or for TMV replication. Little is known about the possible function of the two major non-coat proteins induced by TMV after infection of a plant cell. They appear to comprise functions in early steps of virus multiplication since they are synthesized more rapidly than capsid protein during the first 10 h of detectable virus-specific protein synthesis (Figure 1; Siegel *et al.*, 1978). It has been postulated that the 126-K protein is the viral replicase or one of its subunits (Zaitlin *et al.*, 1973). The evidence for this assumption is rather indirect; however, it would then be reasonable that its synthesis precedes that of the coat protein. One would even like to speculate that the 183-K readthrough protein is also involved in, or even essential for, TMV RNA replication, possibly with a different function. An elegant approach for evaluating the function of the 183-K protein would have been the search for *Nicotiana* variants which do not promote its synthesis by UAG suppression: if the 183-K protein is essential for TMV replication, such variants should be TMV-resistant. Our finding that not a minor host tRNA, but the two normal major cytoplasmic tyrosine tRNAs can act as UAG suppressors indicates that it is most unlikely that such *Nicotiana* variants exist at all. It will, however, now be possible to study these questions by introducing the homologous suppressor tRNAs^{Tyr} into TMV-infected tobacco protoplasts to establish or to exclude a direct relationship between 183-K readthrough protein synthesis and TMV replication.

Materials and methods

RNase T1 and U2 were products from Sankyo, Tokyo; nuclease P1 was a kind gift from Dr.S.Nishimura, Tokyo; endonucleases from *Neurospora crassa* and *Staphylococcus aureus* were from Boehringer, Mannheim and Worthington Biochemical Corp., respectively. T4 polynucleotide kinase and T4 RNA ligase were obtained from Boehringer and P-L Biochemicals, respectively. The vulgare strain of TMV was from the collection of Dr.K.W. Mundry, Stuttgart. RNA was isolated from purified virus by phenol extraction.

Preparation of unfractionated tRNA from tobacco leaves

Unfractionated tobacco tRNA was prepared from fully expanded leaves of *N. tabacum*, cv. Samsun or Xanthi and from *N. rustica* by phenol extraction in a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.01 M $MgCl_2$, 2 mM EDTA, 5 mM mercaptoethanol and 0.1% SDS. High mol. wt. RNA was precipitated with 3 M NaOAc, pH 6.0. tRNA was isolated from this material by the usual DEAE-cellulose fractionation step.

Fractionation and isolation of tRNA^{Tyr} from total tobacco tRNA

Unfractionated tRNA was loaded onto a BD-cellulose column (1.2 x 35 cm) and fractionated by elution with a linear gradient of 0.35 M NaCl (0.01 M $MgCl_2$, 0.02 M NaOAc, pH 4.5) to 1 M NaCl in the same buffer. The tyrosine tRNA-rich fractions were further fractionated by Sepharose 4B chromatography on a column of 0.7 x 28 cm (Holmes *et al.*, 1975). Elution was performed by a reverse gradient of 2 M $(NH_4)_2SO_4$ to 1 M $(NH_4)_2SO_4$ in 0.01 M $MgCl_2$, 0.01 M NaOAc, pH 4.5.

Two-dimensional polyacrylamide gel electrophoresis was used as the final step in purification of tRNA^{Tyr} (Lockard *et al.*, 1978; Walker and RajBhandary, 1978). About 5 μg of the corresponding Sepharose 4B fraction was applied to a 10% polyacrylamide/7 M urea gel at pH 3.5 (thickness, 0.3 mm). Strips of the appropriate section of the gel were cut out and polymerized into a 12.5% polyacrylamide/7 M urea gel (pH 8.3). Individual spots containing tRNA were visualized by staining with 0.4% toluidine blue 'O' in 50% methanol, 1% acetic acid for 15 min and destained in 35% methanol, 1% acetic acid for 20 min. Elution of the RNA was performed in a buffer containing NH_4OAc, EDTA, $MgCl_2$, and SDS (Gross *et al.*, 1982).

Sequencing of tRNA by post-labeling techniques

Nucleotide sequences were determined by two independent methods, the fragment analysis procedure of Stanley and Vassilenko (1978) and rapid 'read off' sequencing gels (Donis-Keller *et al.*, 1977). The Stanley and Vassilenko method was used with some modifications (Gupta and Randerath, 1979). Limited hydrolysis of ~0.5 – 1 μg pure tRNA^{Tyr} was performed in 1 μl H_2O in a sealed glass capillary by incubation in a boiling water bath for 30 s. After 5′-^{32}P labeling the fragments were separated on a 15% polyacrylamide/8 M urea gel (thickness, 0.3 mm; height, 88 cm). The pH of the gel solution was adjusted to pH 8.3 with solid boric acid. On such a gel we could identify ~70 bands corresponding to nucleotide No. 4 up to No. 71. After autoradiography individual bands were eluted (Gross *et al.*, 1982) in the presence of 5 μg carrier tRNA. Complete digestion with 100 ng nuclease P1 in 10 μl 50 mM NH_4OAc (pH 5.3) was performed for 2 h at 50°C. The 5′ nucleotide of each eluted fragment was identified by cellulose t.l.c. using the two solvent system of Nishimura (1979) in parallel runs.

5′-^{32}P- and 3′-^{32}P pCp-labeling with T4 polynucleotide kinase and T4 RNA ligase, controlled enzymatic digestion of 5′- and 3′-labeled tRNA and chemical degradation of 3′-labeled tRNA followed by polyacrylamide gel electrophoresis was as described previously (Peattie, 1979; Krupp and Gross, 1983). The 5′- and 3′-terminal sequences were established by the two-dimensional mobility shift method (Jay *et al.*, 1974; Gillum *et al.*, 1975; Tyc *et al.*, 1983).

Translation in vitro

Translation in the nuclease-treated reticulocyte lysate was performed as described (Pelham and Jackson, 1976; Beier *et al.*, 1980). Unless otherwise stated (Figure 1), the lysate was not supplemented with calf liver tRNA. TMV RNA was added at 100 μg/ml. The reaction mixture was incubated for 60 min at 30°C.

Translation in vivo

Protoplasts were isolated from young leaves of tobacco plants *(N. rustica)* and inoculated with purified TMV preparations as described (Beier *et al.*, 1980). After appropriate periods of incubation, aliquots were removed from the mixture and used for gel electrophoretic analysis.

Analysis of translation products

Proteins were analysed by gel electrophoresis in 10% polyacrylamide gel slabs containing SDS (Laemmli, 1970). Gels were fixed overnight, fluorographed as described by Laskey and Mills (1975) and exposed to RX Fuji X-Ray films for ~8 h at −70°C. Individual bands were excised from the dried gel and the radioactivity was counted directly without adding scintillation fluid.

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References

Beachy,R.N. and Zaitlin,M. (1977) *Virology,* **81**, 160-169.
Beier,H., Mundry,K.W. and Issinger,O.-G. (1980) *Intervirology,* **14**, 292-299.
Bienz,M. and Kubli,E. (1981) *Nature,* **294**, 188-190.
Bossi,L. (1983) *J. Mol. Biol.,* **164**, 73-87.
Bruening,G., Beachy,R.N., Scalla,R. and Zaitlin,M. (1976) *Virology,* **71**, 498-517.
Crick,F.H.C. (1966) *J. Mol. Biol.,* **19**, 548-555.
Diamond,A., Dudock,B. and Hatfield,D. (1981) *Cell,* **25**, 497-506.
Donis-Keller,H., Maxam,A.M. and Gilbert,W. (1977) *Nucleic Acids Res.,* **4**, 2527-2538.
Geller,A.I. and Rich,A. (1980) *Nature,* **283**, 41-46.
Gillum,A.M., Urquhart,N., Smith,M. and RajBhandary,U.L. (1975) *Cell,* **6**, 395-405.
Goelet,P., Lomonossoff,G.P., Butler,P.J.G., Akam,M.E., Gait,M. and Karn,J. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 5818-5822.
Gross,H.J., Krupp,G., Dqmdey,H., Raba,M., Jank,P., Lossow,C., Alberty,

H., Ramm,K. and Sänger,H. (1982) *Eur. J. Biochem.*, **121**, 249-257.
Gupta,R.C. and Randerath,K. (1977) *Nucleic Acids Res.*, **4**, 3441-3454.
Hatfield,D., Diamond,A. and Dudock,B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6215-6219.
Hirsh,D. (1971) *J. Mol. Biol.*, **58**, 439-458.
Holmes,W.M., Hurd,R.E., Reid,B.R., Rimermann,R.A. and Hatfield,G.W. (1975) *Proc. Natl. Acad. Sci. USA*, **92**, 1068-1071.
Huber,R. (1979) PhD Thesis, Meded. Landbouwhogeschool, Wageningen, **79**, 15.
Jank,P., Shindo-Okada,N., Nishimura,S. and Gross,H.J. (1977) *Nucleic Acids Res.*, **4**, 1999-2008.
Jay,E., Bambara,R., Padmanabhan,R. and Wu,R. (1974) *Nucleic Acids Res.*, **1**, 331-353.
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, Inc., NY, pp. 11-58.
Laemmli,U.K. (1970) *Nature,* **227**, 680-685.
Lagerquist,U. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1759-1762.
Laskey,R.A. and Mills,A.D. (1975) *Eur. J. Biochem.*, **56**, 335-341.
Lockard,R.E., Alzner-De Weerd,B., Heckman,J.E., MacGee,J., Tabor, M.W. and RajBhandary,U.L. (1978) *Nucleic Acids Res.*, **5**, 37-56.
Murphy,E.C.,Jr., Wills,M. and Arlinghaus,R.B. (1980) *J. Virol.*, **34**, 464-473.
Nishimura,S. (1979) in Schimmel,P.R., Söll,D. and Abelson,J.N. (eds.), *Transfer RNA: Structure, Properties and Recognition,* Cold Spring Harbor Laboratory Press, NY, pp. 551-552.
Paterson,R. and Knight,C.A. (1975) *Virology,* **64**, 10-22.
Peattie,D.A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1760-1764.
Pelham,H.R.B. (1978) *Nature,* **272**, 469-471.
Pelham,H.R.B. and Jackson,R.J. (1976) *Eur. J. Biochem.*, **67**, 247-256.
Philipson,L., Anderson,P., Olshevsky,U., Weinberg,R. and Baltimore,D. (1978) *Cell,* **13**, 189-199.
Picard-Bennoun,M. (1982) *FEBS Lett.*, **149**, 167-170.
Ryoji,M., Hsia,K. and Kaji,A. (1983) *Trends Biochem. Sci.*, **8**, 88-90.
Sakai,F. and Takebe,I. (1974) *Virology,* **62**, 426-433.
Siegel,A., Hari,V. and Kolacz,K. (1978) *Virology,* **85**, 494-503.
Stanley,J. and Vassilenko,S. (1978) *Nature,* **274**, 87-89.
Tyc,K., Kikuchi,Y., Konarska,M., Filipowicz,W. and Gross,H.J. (1983) *EMBO J.*, **2**, 605-610.
Walker,R.T. and RajBhandary,U.L. (1978) *Nucleic Acids Res.*, **5**, 57-70.
Weissenbach,J., Dirheimer,G., Falcoff,R., Sanceau,J. and Falcoff,E. (1977) *FEBS Lett.*, **82**, 71-76.
Zaitlin,M., Duda,C.T. and Petti,M.A. (1973) *Virology,* **53**, 300-311.
Zaitlin,M. and Hariharasubramanian,V. (1972) *Virology,* **47**, 296-305.

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