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Unusual ciliate-specific codons in *Tetrahymena* mRNAs are translated correctly in a rabbit reticulocyte lysate supplemented with a subcellular fraction from *Tetrahymena*

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The codon usage of *Tetrahymena thermophila* and other ciliates deviates from the 'universal genetic code' in that UAA and probably UAG are not translational termination signals but code for glutamine. Therefore, translation *in vitro* of mRNA from *Tetrahymena* in a reticulocyte lysate is prematurely terminated if a UAA or UAG triplet is present in the reading frame of the mRNA. We show that the addition of a subcellular fraction from *Tetrahymena thermophila* enables a rabbit reticulocyte lysate to translate *Tetrahymena* mRNAs into full-sized proteins. The activity of the subcellular fraction is shown to depend on the combined function of a protein component(s) and a tRNA(s). The subcellular fraction is easily prepared and its usefulness for the identification of isolated mRNAs from *Tetrahymena* by their translation products *in vitro* is demonstrated.

INTRODUCTION

It is now established that ciliates use their own variation of the 'universal' genetic code. TAA, usually considered as a translational stop codon, has been identified as a glutamine-coding triplet in histone H3 genes in *Tetrahymena thermophila* (Horowitz & Gorovsky, 1985), and most probably TAA codes for glutamine also in an α-tubulin gene in *Stylonychia lemnae* (Helftenbein, 1985) as well as in genes of surface proteins of *Paramecium primaurelia* (Caron & Meyer, 1985) and *Paramecium tetraurelia* (Preer *et al.*, 1985). Another translational stop codon, TAG, codes for an amino acid, presumably glutamine, in genes of surface proteins of *Paramecium* (Caron & Meyer, 1985; Preer *et al.*, 1985).

Because of this special codon usage, translation *in vitro* of ciliate mRNAs in rabbit reticulocyte lysates or wheat-germ extracts most often results in polypeptides prematurely terminated owing to the presence of UAA or UAG codons within the coding regions. This failure to obtain full-sized proteins by translation *in vitro* has severely hampered important areas in the study of ciliate molecular biology.

In principle, this technical problem can be solved in different ways.

(1) Theoretically, the most satisfying solution is a homologous message-dependent translation system. A *Tetrahymena* system for translation *in vitro* has been described (David & Smith, 1981), but it is dependent on endogenous mRNA and has a poor initiation capacity.

(2) A translation system based on the normal, or rather 'non-ciliate', genetic code may be supplemented with suppressor tRNAs to obtain amber and ochre suppression activity (Gesteland *et al.*, 1976).

(3) A non-ciliate translation system may be supplemented with ciliate components until faithful translation of ciliate mRNAs is obtained.

Here we show that a *Tetrahymena thermophila* fraction containing tRNA and activating enzymes (aminoacyl-tRNA synthetases, EC 6.1.1.–) suppresses premature termination of translation of *Tetrahymena* mRNA in a nuclease-treated rabbit reticulocyte lysate prepared by the procedure of Pelham & Jackson (1976).

EXPERIMENTAL

Cells

Tetrahymena thermophila inbred strain B1868 (mating type VII) was grown in complex medium, starved in 10 mM-Tris/HCl, pH 7.5, and re-fed with complex medium as previously described (Dreisig *et al.*, 1984*a*,*b*).

Isolation of RNA

Total cellular RNA was isolated from re-fed cells by extractions with guanidinium thiocyanate (Chirgwin *et al.*, 1979) as previously described (Andreasen *et al.*, 1984). Polyadenylated RNA was selected by two successive passages through an oligo(dT)–cellulose column (Collaborative Research) by following the manufacturer's recommendations. tRNA was isolated from a post-ribosomal supernatant (Dreisig *et al.*, 1984*a*) by phenol/chloroform extraction and filtration through Sephadex G-100 in 10 mM-Tris/HCl (pH 7.5)/1 M-NaCl/1 mM-EDTA.

Preparation of the pH 5 fraction

This procedure is a modification of a procedure developed by Rosenbaum & Holz (1966). Cells from an exponentially growing culture (~10^5 cells/ml) were harvested and washed free of medium (Dreisig *et al.*, 1984*a*), washed once more in 10 mM-Hepes/KOH (pH 7.4)/10 mM-potassium acetate/0.5 mM-magnesium acetate, suspended in 2 vol. of 20 mM-Tris/HCl (pH 7.4)/90 mM-KCl/4.5 mM-magnesium acetate/1 mM-$CaCl_2$/10% (v/v) glycerol/1 mM-dithioerythritol/25 μM-haemin to 1 vol. of cells, and lysed at 0 °C by 40–80 strokes in a Potter–Elvehjem homogenizer. The lysate was cleared by centrifugation at 14000 rev./min for 60 min in a Beckman JA20 rotor at 4 °C. The supernatant was centrifuged at 40000

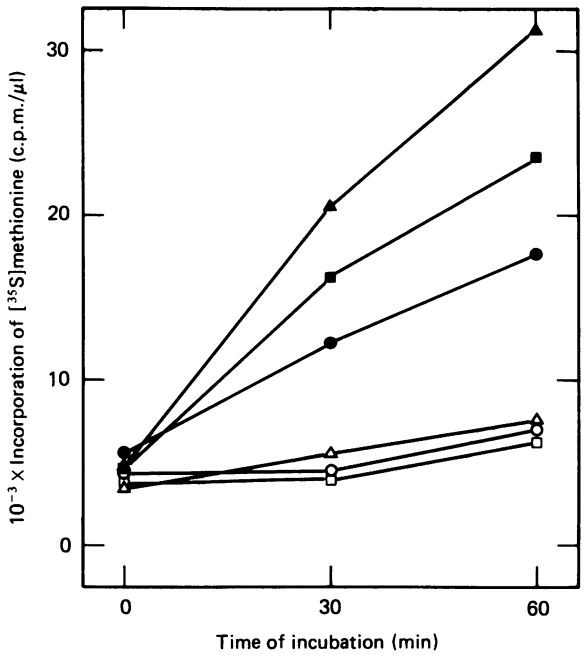

Fig. 1. Time course of translation *in vitro*

Translations were performed in a diluted nuclease-treated rabbit reticulocyte lysate (40% lysate) with (▲, ■, ●) or without (△, □, ○) *Tetrahymena thermophila* polyadenylated RNA (80 μg/ml). ●, ○, No further additions; ▲, △, plus 20% pH 5 fraction; ■, □, plus 20% micrococcal-nuclease-treated pH 5 fraction. The Figure shows incorporation of [^{35}S]methionine into hot-trichloroacetic acid-precipitable material.

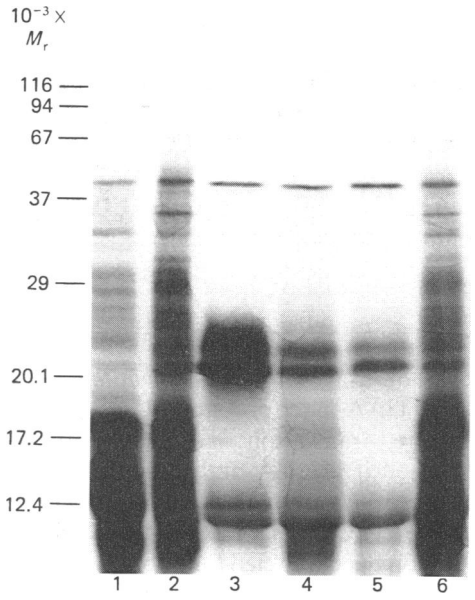

Fig. 2. Effect of pH 5 fraction on the proteins synthesized by translation *in vitro*

Proteins synthesized *in vitro* in a nuclease-treated rabbit reticulocyte lysate (final concn. 40% lysate) were analysed by electrophoresis in 12.5% polyacrylamide gels and fluorography. The translation assays contained: lane 1, *Tetrahymena* mRNA (80 μg/ml); lane 2, *Tetrahymena* mRNA and pH 5 fraction (final concn. 30% pH 5 fraction); lane 3, no addition; lane 4, untreated pH 5 fraction; lane 5, nuclease-treated pH 5 fraction; lane 6, *Tetrahymena* mRNA and nuclease-treated pH 5 fraction. All lanes are loaded with the same amount of radioactivity. The positions and M_r ($\times 10^{-3}$) of co-electrophoresed marker proteins are indicated.

rev./min for 2 h in a Beckman 50 Ti rotor at 4 °C, and the final supernatant (S-100 extract) was transferred to a new tube, adjusted to pH 5.0 with 1 M-acetic acid and left at 0 °C with constant stirring for 15 min. The resulting precipitate (pH 5 fraction) was collected by centrifugation at 14000 rev./min for 10 min in a Beckman JA20 rotor at 4 °C, washed carefully with 10 mM-Hepes/KOH (pH 7.4)/10 mM-potassium acetate/0.5 mM-magnesium acetate, dissolved in the same medium plus 10% (v/v) glycerol, 10 μM-haemin and 1 mM-dithioerythritol (approx. 1 ml/6.7×10^7 initial cells), and stored under liquid N_2. When indicated, total RNA was destroyed in the pH 5 fraction by incubation on ice for 30 min with 1.5 ng of RNAase A (EC 3.1.27.5; Sigma)/μl of pH 5 fraction, followed by specific inactivation of RNAase A with 8 units of RNasin (Promega Biotec)/ng of RNAase A.

Translation *in vitro*

All translations were carried out in the presence of 1.5μCi of [^{35}S]methionine (NEN)/μl in a nuclease-treated rabbit reticulocyte lysate (Pelham & Jackson, 1976), according to the manufacturer's (Amersham) recommendations, with the following exceptions: lysate concentration was decreased to 40% or 20%, spermidine hydrochloride, pH 7.0, was added to 0.5 mM, and a mixture of 19 unlabelled amino acids (minus methionine) was added to a final concentration of 50 μM each. Translation products were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) or two-dimensional gel electrophoresis in the pH 8.6/SDS system (Dreisig *et al.*, 1984*a*) followed by fluorography as previously described (Andreasen *et al.*, 1984).

Hybridization selection

Individual mRNAs were isolated by hybridization selection as described by Maniatis *et al.* (1982), except that the hybridization buffer was 50% (v/v) formamide/20 mM-Pipes/NaOH (pH 6.4)/0.2% SDS/0.6 M-NaCl, containing 100 μg of bovine tRNA (Sigma)/ml, 100 μg of poly(A) (Sigma)/ml, 1 unit of RNasin/μl and 1 mM-dithioerythritol, and that the hybridization reaction was begun at 70 °C, slowly cooled to 42 °C and continued at 42 °C for at least 12 h.

RESULTS

The translational efficiency of a single mRNA species may be determined partly by its codon usage. Therefore, translation *in vitro* in heterologous systems is sometimes improved by the addition of species-specific tRNAs. However, when *Tetrahymena thermophila* mRNA is translated in a message-dependent reticulocyte lysate, the addition of *Tetrahymena thermophila* tRNAs has no effect on the size or distribution of translation products (results not shown). Similar results are obtained when *Paramecium* mRNAs are translated in a tRNA-

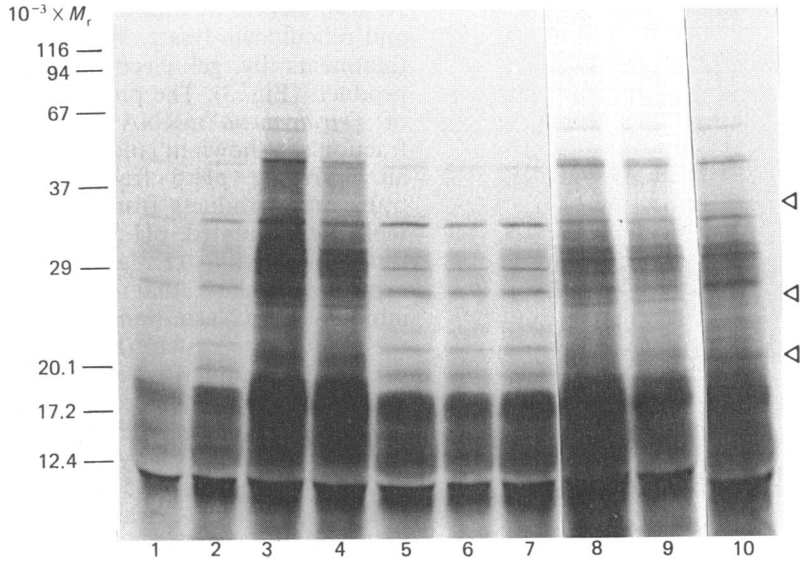

Fig. 3. Effect of treated pH 5 fractions on the synthesis of polypeptides in reticulocyte lysates programmed with *Tetrahymena* mRNA

Gel-electrophoretic analysis of proteins synthesized by translation of *Tetrahymena thermophila* polyadenylated RNA (80 μg/ml) in a nuclease-treated rabbit reticulocyte lysate (final concn. 20% lysate) in the presence of pH 5 fractions treated in various ways as described below. All treatments were performed by mixing three components: (1) pH 5 fraction (or buffer in control experiments), (2) RNAase A (or water in control experiments) and (3) RNasin. Two of the three components were mixed and incubated at 0 °C; 30 min later the third component was added, and incubation continued for another 30 min. In the following, the three components used in each experiment are listed in order of addition: pH 5 fraction, RNAase A, RNasin (lane 1); buffer (pH 5-fraction control), RNAase A, RNasin (lane 2); pH 5 fraction, water (RNAase A control), RNasin (lane 3); RNAase A, RNasin, pH 5 fraction (lane 4); buffer, water, RNasin (lane 5); phenol/chloroform-extracted pH fraction, water, RNasin (lane 6); pH 5 fraction immersed in boiling water for 5 min, water, RNasin (lane 7). Lanes 8–10 show the translation products obtained when an RNAase-A-treated pH 5 fraction is reconstituted with a phenol/chloroform-extracted pH 5 fraction (lane 8), with a heat-inactivated pH 5 fraction (lane 9), or with purified *Tetrahymena thermophila* tRNA (lane 10). The final concentration of pH 5 fraction or buffer was 30% in all experiments. A constant amount of translation assay mixture was loaded on each of the lanes 1–10. The positions and M_r ($\times 10^{-3}$) of co-electrophoresed marker proteins are shown to the left. Arrows point to discrete bands caused by the pH 5 fraction and observed also in the reconstitution experiments (lanes 8–10).

supplemented reticulocyte system (Meyer *et al.*, 1984); most products are prematurely terminated proteins.

The inability of ciliate tRNAs to make the reticulocyte lysate read through UAA and UAG codons can be explained in different ways. Either the reticulocyte system cannot activate the foreign tRNAs, or the reticulocyte ribosomes cannot use the activated ciliate tRNAs. Considering the similarities between tRNAs, and dissimilarities between activating enzymes, the former explanation seems the most plausible. Furthermore, the pool of endogenous release factor in the reticulocyte lysate may have a competitional advantage relative to the added tRNAs. We considered that this might be a problem, and therefore performed all translations in a diluted lysate to lower the concentration of release factor.

A pH 5 fraction from *Tetrahymena thermophila* (see the Experimental section) contains tRNAs and activating enzymes (Rosenbaum & Holz, 1966). Such a pH 5 fraction stimulates the translation of *Tetrahymena* mRNA about 2-fold, as measured by incorporation of [^{35}S]methionine into protein (Fig. 1). This stimulation might be caused by mRNAs in the pH 5 fraction, although this is highly unlikely, because all translations were performed with saturating amounts of exogenous mRNA. To eliminate the putative mRNAs, we incubated the pH 5 fraction with micrococcal nuclease as described by Pelham & Jackson (1976). This nuclease treatment does not abolish the stimulatory effect of the pH 5 fraction, and, since the pH 5 fraction itself (in the absence of added mRNA) does not stimulate translation (Fig. 1), the active components of the pH 5 fraction are most probably tRNAs and activating enzymes. In accordance with this, only tRNA is detected by a gel-electrophoretic analysis of the RNA components of the pH 5 fraction (results not shown).

The alleviation of premature termination leads to larger translation products. This is demonstrated in Fig. 2, which shows a gel-electrophoretic analysis of the proteins obtained by translation of *Tetrahymena* mRNA in the nuclease-treated reticulocyte lysate. A standard translation without the pH 5 fraction gives rise to many small translation products, but only few proteins with M_r higher than 20000 and virtually none above 67000 (lane 1). A translation in the presence of pH 5 fraction increases the size of the translation products, some bands disappear, and new bands are seen (lane 2; see also Fig, 3, lanes 3 and 5).

The gel-electrophoretic analysis of translation products is also a sensitive assay for mRNA activity in the pH 5 fraction. Some preparations of the pH 5 fraction prompted the synthesis of a few proteins in a reticulocyte lysate in the absence of extraneously added mRNA. To circumvent this batch variability, which may be distur-

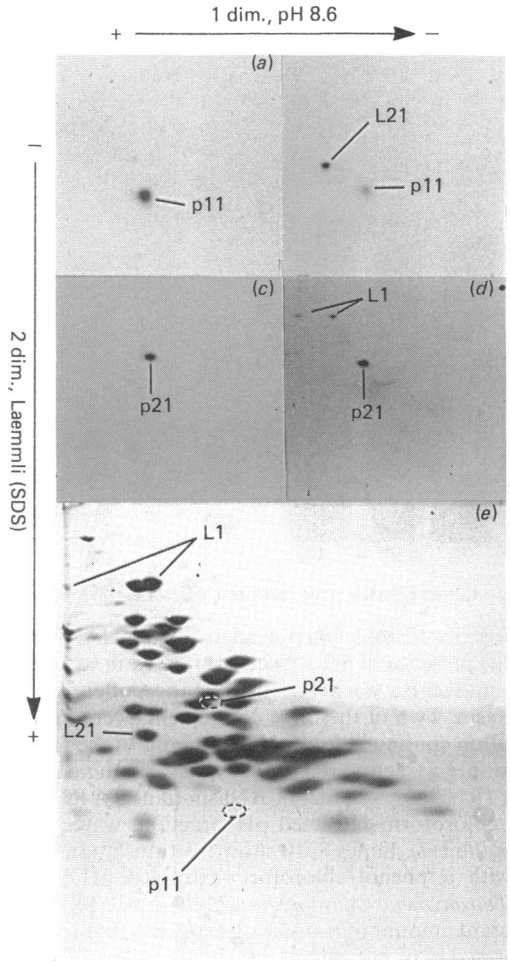

Fig. 4. Translation of individual mRNAs in the absence or presence of the pH 5 fraction

mRNAs corresponding to two different *Tetrahymena thermophila* cDNA clones, C7 (*a*, *b*) and C12 (*c*, *d*), were isolated by hybridization selection and translated in the absence (*a*, *c*) or presence (*b*, *d*) of the pH 5 fraction. The translation products were analysed by gel electrophoresis in the pH 8.6/SDS system (Dreisig *et al.*, 1984*a*) followed by fluorography. For comparison, the stained pattern of isolated ribosomal proteins is shown in panel (*e*). The approximate locations of p11 and p21, the prematurely terminated translation products, are indicated in panel (*e*). The correctly terminated ribosomal protein L1 appears as two spots in panel (*d*), because much of the protein remains at the application site of the first-dimension gel. This is a commonly observed phenomenon for some proteins (notably L1). Abbreviation: dim., dimension.

bing in analyses of individual mRNAs (cf. Fig. 4), we now routinely treat our pH 5 fractions with micrococcal nuclease as described by Pelham & Jackson (1976). This treatment destroys a potential mRNA activity in the pH 5 fraction (Fig. 2, lanes 3–5) without affecting the read-through activity (lane 6).

If the activity of the pH 5 fraction is caused by the combination of tRNA and activating enzymes, any treatment harmful to either RNA or protein should abolish the activity. We degraded the RNA components of the pH 5 fraction by incubation with RNAase A. The RNAase A was then inhibited by the specific inhibitor, RNasin, before we mixed the pH 5 fraction with mRNA and reticulocyte lysate. We analysed the effect of these treatments by gel electrophoresis of the translation products (Fig. 3). The products obtained by translation of *Tetrahymena* mRNA with and without the pH 5 fraction are shown in lanes 3 and 5 respectively. Clearly, an untreated pH 5 fraction shifts the M_r of the translation products from lower to higher values. An RNAase A-treated pH 5 fraction has lost the read-through activity (cf. lanes 1 and 3). The control experiments show that the RNAase A is effectively inhibited by RNasin (lane 4) and then has no effect on the reticulocyte system (lane 2). The protein moiety of the pH 5 fraction was removed by phenol/chloroform extraction or denatured by heat treatment. Both treatments abolished the activity of the pH 5 fraction (lanes 6 and 7). Finally, we tried to reconstitute an RNAase A-treated pH 5 fraction by the addition of a phenol/chloroform-extracted pH 5 fraction (lane 8), a boiled pH 5 fraction (lane 9) or isolated *Tetrahymena thermophila* tRNA (lane 10). All three types of reconstitution resulted in partial re-establishment of the suppressing activity. Thus a protein component as well as an RNA component is essential for the suppressing activity of the pH 5 fraction.

The ultimate proof for the suppressing activity of the pH 5 fraction is the demonstration that a single mRNA containing an internal UAA or UAG codon is translated into a prematurely terminated protein in the absence of the pH 5 fraction, but into a recognizable full-sized product in the presence of the pH 5 fraction. Therefore we tested the effect of the pH 5 fraction on the translation of individual mRNAs. From a *Tetrahymena thermophila* cDNA library we chose two clones, C7 and C12. Several lines of evidence suggest that these clones are specific for ribosomal proteins: the cDNA library is enriched for ribosomal protein sequences (Nielsen *et al.*, 1986), hybridization experiments show that during a starvation–re-feeding cycle the abundance of mRNA sequences homologous to C7 and C12 varies in a manner characteristic of ribosomal proteins (P. H. Andreasen, H. Dreisig & K. Kristiansen, unpublished work), and the translation products corresponding to C7 and C12 are clearly basic (cf. Fig. 4), as would be expected for ribosomal proteins. We isolated the mRNAs corresponding to C7 and C12 by hybridization selection, precipitated the selected mRNAs individually with *Tetrahymena* tRNA as carrier, and translated the isolated mRNAs *in vitro* with and without the pH 5 fraction in the reticulocyte lysate (Fig. 4).

We found it favourable to use *Tetrahymena* tRNA as carrier in the precipitations of mRNA because bovine tRNA decreased the suppressing activity of the pH 5 fraction (results not shown). In the absence of the pH 5 fraction, the mRNA selected by the C7 cDNA was translated into an unidentifiable basic polypeptide, p11 ($M_r \sim 11000$), smaller than most ribosomal proteins (Fig. 4*a*). The alleviation of premature termination by the addition of the pH 5 fraction prompted the synthesis of a protein which co-migrated exactly with ribosomal protein L21 in addition to p11 (Figs. 4*b* and 4*e*). Similarly, in the absence of the pH 5 fraction the mRNA selected by the C12 cDNA was translated into an unidentifiable basic polypeptide, p21, with M_r 21500 (Fig. 4*c*), but into p21 and ribosomal protein L1 (M_r 48000) in the presence of the pH 5 fraction (Figs. 4*d* and

4*e*). All estimates of M_r are based on the electrophoretic mobilities in the second dimension of pH 8.6/SDS gels such as those shown in Fig. 4.

The nucleotide sequence of the C12 cDNA (P. H. Andreasen, H. Dreisig, K. Kristiansen, J. Engberg & H. Nielsen, unpublished work) shows that the corresponding mRNA indeed contains an internal UAA codon, and that termination of translation at this codon will give rise to a protein with M_r 20850, whereas the full-sized product has M_r 44353. Thus there is a good agreement between the expected and the observed M_r especially when it is taken into consideration that determinations of M_r based on migration in SDS/polyacrylamide gels usually lead to erroneously high values for positively charged basic proteins such as ribosomal proteins and histones (Panyim & Chalkley, 1971).

DISCUSSION

We have shown that the addition of a subcellular fraction from *Tetrahymena thermophila* enables a reticulocyte lysate to translate *Tetrahymena* mRNAs into full-size proteins. The active components of the fraction are tRNA and activating enzymes. At present we do not know how many tRNAs and enzymes participate in the read-through activity. In *Paramecium*, both UAA and UAG code for amino acids (Caron & Meyer, 1985; Preer *et al.*, 1985), whereas in *Tetrahymena* (Horowitz & Gorovsky, 1985) and in *Stylonychia* (Helftenbein, 1985) until now only UAA has been found to code for an amino acid. According to the wobble hypothesis (Crick, 1966) a tRNA recognizing UAA will have one of the anticodons $^{3'}$AUU$^{5'}$ or $^{3'}$AUI$^{5'}$, which recognize the codons $UA\begin{matrix}A\\G\end{matrix}$ and $UA\begin{matrix}U\\C\\A\end{matrix}$ respectively. Since UAU and UAC are the only two tyrosine codons, the unusual ciliate $tRNA^{Gln}$ probably has the anticodon $^{3'}$AUU$^{5'}$ and recognizes UAA as well as UAG. In accordance with this, Nishimura's group (Kuchino *et al.*, 1985; Hanyu *et al.*, 1986) has isolated two unusual $tRNA^{Gln}$ species from *Tetrahymena thermophila*, one recognizing UAA and UAG, and another recognizing only UAG.

Technically, supplementation of a reticulocyte lysate with the pH 5 fraction facilitates ciliate molecular biology by making it possible to identify cDNA clones by the translation products *in vitro* of their corresponding mRNAs. In this kind of analysis, incomplete suppression is not an obstacle for the interpretation. Translations of complex mixtures of *Tetrahymena* mRNA yield extremely complex patterns of proteins, consisting of full-sized products and prematurely terminated polypeptides, and may be very difficult to interpret directly. However, meaningful interpretation should be possible by employing, e.g., immunological techniques. Finally, it should be noted that the pH 5 fraction may be of general use for amber and ochre suppression *in vitro*, regardless of the origin of the mRNAs.

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REFERENCES

Andreasen, P. H., Dreisig, H. & Kristiansen, K. (1984) Eur. J. Biochem. **140**, 485–492
Caron, F. & Meyer, E. (1985) Nature (London) **314**, 185–188
Chirgwin, J. W., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry **18**, 5294–5299
Crick, F. H. C. (1966) J. Mol. Biol. **19**, 548–555
David, E. T. & Smith, K. E. (1981) Biochem. J. **194**, 761–770
Dreisig, H., Andreasen, P. H. & Kristiansen, K. (1984*a*) Eur. J. Biochem. **140**, 469–475
Dreisig, H., Andreasen, P. H. & Kristiansen, K. (1984*b*) Eur. J. Biochem. **140**, 477–483
Gesteland, R. F., Wolfner, M., Grisafi, P., Fink, G., Botstein, D. & Roth, J. R. (1976) Cell **7**, 381–390
Hanyu, N., Kuchino, Y., Nishimura, S. & Beier, H. (1986) EMBO J. **5**, 1307–1311
Helftenbein, E. (1985) Nucleic Acids Res. **13**, 415–433
Horowitz, S. & Gorovsky, M. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2452–2455
Kuchino, Y., Hanyu, N., Tashiro, F. & Nishimura, S. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4758–4762
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, pp. 331–333, Cold Spring Harbor Laboratory, New York
Meyer, E., Caron, F. & Guiard, B. (1984) Biochimie **66**, 403–412
Nielsen, H., Andreasen, P. H., Dreisig, H., Kristiansen, K. & Engberg, J. (1986) EMBO J. **5**, 2711–2717
Panyim, S. & Chalkley, R. (1971) J. Biol. Chem. **246**, 7557–7560
Pelham, H. R. B. & Jackson, R. J. (1976) Eur. J. Biochem. **67**, 247–256
Preer, J. R., Jr., Preer, L. B., Rudman, B. M. & Barnett, A. J. (1985) Nature (London) **314**, 188–190
Rosenbaum, J. L. & Holz, G. G., Jr. (1966) J. Protozool. **13**, 115–123

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