Functional assay of tRNA molecules transcribed from a purified gene

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ABSTRACT

Purified tRNA genes are expressed when microinjected into the nucleus of X. laevis oocytes. In this paper we describe a method to assay the capacity to be aminoacylated of the tRNA transcribed in the frog oocytes. The method exploits the radiochemical purity of the transcript and relies on the binding of aminoacyl-tRNA but not of uncharged tRNA to purified elongation factor EF-Tu. We also present some preliminary results on several single point mutants of tRNA Pro from Caenorhabditis elegans. We show that nucleotide 73 of tRNA Pro can be substituted by any other nucleotide without loss of acceptor activity. A double mutant, causing transition from G45G46 to A45A46 has lost acceptor activity. Also inactive is a mutant carrying an insertion of a single base in the anticodon loop.

INTRODUCTION

Purified genes have been used for studies of gene expression in vivo and in vitro (1-4). In the last few years we have studied the expression of tRNA genes by microinjection in the nucleus of the Xenopus laevis oocytes (5-11). This system has many convenient properties: following DNA microinjection, tRNA precursors are transcribed and then converted, via a series of processing reactions, to what appears to be mature tRNA.

In our previous studies we have shown that a segment of DNA comprising only the tDNA Pro sequence from Caenorhabditis elegans was sufficient to direct transcription of tRNA precursors which were efficiently matured (8,11). The sequence of this relatively short DNA segment (72 base pairs) has therefore at least three functions: i) it contains the transcriptional signals responsible for the interaction with the transcriptional machinery; ii) it codifies for an RNA precursor which is the specific substrate of several maturation enzymes; iii) and, of course, is responsible for the synthesis of a functional tRNA molecule, capable of being aminoacylated and of participating in the various steps of protein synthesis.

Using a variety of site-directed mutagenesis procedures we have constructed in vitro, a series of mutants altered in transcription or in maturation.
There are however mutations which influence neither transcription nor maturation giving thus rise to a mature but altered tRNA molecule. In this case it is of interest to determine the functional competence of these molecules, in order to identify those regions which are essential or important for the interaction with the various components of the protein synthesis apparatus.

In this paper we describe a method to analyze the functional properties of tRNA^Pro synthesized from tDNA^Pro microinjected in *Xenopus laevis* oocytes, and present preliminary results obtained with mutants altered in the aa stem, or in the extra-arm, or in the anticodon loop.

**MATERIALS AND METHODS**

**Bacterial strains and plasmid vectors**

Plasmids and phages used in this work have all been previously described (8,10-13).

**Chemicals and enzymes**

All radioactive compounds were from Amersham; all restriction endonucleases used were from B.R.L.; purified EFTu elongation factor from *E.coli* was a gift of Dr. Ottavio Fasano.

**Purification of prolyl-tRNA synthetase**

Prolyl-tRNA synthetase was prepared from wheat germ. 20 gr. of wheat germ were homogenized with alumina and then suspended in 100 ml of 0.01 M Tris-Cl pH 7.5, 0.01 M Mg acetate, 0.01 M β-mercaptoethanol, 0.25 M Sucrose, 0.05 M KCl and 10% glycerol. The high-speed supernatant was then precipitated with ammonium sulfate to reach 70% saturation and then applied on a Sephadex G100 column equilibrated with: 0.01 M K_2HPO_4 pH 7.5, 0.02 M β-mercaptoethanol, 0.005 M KCl and 10% glycerol (2 x 80 cm; flow-rate 12 ml/h). The column was eluted with the same buffer and the active fractions were pooled and applied on a DE-52 column equilibrated with the same buffer (2 x 20 cm; flow-rate 20 ml/h). After washing with buffer, a linear KCl gradient (0.005-0.3 M) was applied and the active fractions were pooled and precipitated with ammonium sulfate to reach 70% saturation. The active pool was dialyzed against 50 mM Tris-Cl pH 7.5, 20 mM NaCl, 2 mM β-mercaptoethanol, 0.5 mM EDTA and 10% glycerol and then applied on a phosphocellulose column (1.5 x 12 cm; flow-rate 16 ml/h). After washing with the same buffer, a linear KCl gradient (0.0-0.35 M) was applied and the active fractions were pooled and concentrated by Amicon ultrafiltration (membrane cone type CG25). The enzyme preparation was dialyzed against 0.01 M K_2HPO_4 pH 7.5, 0.02 M β-mercaptoethanol, 0.005 M KCl and 50% glycerol. At this stage the enzyme was stored at -20°C.
All the buffers used in this preparation contained the protease inhibitor phenylmethanesulfonylfluoride (PMSF) at the concentration 0.1 mM.

Microinjection

Microinjections into the nucleus of Xenopus laevis oocytes were performed as described (5) using 50 nl solution of DNA (200 µg/ml) and as radioactive precursor \(^{32}\)P-GTP (410 Ci/mmol, 10 Ci/ml). 20-40 oocytes were injected for each sample and then incubated in Barth solution for 6 hours. RNA was extracted as described (5) and fractionated on TB 10% polyacrylamide gel (5). For tRNA analysis the radioactive bands were extracted as described (6).

Assay for interaction of Pro-tRNA with EF-Tu-GTP

a) Aminoacylation reaction. The reaction mixture contained 0.01 M ATP-Tris pH 7.5, 0.016 M MgCl\(_2\), 0.1 mM GTP, 40 µM proline, tRNA extracted from gel, and purified synthetase from wheat germ in a final volume of 40 µl. This mixture was incubated for 15 minutes at 37°C.

b) Ternary complex formation. After aminoacylation, ternary complex between EF-Tu-GTP and aminoacyl-tRNA was formed in the following conditions: 10 µl of a solution containing 25 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 1 mM DTT, 0.75 M NH\(_4\)Cl, 1 mM GTP, 50 µM EF-Tu, were added to the 40 µl aminoacylation mixture and incubated for 5 minutes at 37°C.

The sample was then chilled at 0°C and immediately applied on a Sephadex G-100 column (0.7 x 15 cm) equilibrated with 25 mM Tris-HCl pH 7.5, 10 mM MgCl\(_2\), 1 mM DTT, 150 mM NH\(_4\)Cl. The column was eluted with the same buffer at + 4°C and fractions of 0.1 ml were collected and counted in Instagel.

RESULTS

Microinjected tDNA\(^{35}\)P directs the synthesis of negligible amounts of tRNA\(^{35}\)P labelled at high specific activity. In order to assay its biological activity we developed a method which could exploit its radiochemical purity.

The aminoacylation reaction detected by binding of the aa-tRNA to EF-Tu

Aminoacylated tRNA (but not free tRNA) can form a ternary complex with purified EF-Tu and GTP (14). On a Sephadex G-100 column the elution profile of prolyl-tRNA\(^{35}\)P bound to E.coli EF-Tu and free prolyl-tRNA are different as shown in Fig. 1. This property can be exploited for an indirect assay of aminoacylation of \(^{35}\)P-tRNA synthesized in the oocyte. \(^{35}\)P-tRNA\(^{35}\)P is extracted from polyacrylamide gel and incubated in the presence of aminoacyl-tRNA synthetase and cold proline; after 20 min EF-Tu and GTP are added and the formation of the ternary complex occurs almost instantaneously. The material is then chromatographed on a Sephadex G-100, as shown in Fig. 2 (continuous
If the aminoacylation reaction is prevented by omission of proline (Fig. 2, dotted line) or ATP (data not shown), ternary complex is not formed and radioactive tRNA Pro shows a different elution profile.

Note however that C. elegans tRNA Pro is one nucleotide shorter than yeast tRNA Pro.

A series of tDNA Pro mutants were constructed (8,11,12) carrying changes in the sequence coding for part of the aa-stem or for the base (nucleotide 73) preceding the posttranscriptionally added -CCA (Fig. 3) (Throughout the paper we have numbered nucleotides according to the conventional system (19). Note however that C. elegans tRNA Pro is one nucleotide shorter than yeast tRNA Pro.

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**Fig. 1.** Sephadex G-100 gel filtration of $^3$H-pro-tRNA and Tu-GTP-$^3$H-pro-tRNA complex. 1 OD 260 of w. germ tRNA and 5 pmoles of $^3$H-proline were used in the same conditions described in the methods section. Each fraction was acid-precipitated, filtered and counted.

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**Fig. 2.** Sephadex G-100 gel filtration of $32^P$-tRNA Pro and Tu-GTP-$^32^P$ tRNA Pro. $32^P$-tRNA Pro obtained by microinjection of tDNA Pro (plasmid Mct1) into the nucleus of X. laevis oocytes was incubated with aminoacyl-tRNA synthetase in presence (+aa) or absence (-aa) of cold proline. After 20 min incubation time EF-Tu and GTP were added and the tertiary complex fractionated on Sephadex G-100 column as described in the methods section.
Fig. 3. Sequence of the amino acid acceptor stem of tRNAPro codified by wild-type tDNAPro and some mutants. Experimental details concerning the construction of the various mutants are described (8,11). For each of the mutant tRNAs the altered nucleotide is boxed.

Following microinjection, these tDNAs direct the synthesis of mature tRNAPro including CCA addition (8,11). 32P-tRNAPro was purified and subjected to the aminoacylation-EF-Tu binding assay. None of these mutations interferes (at least qualitatively) with the aminoacylation reaction (patterns were identical to that shown in Fig. 2). This is interesting because it has been frequently suggested that the base preceding the CCA (nucleotide 73) plays a role in the recognition of a specific tRNA by the cognate synthetase.

Mutants in the extra arm of tRNAPro. Construction of these mutants has been described (13). The extra arm sequence of the corresponding tRNA (as deduced by DNA sequence and fingerprint analysis) is shown in Fig. 4. The results of the aminoacylation-EF-Tu binding assay are shown in Fig. 5. The elution profile in the case of mCetB2 or mCetB28 is identical to that of wild-type...
Fig. 5. Sephadex G-100 gel filtration of $^{32}$P-tRNA$_{Pro}$ and Tu-GTP-$^{32}$P-tRNA$_{Pro}$. $^{32}$P-tRNA$_{Pro}$ obtained by microinjection of mutants mCet1B28 (panel A) and mCet1B23 (panel B) was subjected to aminoacylation assay, binding to EF-Tu and then fractionated on Sephadex G-100 column as described in the materials and methods section. +aa: aminoacylation reaction in presence of cold proline; -aa: aminoacylation reaction in absence of cold proline.

Prolyl tRNA$_{Pro}$ (Fig. 5, panel A) whereas the elution profile obtained with mCetB23 (Fig. 5, panel B) is corresponding to that of uncharged tRNA$_{Pro}$, indicating that this mutant tRNA cannot be aminoacylated in these experimental conditions.

We conclude that transitions of $G_{45}$ to $A_{45}$ or $G_{46}$ to $A_{46}$ do not interfere with the aminoacylation reaction. On the contrary, the double $G_{45}G_{46}$ to $A_{45}A_{46}$ transition results in a complete lack of aminoacylation.

A single base insertion in the anticodon loop. Mutant B78RI$_2$ was constructed

![Diagram of tRNA structures](image)

Fig. 6. Sequence of the anticodon stem and loop of wild-type and B78RI$_2$ tRNA$_{Pro}$. Construction of B78RI$_2$ has been described (10).
by using a procedure described elsewhere (10) and carries an insertion of one base, a G residue, in the region coding for the anticodon loop. Following microinjection into the nucleus of the frog oocyte this DNA directs the synthesis of a mature tRNA\textsuperscript{Pro} whose sequence (deduced by DNA sequence and fingerprint analysis) is shown in Fig. 6. The anticodon loop of this mutated tRNA is formed by 8 rather than the usual 7 bases. This sequence change prevents aminoacylation (data not shown).

**DISCUSSION**

The method. *In vitro* constructed mutant tRNA genes, when microinjected into the nucleus of frog oocytes, direct the synthesis of altered gene products. We have been interested in developing simple assays to characterize the capacity of these altered tRNAs to function in the various reactions of protein synthesis. The method presented in this paper is a first preliminary attempt for an indirect aminoacylation assay which relies on the radiochemical purity of the tRNA species synthesized in the living oocytes. By using the EF-Tu binding assay we measure at the same time the capacity of tRNA of being aminoacylated and the capacity of aminoacyl-tRNA to bind to EF-Tu. It is probable that in all cases presented mutant tRNAs which fail to bind to EF-Tu are, in fact, defective in the aminoacylation reaction because it is well established that, even short oligonucleotides including the terminal CCA, when aminoacylated, can bind to EF-Tu (15). It is in principle possible to measure independently the aminoacylation reaction, for example using boronic resins (16); so far, however, in our hands this procedure has not given satisfactory results.

In the conditions used in this paper we could only obtain qualitative results: we could detect charging or lack of charging in an all or none fashion. More quantitative results and more detailed informations about the reaction kinetics could, in principle, be obtained by adjusting enzyme concentrations and reaction times. All assays have been performed in a doubly heterologous system: elongation factor Tu was from *E.coli*, whereas prolyl-tRNA\textsuperscript{Pro} synthetase was purified from wheat germ (a preliminary study had shown that the wheat germ enzyme could efficiently aminoacylate tRNA\textsuperscript{Pro} from *C.elegans*). In the future it will be possible to use homologous systems. In order to obtain significant and precise information about the role of specific nucleotides in the various functions of tRNA it will be necessary to extend our collection of mutants. We believe, however, that some of the results presented in this paper are of considerable interest:
Base-pair substitution mutants in the aa-acceptor stem. Sequence comparison (17) and the properties of some mutant tRNAs (18) suggested that nucleotide 73 (according to the conventional numbering (19)) plays a role in tRNA-synthetase recognition. It was for instance observed that E.coli tRNA<sub>Tyr</sub> mutants in nucleotide 73 were misacylated (18). We have constructed mutant tRNA<sub>Pro</sub>'s, which have any of the four possible nucleotides in position 73 and all of them are normally aminoacylated. The role of nucleotide 73 cannot therefore be crucial for recognition by the cognate synthetase, even though it is possible that the specificity is somehow relaxed. It must be stressed that our assay cannot be easily used to test for misacylation because, in order to obtain reproducible results, we need to use purified synthetases.

A single base insertion in the anticodon loop. There are several evidences that for many synthetases the anticodon of the cognate tRNA is an important point of contact. Loss of acceptor activity of a yeast tRNA<sub>Val</sub> was observed following removal of bases from the anticodon (20). Squires and Carbon (21) reported that a mutation in the anticodon of an E.coli tRNA<sub>Gly</sub> species results in a 10<sup>4</sup> fold depression in the rate of aminoacylation. Yaniv et al. (22) discovered that a mutation in the anticodon of E.coli tRNA<sub>Trp</sub> enables this tRNA to accept glutamine. Studies on chemically modified E.coli tRNAs showed that C→U changes in any of the nucleotides of the anticodon lead to loss of the acceptor activity (23-26). Prat et al. (27) reported that a single nucleotide insertion in the anticodon loop of a E.coli tRNA<sub>Gly</sub> causes missense suppression. These authors did not establish whether the suppressor tRNA<sub>Gly</sub> was still charged with glycine, but, on the basis of RPC-5 column chromatography analysis, they suggested that the change in the anticodon may lead to a decreased rate of aminoacylation. The same authors, in order to explain the missense suppressor property of this Gly-T derived mutant carrying an insertion in the anticodon, hypothesized an anticodon shift of one nucleotide to the 3' side (27).

Our mutant B78R I<sub>2</sub> carries the insertion of one base which does not change the anticodon triplet and in a hypothetical anticodon shift the new anticodon will still be specific for proline. In line with the data discussed above this tRNA has lost its capacity to be aminoacylated. Also in this case, of course, we have not explored the possibility for misacylation.

Mutants in the extra-arm. There are several indirect evidences that the extra-arm may not be a primary contact point with the synthetase (28). Our data, on the contrary, suggest that the extra-arm nucleotide sequence may be important. We have constructed three different mutants all of which involve
nucleotides that are engaged in tertiary interactions with corresponding bases in the D-stem. We observe normal aminoacylation when only a single G at either position is substituted. When, however, both Gs are converted to As, then the capacity to be aminoacylated is lost. The non Watson-Crick tertiary interactions G₄₅₋G₁₀ and G₄₅₋G₂₂ are important for the overall tRNA tridimensional structure. If only one of these two bonds is missing the structure is not significantly altered, but when both of them are missing the alteration is probably more dramatic with consequent loss of the capacity to be aminoacylated.

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