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In vivo repair of the 3' terminus of transfer RNA injected into amphibian oocytes

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ABSTRACT

Yeast transfer RNA specific for phenylalanine has been treated chemically to remove either one or two nucleotides of its 3' terminus and has been injected into Xenopus laevis oocytes to test whether this RNA can be repaired in vivo. The results obtained showed that oocytes could aminoacylate and thus repair $tRNA^{Phe}$ that has lost both its terminal adenosine and 3' phosphate. A similar result was obtained with $tRNA^{Phe}$ that had undergone two full cycles of 3' terminal nucleotide removal. The oocytes cannot aminoacylate $tRNA^{Phe}$ whose 3' terminal ribose has been oxidized with periodate or the derivative that retains a 3' phosphate after adenosine removal. In vitro assays show that the Xenopus ovary contains a tRNA nucleotidyl transferase with the properties similar to enzymes obtained from other sources which may be responsible for the 3' terminal repair observed in vivo.

INTRODUCTION

One of the mechanisms that has been postulated for the regulation of transfer RNA activity in the cell has been the removal and addition of the nucleotides that comprise the CCA sequence that is common to the 3' terminus of all tRNA. Although an enzyme, tRNA nucleotidyl transferase, that is capable of catalyzing both the removal and the reincorporation of these nucleotides has been known for a number of years, there is some uncertainty as to its physiological role (1).

It seemed interesting, therefore to use the microinjection technique to answer directly the question whether tRNA missing its 3' nucleotides could be repaired under in vivo conditions by an activity with the characteristics of tRNA nucleotidyl transferase. Using Xenopus laevis oocytes it has been possible to study the translation of mRNA and the aminoacylation of tRNA introduced into these cells (2-5).

The present report includes data that demonstrates that tRNA that has been chemically treated to remove 3' terminal nucleotides prior to injection can be repaired and aminoacylated inside Xenopus laevis oocytes. Data is also presented to show that oocyte homogenates contain an activity with the characteristics of tRNA nucleotidyl transferase which may be responsible for the observed in vivo tRNA repair.

MATERIALS AND METHODS

Adult Xenopus laevis females were obtained from the South African Snake Farm, Cape Providence, South Africa. Full grown oocytes of approximately 1.2 mm diameter were dissected out manually and cultured in an amphibian saline solution (63 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM Tris HCl pH 7.4 and 10 mg/l each of penicillin and streptomycin sulfate), a modification of Holtfreter's solution (6).

Microinjections were carried out by the method of Gurdon (7) with needles calibrated to 30 to 50 nl. All incubations of the injected oocytes were carried out at 21°C.

The aminoacylation assay of the injected $tRNA^{Phe}$ was carried out by preincubating duplicate groups of 5 injected oocytes each with 20 µM [^{14}C]phenylalanine in 50 µl of the amphibian saline for two hours. After the injection of tRNA, incubation was continued in fresh saline containing 20 µM [^{14}C]phenylalanine for the times indicated in each experiment and the reaction was stopped by the addition of 4 ml of 50% phenol in a buffer containing 10 mM $MgCl_2$, 20 mM sodium acetate pH 5.0, 1 mM EDTA and 0.1% Triton. The oocytes which contained aminoacyl-tRNA were homogenized in the phenolic mixture and the water phase was separated by centrifugation at 1500 x g for 15 minutes. Duplicate aliquots of 0.5 ml were precipitated with cold 5% trichloracetic acid in the presence of 0.1 mg of carrier yeast tRNA. The precipitate was collected on glass fiber filters which were dried and counted.

The removal of the 3' terminal nucleotides of yeast $tRNA^{Phe}$ was carried out essentially as described by Daniel and Littauer (8). Pure yeast $tRNA^{Phe}$, purchased from Boehringer, was oxidized with $NaIO_4$ using a ratio of 0.3 µmol of periodate

per mg of $tRNA^{Phe}$ in 50 mM sodium acetate buffer pH 5.0 during 20 minutes in the dark at 20°C. The reaction was stopped by addition of a 10 fold excess of glycerol to eliminate the unreacted $NaIO_4$ and the oxidized tRNA ($tRNA^{Phe}$-CCA_{ox}) was recovered by precipitation with cold 70% ethanol and redissolved in water. To eliminate the terminal adenosine the material was treated with 0.3 M lysine at pH 8.0 for 20 minutes. The product which corresponds to a tRNA lacking adenosine and having a 3' terminal phosphate ($tRNA^{Phe}$-CC_p) was treated with bacterial alkaline phosphatase (Worthington) at a concentration of 0.06 units/mg of tRNA in a buffer containing 10 mM $MgCl_2$ in 100 mM Tris-HCl pH 8.5 during 90 minutes at 37°. The reaction was stopped with 50% phenol in acetate buffer and the ($tRNA^{Phe}$-CC) recovered as described above.

In order to remove the second 3' nucleotide and obtain $tRNA^{Phe}$-C, the whole cycle was repeated once again. The materials prepared thus were controlled enzymatically by testing their capacity to accept amino acids and nucleotides using partially purified phenylalanyl-tRNA synthetase and tRNA nucleotidyl transferase from wheat embryos. The purity of the preparations was better than 90%.

The in vitro assay for tRNA nucleotidyl transferase contained in a total volume of 0.2 ml: 50 mM Tris HCl pH 8.5, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.3 mg of unfractionated yeast tRNA (Schwarz), 0.5 mM [^{3}H]pyrimidine nucleoside 5' triphosphate or 4 mM [^{3}H]purine nucleoside 5' triphosphate of a specific activity of 20 mCi/mmol, and approximately 25 µg of protein of the enzyme preparation. The incubation was carried out for 15 minutes at 37° and the reaction was stopped by the addition of cold 5% trichloroacetic acid. The mixture was filtered, dried and counted in a liquid scintillation counter. Partially purified tRNA nucleotidyl transferase from Xenopus laevis ovary was prepared by homogenizing 38 g of ovary with 40 ml of buffer solution containing 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 2 mM mercaptoethanol and 0.2 M sucrose. The homogenate was centrifuged at 12,000 x g for 15 minutes and the resultant supernatant was centrifuged at 105,000 x g for 1 hour. The soluble fraction was precipitated between 40

and 80% saturation with ammonium sulfate. The precipitate was redissolved and passed through a Sephadex G-25 column (20 x 15 cm) equilibrated with buffer containing 10 mM Tris HCl pH 7.5, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.1 mM EDTA. The fraction excluded from the column was chromatographed on a DEAE-cellulose column (20 x 3 cm) equilibrated with the same buffer as used for gel filtration. The ion exchanger was subsequently eluted with a NaCl gradient from 0 to 0.3 M. The tRNA nucleotidyl transferase activity was eluted at a 0.15 M salt concentration. The active fractions were concentrated by ultrafiltration and kept at -20° with 20% glycerol. The enzyme was stable for several weeks.

RESULTS AND DISCUSSION

Early attempts to measure in vivo repair of tRNA whose terminal nucleotides have been removed by direct incorporation of co-injected [^{3}H]ATP did not give conclusive results. The presence of very large endogenous pools of nucleoside triphosphates in the oocyte (9) which dilute the radioactive compounds introduced into the cell made this experimental approach unfeasible.

The capacity of the oocyte to aminoacylate microinjected deficient tRNA was taken as an alternative means of measuring the in vivo repair. It is well established that the tRNAs which lack terminal nucleotides are not acceptors for their respective amino acids. Efficient aminoacylation of unmodified yeast $tRNA^{Phe}$ microinjected into Xenopus laevis oocytes was reported in a previous publication from this laboratory (5).

Table I shows the results obtained in two different experiments in which the aminoacylation of the different $tRNA^{Phe}$ injected into oocytes was measured. The results are clear cut in that the $tRNA^{Phe}$ that has the terminal adenosine and 3' phosphate removed (tRNA-CC) has full capacity for aminoacylation in the oocyte. A similar result is obtained with $tRNA^{Phe}$ that has undergone two full cycles of 3' terminal nucleotide removal (tRNA-C). The oocytes are obviously capable of repairing these tRNAs prior to their aminoacylation. The efficiency of the chemical modification of the terminal

TABLE I

Aminoacylation of tRNAs with Different 3' Termini Injected into Xenopus Laevis Oocytes

Experiment	3' terminus of injected $tRNA^{Phe}$	[^{14}C]Phe-tRNA formed (pmol/oocyte)
1.	tRNA-CCA	1.6
	tRNA-CCA_{ox}	0.1
	tRNA-CC_p	0.4
	tRNA-CC	1.9
2.	tRNA-CCA	3.0
	tRNA-CCA_{ox}	0.4
	tRNA-CC_p	0.4
	tRNA-CC	2.5
	tRNA-C	2.7
3.	uninjected control	0.08

Equal concentrations of the different tRNA preparations were microinjected into duplicate groups of 5 oocytes and the amount of [^{14}C]Phe-tRNA formed after 90 minutes incubation in 20 µM [^{14}C]Phe was determined. In experiments 1 and 2, 5 and 10 pmol of tRNA, respectively, were injected.

nucleoside can be seen by the fact that the tRNA-CCA_{ox} cannot be significantly aminoacylated. This same preparation was used to make tRNA-CC and tRNA-C. It is interesting to note that tRNA-CC_p is also inactive in aminoacylation indicating that the repair system has a specificity similar to the known tRNA nucleotidyl transferases which cannot use tRNA with 3' phosphates as nucleotide acceptors. It is somewhat surprising that the oocytes do not contain phosphatases active enough to remove the 3' terminal phosphate under the conditions employed. It is possible that the tRNA is not readily available to phosphatase action. A similar resistance of tRNA to degradative enzymes was observed previously in oocytes (10).

In the calculations of pmoles of [^{14}C]Phe-tRNA formed presented in this table no correction has been introduced for the dilution of the labelled phenylalanine by the endogenous

pool since the oocytes had been preincubated in the solution containing [^{14}C]phenylalanine two hours prior to the tRNA injection. For a discussion of the problem in considering the internal amino acid pools for this reaction see reference 5.

Figure I shows the kinetics of aminoacylation of tRNA^{Phe}-CC compared to the reaction of untreated tRNA^{Phe}-CCA. It is evident that the aminoacylation reaction of the deficient tRNA shows an initial lag which is probably due to its requirement for repair prior to aminoacylation.

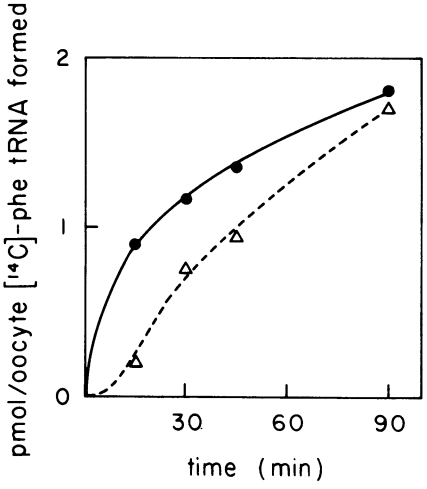

Figure I. Time course of aminoacylation of tRNA^{Phe}-CCA and tRNA^{Phe}-CC injected into Xenopus laevis oocytes. Oocytes were preincubated for 2 hours in 20 μM [^{14}C]-phenylalanine and subsequently injected with 5 pmoles of yeast tRNA^{Phe}-CCA (-●-●-) or with tRNA^{Phe}-CC (--Δ--Δ--) per oocyte.

Table II shows that a crude preparation of the supernatant fraction of a Xenopus laevis ovary homogenate contains an activity which incorporates radioactive ATP and CTP into yeast tRNA. As observed with well-characterized tRNA nucleotidyl transferases, this enzyme is also able to use UTP as substrate but not GTP (1). The reaction requires magnesium ion and is stimulated by 2-mercaptoethanol. It is noteworthy that Xenopus ovary tRNA is a much less efficient acceptor than yeast tRNA for ATP and almost inactive for CTP, indicating that this tRNA is mostly complete in its 3' end and that the deficient fraction is largely tRNA-CC.

Using an ovarian tRNA nucleotidyl transferase preparation which has been partially purified by DEAE cellulose chromatography, the apparent K_m for ATP and CTP were determined to be 2 mM and 0.2 mM respectively. These values are similar to those obtained for the tRNA nucleotidyl transferase from rabbit

TABLE II

In vitro Characteristics of tRNA Nucleotidyl Transferase Activity Obtained from Xenopus Laevis Ovary

Nucleotide Substrate	Conditions	Incorporation into TCA precipitable material, pmol
[^{3}H]CTP	Complete system	720
	Minus $MgCl_2$	61
	Minus 2-mercaptoethanol	520
	Minus tRNA	30
	Minus tRNA + oocyte tRNA	70
	Minus tRNA + poly U	54
	Complete + RNase	45
[^{3}H]ATP	Complete system	1060
	Minus tRNA + oocyte tRNA	320
[^{3}H]GTP	Complete system	38
[^{3}H]UTP	Complete system	270

The enzyme source was the fraction of the 105,000 x g supernatant of an ovarian homogenate that precipitates between 40 and 80% saturation of ammonium sulfate.

liver (11). A previous report has appeared in which tRNA nucleotidyl transferase activity of Xenopus eggs and early embryos was detected (12). Using E. coli mutants deficient in tRNA nucleotidyl transferase Deutscher et al. (13) have established the importance of this enzyme for tRNA repair in vivo in bacteria.

In conclusion the experiments presented above demonstrate that under in vivo conditions a tRNA whose terminal nucleotides have been removed chemically can be repaired and aminoacylated in amphibian oocytes. Homogenates of Xenopus ovary contain tRNA nucleotidyl transferase which has properties similar to enzymes from other systems which could be responsible for the repair observed in vivo.

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REFERENCES

1 Deutscher, M.P. (1973) Prog. Nucleic Acid Res. Mol. Biol. 13, 15-92.
2 Lane, C.D., Marbaix, G. and Gurdon, J.B. (1971) J. Mol. Biol. 61, 73-91.
3 Laskey, R.A., Gurdon, J.B. and Crawford, L.V. (1972) Proc. Natl. Acad. Sci. USA 69, 3665-3669.
4 Vander Konk, J.A.W.M. (1975) Nature 256, 674-675.
5 Gatica, M., Tarrago, A., Allende, C. and Allende, J. (1975) Nature 256, 675-678.
6 Hamburger, V. (1960) A Manual of Experimental Embryology, Rev. Edition (Chicago, University of Chicago Press) p 85.
7 Gurdon, J.B. (1968) J. Embryol. Exp. Morph. 20, 401-414.
8 Daniel, V. and Littauer, U.Z. (1965) J. Mol. Biol. 11, 692-705.
9 Woodland, H.R. and Pestell, Q.W. (1972) Biochem. J. 127, 597-605.
10 Allende, C., Allende, J. and Firtel, R.A. (1974) Cell 2, 189-196.
11 Deutscher, M.P. (1972) J. Biol. Chem. 247, 459-468.
12 Paradiso, P. and Schofield, P. (1976) Exp. Cell Res. 100, 9-14.
13 Deutscher, M.P., Foulds, J. and McClain, W.H. (1974) J. Biol. Chem. 249, 6696-6699.