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Studies on aminoacyl tRNA synthetases and transfer RNA in living *Xenopus laevis* oocytes

Estudios de las aminoacil-tRNA sintetasas y del RNA de transferencia en oocitos de *Xenopus laevis*

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The microinjection of transfer RNA into amphibian oocytes permits one to study under *in vivo* conditions the reactions that affect this important macromolecule. A comparative study has been carried out between the *in vivo* and *in vitro* specificity of the aminoacylation reaction. The results obtained show that modifications of the tRNA structure affect aminoacyl-tRNA synthetase recognition in the same fashion in both conditions. The *in vivo* aminoacylation was not affected by the presence of puromycin (0.5mM) or cycloheximide (0.1 mM) which completely inhibited oocyte protein synthesis. An interesting difference was obtained between the *in vivo* and *in vitro* aminoacylation of tRNA with regards to temperature requirements. While the *in vivo* reaction was optimal at 25° and was totally inhibited at 37°, the *in vitro* was optimal, at the latter temperature. The inhibition of the *in vivo* reaction at 37° was not due to inactivation of the enzyme. The transfer of the amino acid moiety to nascent proteins was studied by measuring the transfer of radioactivity from injected (^{14}C) phenylalanyl-tRNA into hot trichloroacetic acid precipitable material. It was found that 30% or more of the amino acid became incorporated into oocyte proteins and that this incorporation was due to direct transfer from the aminoacyl-tRNA and was inhibited by puromycin and cycloheximide.

TRANSFER-RNA AMPHIBIAN OOCYTES AMINOACYLATION and TRANSFER REACTIONS

In our laboratory we have been interested for a number of years in the aminoacyl-tRNA synthetases and in the main products of the reaction catalyzed by these enzymes, the aminoacyl-tRNAs

After directing our initial efforts towards the study of the characteristics of these enzymes and their reaction mechanism (1-3), we have more recently become interested in determining whether these enzymes and the transfer RNAs play a significant regulatory role in protein biosynthesis. We have approached this problem by the use of the amphibian oocyte system. This system has the important advantage of allowing, through direct microinjection, the modification of the internal concentrations of tRNAs or enzymes of a living cell. This property tends to lend more physiological significance to the possible effects that may be observed.

In this communication we will present some of the experiments which we have carried out with the microinjection of tRNA and aminoacyl-tRNA into *Xenopus laevis* oocytes.

MATERIALS AND METHODS

Adult Xenopus laevis females were obtained from the South African Snake Farm, Fish Hoek, South Africa. Full grown oocytes (1.0 to 1.2 mm diameter) were obtained as described previously (4). Oocytes of earlier stages were dissected from the ovary manually and sized using a micrometer lens on a dissecting microscope.

Microinjections where carried out essentially as described by Gurdon (5) using 20 to 50 nl volumes per oocyte. All materials microinjected were dissolved in sterile water and in all cases duplicate groups of five oocytes were used. Incubations were carried out in amphibian saline (63 mM NaCl, 1 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH and 10 μg/ml each of penicilin and streptomycin sulfate) and at 22° unless otherwise specified.

Yeast $tRNA^{phe}$ was purchased from Boeringer and at the time of use had an amino acid acceptor capacity of 400 pmol per OD_{260} unit *in vitro*. Unfractionated yeast tRNA was from Calbiochem.

^{14}C-phenylalanyl-$tRNA^{phe}$ was prepared using a partially purified wheat germn enzyme preparation obtained essentially as described by Muench and Berg (6).

In vivo aminoacylation. Full grown oocytes were preincubated for three hours in amphibian saline containing 20 μM ^{14}C-phenylalanine, specific activity 270 or 494 μCi/umol. Individual oocytes were injected with 4 to 15 pmoles of yeast $tRNA^{phe}$ per cell, and transfered to a fresh solution (50 μl) containing 20 μM ^{14}C-phenylalanine for incubation. The reaction was stopped by the addition of 2 ml of buffer (0.2 M sodium acetate pH 5.0 mM $MgCl_2$, 5 mM EDTA and 0.1% Triton X-100) and 2 ml of phenol. The oocytes were broken by homogenization and the water phase was separated by centrifugation at 10.000 × g for 10 minutes and duplicate aliquots of 0.5 ml were added to 5 ml of cold trichloroacetic acid containing 20 mM ^{12}C-phenylalanine. The precipitate was collected on glass fiber discs and counted.

The incubation mixture to assay *in vitro* aminoacylation contained in a total volume of 50 μl: 5 mM ATP, 7.5 mM $MgCl_2$, 10 μM ^{14}C-phenylalanine (specific activity 270 μC/μmol) 5 mM dithiothreitol, 10 mM Tris-HCl pH 7.5, 300 pmoles of yeast $tRNA^{phe}$ and 25 μg of bovine serum albumin, and between 3-20 μg of a crude enzyme preparation. Unless otherwise specified, the incubations were carried out at 22° for 15 minutes and the reaction was stopped by the addition of 5% cold trichloroacetic acid. The ^{14}C-phe-tRNA formed was retained on glass fiber filters which were subsequently dried and counted in a liquid scintillation spectrometer.

The crude extract containing aminoacyl-tRNA synthetases was prepared as follows: 20 full-grown oocytes or 20-60 oocytes of smaller sizes were homogenized with a glass homogenizer in 0.5 ml of amphibian saline solution containing 1 mM dithiothreitol. The homogenate was centrifuged at 10.000 × g for 10 minutes and the supernatant fraction was removed carefully to avoid contamination with the top layer of lipid material. This fraction was used as a source on enzyme in assays measuing total *in vitro* activity.

In other assays the enzyme was further purified by centrifugation of the fraction at 100,000 × g for three hours. The high speed supernatant liquid was precipitated between 40-80% saturation with ammonium sulfate and the precipitate was dissolved and desalted on Sephadex G-25. The solution was applied to a DEAE-cellulose column (1.2 × 15 cm) equilibrated with 70 mM KCl and 20 mM Tris-HCl, pH 7.5. The enzyme was eluted with 0.2 M KCl and used directly.

Periodate oxidized $tRNA^{phe}$ ($tRNA^{phe}_{ox}$) was prepared by the procedure of Daniel and Littauer (7) as described previously (8). Removal of the Y base to yield $tRNA^{phe}_{-y}$ was achieved by the methods of Thiebe and Zachau (9).

In vivo deacylation of injected ^{14}C-phe-$tRNA^{phe}$ was assayed as follows: oocytes were injected with 2-6 pmoles of ^{14}C-phe-$tRNA^{phe}$ per cell, and incubated for varying periods. The cells were subsequently extracted with a phenol-buffer mixture as described for the *in vivo* aminoacylation assay. The amount of ^{14}C-phe-tRNA remaining in the oocyte was determined by counting the radioactivity precipitable with cold 5% tricholoroacetic acid present in the water phase.

The aminoacyl transfer from ^{14}C-phe-tRNA to oocyte proteins was measured by injecting oocytes with [^{14}C]-phe-tRNA and incubating them in 50 μl of amphibian saline for the specified times. The reaction was stopped by addition of 2 ml of cold 5% trichloroacetic acid and the oocytes were homogenized with a glass rod and the suspension was treated for 15 minutes at 90°, filtered on glass fiber discs which were subsequently dried and counted.

RESULTS AND DISCUSSION

Specificity of aminoacylation of microinjected tRNAs

Evidence has been previously presented (10) to show that microinjected yeast $tRNA^{phe}$ can be aminoacylated inside *X. laevis* oocytes. Table I shows the *in vivo* acceptor capacity of $tRNA^{phe}$ and chemically modified derivatives of this nucleic acid. It is apparent that the reaction *in vivo* shows very similar requirements to the *in vitro* process in that periodate oxidated $tRNA^{phe}$ cannot accept amino acid while the $tRNA^{phe}$ whose "Y" base has been chemically removed is a good amino acid acceptor. An interesting difference from the purified system, however, is observed with the $tRNA^{phe}_{cc}$ which has lost the terminal 3' adenosine needed for aminoacylation and

which is seen to have acceptor capacity *in vivo*. A more detailed of the *in vivo* repair of the 3' end of injected tRNA has been published. (8).

TABLE I

Aminoacylation of different tRNA derivatives microinjected into Xenopus laevis oocytes

tRNA microinjected	^{14}C-Phe- *tRNA formed per 5 oocytes in 15 minutes*	
	cpm	pmoles
None	120	0.24
$tRNA^{phe}$	1866	4.9
$tRNA^{phe}_{CC}$	706	1.86
$tRNA^{phe}_{CCp}$	262	0.68
$tRNA^{phe}_{ox}$	266	0.70
$tRNA^{phe}_{Y}$	1535	4.04
tRNA (Bulk)	223	0.45
tRNA (Bulk)	223	0.45

Oocytes were microinjected with 7.6 pmoles of $tRNA^{phe}$, except where 1 pmol of $tRNA^{phe}_{Y}$ was used. Ten pmoles of unfractionated or bulk tRNA were used.

In vivo aminoacylation was essayed as detailed in Methods.

Figure 1 shows the rate of aminoacylation of tRNA injected in oocytes that are incubated in the presence and absence of cycloheximide and puromycin, potent inhibitors of endogenous oocyte protein synthesis (10). The results show that the reaction which proceeds linealy during the first 10 minutes is not affected by cellular protein synthesis since the same amount of aminocylation have been obtained in the presence or absence of inhibitors.

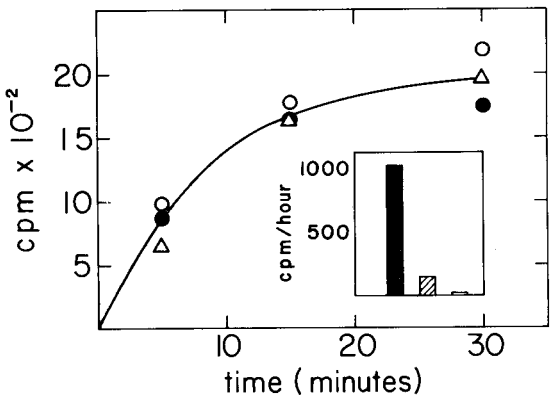

Fig. 1. Aminoacylation of $tRNA^{Phe}$ microinjected into oocytes treated with protein synthesis inhibitors. Duplicate groups of 5 oocytes were injected with 20 pmoles of yeast $tRNA^{Phe}$ and incubated in the presence of 20 μM ^{14}C phenylalanine in amphibian saline at 22°. At the times indicated after the injection, the amount of *in vivo* aminoacylation was determined by the assay described in Methods in oocytes incubated in the absence of inhibitors (o), and in group of oocytes incubated in the presence of 0.1 mM cycloeximide (o) or 0.5 mM puromycin ( ).

The ordinate shows the amount of ^{14}C Phe incorporated into tRNA *per oocyte*.

The insert shows the amount of ^{14}C Phe incorporated into proteins per hour per oocyte in control cells (black bar) and in the oocytes treated with puromycin (crossed bar) with cycloheximide (white bar).

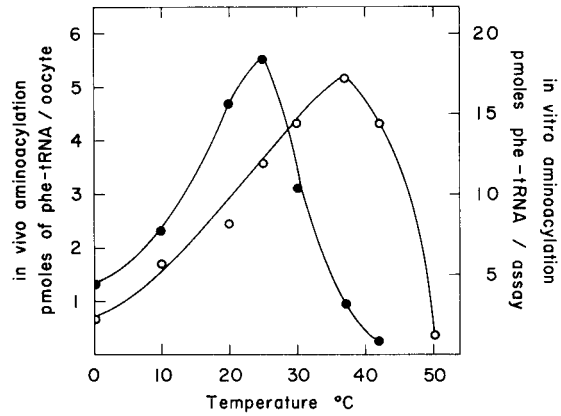

Fig. 2. The effect of temperature on *in vivo* and *in vitro* aminoacylation of $tRNA^{Phe}$. For the *in vivo* assay (●-●) duplicate groups of 5 *X. laevis* oocytes were pre-incubated for 3 hours at 22° with 20 μM ^{14}C Phe in amphibian saline, injected with 20 pmoles per oocyte of yeast $tRNA^{Phe}$ and incubated for 40 minutes at the temperature indicated. The formation of ^{14}C Phe-tRNA was measured as described in Methods. In the *in vitro* determination (o-o), a partially purified phenylalanyl-tRNA synthetase from *X. laevis* ovary that had been prepared through the DEAE cellulose chromatography step was used. The preparation of the enzyme and the assay conditions are detailed in Methods. These incubation were carried out for 30 minutes at the specified temperatures.

The effect of temperature on the in vivo and in vitro aminoacylation

Temperature has been shown to have a drastic effect on *in vivo* amphibian oocyte protein synthesis(10). For this reason it seemed pertinent to study the effect of this parameter in the *in vivo* and *in vitro* aminoacylation of tRNA.

An interesting difference is observed between the reaction carried out in the living cell and the one performed in the test tube. From the results shown in figure 2 it is evident that the

in vivo reaction has an optimum at 25° and is seriously inhibited at higher temperatures. At 37° the reaction has a slower rate than is observed at 0°. The inhibition of the reaction occuring in the cell is obviously not due to an intrinsic lability of the oocyte phenylalanyl-tRNA synthetase or of the yeast $tRNA^{phe}$ because these same components participate in the *in vitro* reaction which has an optimun at 37°. The possibility still persisted that the phenylalanyl-tRNA synthetase is labilized by the *in vivo* environment. To test this possibility an experiment was performed in which oocytes were pre-incubated at 0° 22° and 37° for 40 minutes before homogenizing the cells and assaying for phenylalanyl-tRNA synthetase. The results shown in table II indicate

TABLE II

Determination of Aminoacylation Activity After Preincubation at Different Temperatures

Temperature of preincubation	*Volume of crude enzyme extract assayed, ul*	*Phe-tRNA formed (cpm)*
0	2	1046
	4	2036
22	2	1036
	4	2032
37	2	945
	4	2140

Duplicate groups of 20 oocytes each were pre-incubated for 40 minutes at the temperatures indicated. Subsequently the oocytes were homogenized as described in Methods for the preparation of a crude extract (10000 × g supernatant) containing aminoacyl-tRNA synthetases.

The activity of these enzyme extracts was assayed using two levels of added enzyme to test linearity of reaction. The assay was carried out as described for *in vitro* conditions in the Methods section.

that the same enzyme activity was present in oocytes pre-incubated at 37° than at the lower temperature. Thus, it is clear that the *in vivo* inactivity at the higher temperature is not due to an irreversible inactivation of the enzyme.

In analyzing these experiments is should be noted that the effect of temperature on that uptake of the exogenous amino acid for the *in vivo* reaction was minimized by the fact that the oocytes had been pre-incubated with the labelled amino acid for three hours at 21° prior to their injection and incubation at the different temperatures.

The fate of ^{14}C phenylalanine microinjected as ^{14}C-phe-$tRNA^{phe}$

If instead of unacylated $tRNA^{phe}$, we inject ^{14}C-phe-$tRNA^{phe}$ into *X. laevis* oocytes, it is possible to follow the fate of the amino acid moiety introduced into the cell.

Figure 3A shows that the injected radioactive phenylalanine gradually leaves the tRNA after its injection as evidenced by the decrease of radioactivity extractable with phenol. It can also be observed in this figure that the presence of puromycin or cycloheximide at concentrations known to inhibit oocyte protein synthesis does not affect the rate of phe-tRNA deacylation. In figure 3B we can see a similar

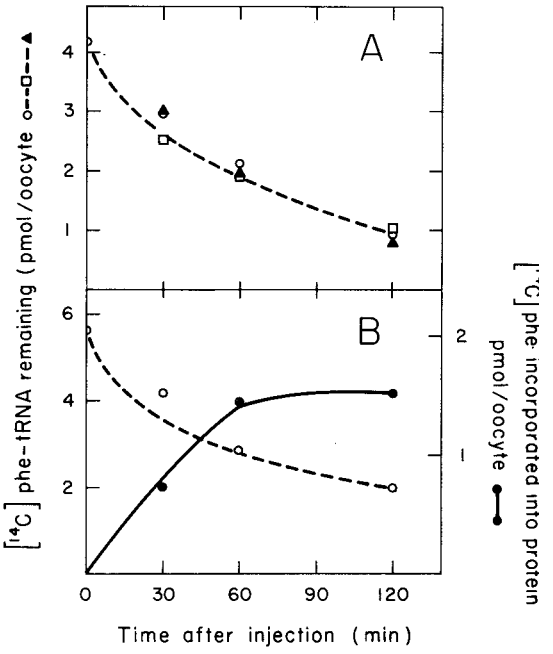

Fig. 3. Deacylation and aminoacyl transfer from [^{14}C]-phe-tRNA injected in oocytes. Duplicate groups of 5 oocytes each were injected with 4.5 (in A) and 5.5 (in B) pmol of [^{14}C]-phe-tRNA per oocyte and incubated for the times indicated. Deacylation of injected [^{14}C]-phe-tRNA and incorporation of ^{14}C Phe into oocyte proteins was measured as detailed in Methods. In A, the deacylation of [^{14}C]-phe-tRNA was measured in cocytes incubated in amphibian saline (control 0) and in cells that were pre-incubated for 1 hour prior to injection in the presence of 0.1 nM cycloheximide ( ) or 0.5 mM puromycin ( ) and subsequently incubated in the continued presence of the protein synthesis inhibitors. In B, parallel groups of the same batch of injected oocytes were used to measure deacylation ((o---o) and incorporation into oocyte proteins (●---●).

experiment in which concomitant to the disappearance of radioactivity from the tRNA, measurements were made on the incorporation of the ^{14}C-phenylalanine into proteins as measured by hot trichloroacetic acid precipitable material. In this experiment approximately 30% of the radioactivity that leaves the tRNA is incorporated into oocyte proteins. The remainder of the labelled phenylalanine presumably enters the cellular free amino acid pool.

In experiments in which different amounts of ^{14}C-phe-tRNA are injected into the oocyte, different proportions of the radioactivity that is deacylated from the tRNA is transferred into proteins. Injecting low amounts of ^{14}C-phe-tRNA (2 pmoles/oocyte), it has been possible to obtain as high as 64% of label transferred into protein in 1 hour (not shown).

In experiment 1 of Table III, it is observed that puromycin and cycloheximide are also potent inhibitors of the transfer or radioactivity from injected ^{14}C-phe-tRNA into oocyte proteins.

TABLE III

Conditions Affecting the Transfer of Amino Acid from tRNA to Protein in Microinjected oocytes

Experiment	[^{14}C] *Phe-tRNA microinjected*	*Treatment*	[^{14}C] *Phe incorp. into protein*	
	pmol		pmol	
			1 hr	2 hrs
1.	12	None	—	4.7
	12	+ Cycloheximide	—	0.6
		+ Puromycin	—	0.3
2.	3.7	None	0.9	1.4
	3.8	Coinjection of [^{12}C] Phe	0.7	0.8

Duplicate groups of 5 oocytes each were injected with [^{14}C] phe-tRNA as indicated and after 1 or 2 hours incubation the amount of [^{14}C] pehylalanine transferred into oocyte protein was determined as detailed in Methods. Where indicated oocytes were preincubated for 1 hour in the presence of 0.1 mM cycloheximide or 0.5 mM puromycin in amphibian saline prior to the injection and subsequently the cells were again incubated in the presence of the protein synthesis inhibitors. In experiment 2, as specified, some oocytes were coinjected with 3.8 pmoles of [^{14}C] phe-tRNA and 4000 moles of [^{12}C] phenylalanine.

The second experiments reported in Table III attempts to answer the question whether the ^{14}C-phenylalanyl moiety injected as ^{14}C-phe-tRNA is transferred directly from the phe-tRNA into the nascent protein or whether it goes through the free amino acid pool before becoming incorporated into the cellular proteins. In this experiments ^{12}C-phenylalanine at a concentration 75-100 times larger that that of ^{14}C-phe-tRNA was co-injected with the radioactive compound. If the radioactive amino acid were deacylated from the tRNA and entered the free amino acid pool before becoming incorporated into protein under these conditions it would suffer such a large dilution of the label that no incorporation of radioactivity should be observed. This is clearly not the case because most of the radioactivity still labels the protein in the oocytes co-injected with the cold amino acid. At longer incubation periods, when deacylation increases, the inhibition caused by the ^{12}C-phe-co-injection upon incorporation of radioactivity into protein also increases as would be expected.

These experiments demonstrate that tRNAs introduced into these cells are not only functional in their capacity to be aminoacylated but also are operative in the endogenous protein synthesis machinery of the oocyte.

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