Subcellular localization of the tRNA processing enzyme, tRNA nucleotidyltransferase, in *Xenopus laevis* oocytes and in somatic cells*

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ABSTRACT

Earlier studies of the tRNA processing enzyme, tRNA nucleotidyltransferase, in rat liver cells suggested that the enzyme was absent from nuclei, whereas more recent studies with tRNA transcription-processing systems from *Xenopus laevis* oocytes indicated that the enzyme was present in germinal vesicles of these cells. In order to resolve this apparent discrepancy, the subcellular localization of tRNA nucleotidyltransferase in oocytes was directly determined, and its distribution in somatic cells was re-examined. Oocyte germinal vesicles, isolated manually, contained about 25% of the total tRNA nucleotidyltransferase activity of the cell, but the enzyme rapidly leaked out of these nuclei during storage. In contrast, purified nuclei isolated from rat or *Xenopus* liver using a variety of aqueous procedures were devoid of tRNA nucleotidyltransferase activity. Since rapidly-isolated, crude nuclear preparations from liver contained substantially more of the enzyme than could be accounted for by cytoplasmic contamination, it appears that the enzyme leaks out of the somatic cell nuclei during the lengthy times required for their purification. The implications of these results for the development of tRNA processing systems from somatic cell nuclei are discussed.

INTRODUCTION

All eukaryotic tRNA genes sequenced to date lack the common -C-C-A sequence found at the 3' terminus of the mature tRNA (1). Thus, these residues must be added post-transcriptionally during processing of tRNA precursors, presumably by the enzyme, tRNA nucleotidyltransferase (2). An important question related to tRNA maturation in eukaryotic cells has to do with the intracellular location of this process. Since -C-C-A addition is a common feature of tRNA processing in all eukaryotic systems, and is carried out by a well-characterized enzyme, elucidation of the subcellular localization of tRNA nucleotidyltransferase can serve as a useful marker for determination of the cellular site(s) of tRNA processing.

An early study of tRNA nucleotidyltransferase distribution in rat liver cells (3) indicated that the enzyme was present in both the cytoplasm and mitochondria of these cells, but was absent from nuclei, although leakage

from the latter organelle during preparation was considered a possibility. In recent years, germinal vesicle (nuclear) extracts prepared from Xenopus laevis oocytes have been used to study tRNA transcription and processing in vitro (4-6). This system is capable of synthesizing and processing tRNA precursors, including -C-C-A addition, implying the presence of tRNA nucleotidyltransferase in the nucleus. Microinjection of cloned tRNA genes into intact Xenopus oocytes has also suggested that tRNA processing occurs in the nucleus (7,8). On the other hand, direct injection of tRNA-C-C into the cytoplasm and nucleus of oocytes indicated that both compartments could repair the 3' end of tRNA (8,9), although redistribution of the tRNA after injection was not ruled out.

In view of the apparent differences found between oocytes and liver cells in the location of tRNA nucleotidyltransferase, and the importance of this issue for determining whether the nuclear tRNA processing found in oocytes also applies to somatic cells, we felt it would be worthwhile to directly determine the localization of tRNA nucleotidyltransferase in oocytes, and to re-examine this question in somatic cells. Our results indicate that tRNA nucleotidyltransferase is present in both the cytoplasmic and nuclear compartments of mechanically-fractionated Xenopus oocytes, but that it readily leaks out of nuclei during storage. In contrast, tRNA nucleotidyltransferase was essentially undetectable in purified Xenopus or rat liver nuclei isolated under a variety of conditions, but this was most likely due to leakage during preparation of the nuclei.

MATERIALS AND METHODS

Materials

[¹⁴C]ATP, [¹⁴C]CTP and yeast tRNA were obtained from Schwarz/Mann. Unlabeled nucleoside triphosphates, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF) and polyvinylpyrrolidone (PVP) were from Sigma. Hexylene glycol (2-methyl-2,4-pentanediol) was purchased from Baker Chemical Co. All salts were reagent grade. Rabbit liver tRNA-C-C and tRNA-C were prepared by periodate oxidation and alkaline phosphatase treatment as described earlier (10).

Adult female Xenopus laevis were obtained from Nasco, Fort Atkinson, Wisconsin.

Isolation of oocyte germinal vesicles

The germinal vesicles were isolated from washed stage VI oocytes (11) by puncturing and squeezing the oocytes according to the procedure of Merriam and Hill (12) using the J buffer described by Birkenmeier et al. (13) except

that glycerol was omitted (70 mM NH_4Cl , 7 mM MgCl_2 , 0.1 mM EDTA, 2.5 mM dithiothreitol, 10 mM Hepes, pH 7.4). The cleaned germinal vesicles were stored in the same buffer at a concentration of 1-2 per μl . Using this procedure, about 80 germinal vesicles could be isolated in 30 min.

Preparation of oocyte fractions for tRNA nucleotidyltransferase assay

The total oocyte fraction was prepared by gently homogenizing oocytes with a Potter-Elvehjem homogenizer in the presence of J buffer, followed by centrifugation at 12,000 xg for 30 min. The supernatant fraction from this procedure was used as the source of oocyte enzyme. Isolated germinal vesicles were ruptured by centrifugation in an Eppendorf Zentrifuge 3200 for 15 sec at 4°C. The supernatant fraction from this centrifugation was used as the source of nuclear enzyme. The pellet, which represented nuclear membrane and nuclear gel (12) was devoid of tRNA nucleotidyltransferase activity. The enucleated oocytes remaining after removal of the germinal vesicles were used as the source of cytoplasmic enzyme. All fractions were tested for tRNA nucleotidyltransferase activity within a few hours of preparation.

Determination of tRNA nucleotidyltransferase leakage from oocyte nuclei

Duplicate batches of about 40 germinal vesicles each were rapidly prepared in about 30 min, and suspended in J buffer without glycerol to give 100 μl of suspension. Samples were stored in ice in a plastic centrifuge tube. After the nuclei had settled, a 20 μl aliquot of the supernatant fluid from each tube was taken as the zero time sample to correct for leakage during preparation. Separate batches were prepared for each time point beginning with the longest incubation. At the indicated times the suspensions were gently stirred and a 20 μl aliquot of the supernatant again taken for assay. Total activity in each tube was determined from an aliquot of the nuclear suspension ruptured and assayed as described above. The percent leakage was calculated from the activity of the supernatant and the activity of the total suspension after correcting each value for the activity of the zero time sample. No inactivation of the enzyme occurred during the course of the experiment.

Preparation of somatic cell nuclei

Crude nuclear fractions were prepared by homogenizing 3 to 10 g of rat liver in a Potter-Elvehjem homogenizer in 5 volumes of the indicated medium. The homogenate was filtered through cheese cloth, and the filtrate centrifuged at 600 xg for 10-12 min. The supernatant fraction was again centrifuged under the same conditions, and the two pellets were resuspended in the homogenizing medium and centrifuged as above. The washed, crude nuclear pellets were resuspended in homogenizing medium, and used directly for assay. In some ex-

periments the crude nuclear fraction was further purified in 2.3 M sucrose by the method of Chauveau *et al.* (14). Details of the various homogenizing media used are in Table 1.

Xenopus liver nuclei were prepared under the same conditions using a homogenizing medium consisting of 0.3 M sucrose, 20 mM Tris-Cl, pH 7.5, 75 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂.

Enzyme assays

tRNA nucleotidyltransferase activity was determined by incorporation of [¹⁴C]ATP or [¹⁴C]CTP into acid-precipitable material as described previously (10). Reaction mixtures contained in 0.2 ml: 50 mM glycine-NaOH, pH 9.4, 5 mM MgCl₂, 150 µg of the appropriate tRNA acceptor and 0.5 mM radioactive nucleoside triphosphate (~ 100 cpm per nmol).

RESULTS

Location of tRNA nucleotidyltransferase in oocytes

An earlier study examining repair of tRNA-C-C microinjected into the cytoplasm and nucleus of oocytes suggested that tRNA nucleotidyltransferase might be present in both cellular compartments (8); however, the actual cellular site of the AMP addition to the tRNA was only inferred since redistribution of the tRNA could have taken place. In order to determine whether tRNA nucleotidyltransferase was present in each compartment and to quantitate the distribution of the enzyme in the cell, oocytes, enucleated oocytes and isolated nuclei were each assayed directly for tRNA nucleotidyltransferase activity.

The data in Fig. 1 show that tRNA nucleotidyltransferase activity is present in both the nucleus and cytoplasm (enucleated oocytes) of Xenopus oocytes. Quantitation of AMP and CMP incorporation indicated that the activity in these two compartments accounts for all the activity detectable in an oocyte extract (Table I). In the experiment shown in Table I, 21% of the total tRNA nucleotidyltransferase activity, based on AMP or CMP incorporation, was found in the nucleus. In 5 experiments this percentage varied from 17-28% with an average of 23% of AMP-incorporating activity being found in the nucleus. Likewise, 20% of the CMP-incorporating activity was found in the nuclear fraction.

Both AMP and CMP incorporation in each of the cellular fractions was dependent on the addition of a tRNA substrate (Fig. 2, only intact oocytes shown), indicating that the reaction measured was, in fact, due to tRNA nucleotidyltransferase. This was confirmed by alkaline hydrolysis of the

Fig. 1. Distribution of tRNA nucleotidyltransferase between nucleus and cytoplasm in *Xenopus* oocytes. Oocytes, enucleated oocytes and nuclei (germinal vesicles) were isolated and assayed as described in "Methods". Assays were for 30 min at 37° using [14 C]ATP and yeast tRNA as substrates. The amount of intact oocytes, enucleated oocytes and nuclei have been normalized to the number of cell equivalents. Oocytes (■—■), enucleated oocytes (○—○), nuclei (●—●).

Table 1. Subcellular distribution of tRNA nucleotidyltransferase in *Xenopus* oocytes

Fraction	AMP incorporation		CMP incorporation	
	Activity (milliunits)	Specific activity (milliunits/ μ g)	Activity (milliunits)	Specific activity (milliunits/ μ g)
Oocyte	58	4.3	62	4.6
Enucleated oocyte	46	4.5	46	4.5
Germinal vesicle (nucleus)	12	8.0	13	8.7

Intact oocytes, enucleated oocytes and germinal vesicles were isolated and assayed as described in "Methods". Assays were for 15 min at 37° using [14 C]ATP and liver tRNA-C-C or [14 C]CTP and liver tRNA-C. tRNA nucleotidyltransferase activity was calculated from an experiment like that shown in Fig. 1. Each value represents the average of assays carried out at three different amounts of fraction. Activities have been normalized to that in one cell or cell equivalent. Specific activities have been calculated based on 13.5 μ g protein per oocyte, 10.2 μ g protein per enucleated oocyte, and 1.5 μ g protein per germinal vesicle in the fractions prepared for assay.

Fig. 2. Dependence of AMP incorporation in oocyte extracts on tRNA. Oocytes were prepared and assayed as described in "Methods" in the presence and absence of tRNA-C-C and [^{14}C]ATP. Assays were for the indicated times at 37° using the equivalent of 8 oocytes.

tRNA product after AMP incorporation which showed that approximately 95% of the incorporated radioactivity was present as a single residue at the 3' terminus. Since the direct isolation procedure for oocyte germinal vesicles should not lead to redistribution of proteins, these results demonstrate that tRNA nucleotidyltransferase is present in both the nuclear and cytoplasmic compartments of oocytes. In addition, its level in the nucleus indicates that the enzyme is enriched in this organelle (see Discussion).

Localization of tRNA nucleotidyltransferase in somatic cells

A previous examination of the subcellular distribution of tRNA nucleotidyltransferase in rat liver cells suggested that the enzyme was absent from the nuclei of this tissue (3). However, our current results with oocytes, and the possibility of protein leakage during the earlier preparations of liver nuclei, prompted us to re-examine the rat liver system using a variety of conditions for isolation of nuclei. Generally, crude nuclear fractions, prepared by two cycles of low-speed centrifugation (see "Methods"), were assayed for tRNA nucleotidyltransferase activity. Assay of the crude nuclear fraction was used to ascertain an upper limit for nuclear activity since these fractions were prepared relatively rapidly. In some cases the crude nuclear fractions were further purified through 2.3 M sucrose and assayed in order to determine the enzyme level in purified nuclei.

The data in Table II indicate, in agreement with earlier results (3), that purified rat liver nuclei are essentially devoid of tRNA nucleotidyltransferase activity. On the other hand, crude nuclei prepared in a variety of media contained between 10 and 20% of the activity originally present in the homogenate. Since the crude nuclear fractions had been washed with the homogenization buffer, it is unlikely that the high levels of enzyme activity present in this fraction were due solely to cytoplasmic contamination.

Table 2. tRNA nucleotidyltransferase in rat liver nuclei prepared in a variety of homogenization media

Homogenization medium	Enzyme distribution	
	Crude nuclei	Purified nuclei
		%
1. 0.25 M Sucrose, 1 mM MgCl ₂ , 1 mM CaCl ₂	10	1
2. 10 mM Tris-Cl (pH 7.5), 15 mM MgCl ₂ , 5 mM CaCl ₂ , 80 mM NaCl, 120 mM KCl, 0.1 mM DTT	11	-
3. 10 mM Tris-Cl (pH 7.3), 10 mM MgCl ₂ , 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol	17	2
4. 30 mM Tris-Cl (pH 7.5), 5 mM MgCl ₂ , 25 mM KCl, 1 mM DTT, 25% glycerol	8	-
5. 0.1 M Tris-Cl (pH 7.4), 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol	14	-
6. 10 mM Tris-Cl (pH 7.4), 10 mM MgCl ₂ , 0.1 mM DTT, 0.1 mM EDTA, 15% Ficoll	21	3
7. 10 mM Tris-Cl (pH 7.4), 10 mM MgCl ₂ , 0.1 mM DTT, 0.1 mM EDTA, 10% hexylene glycol	16	2
8. 20 mM Tris-Cl (pH 7.4), 2 mM MgCl ₂ , 1 mM DTT, 2% PVP	-	3

Crude and purified nuclear fractions were prepared in the indicated medium as described in "Methods". The amount of nuclear activity in each case is expressed as a percentage of the activity in the original homogenate.

Nevertheless, in order to be sure that the different results between Xenopus oocytes and rat liver were not due to species differences, purified nuclei were also isolated from Xenopus liver. Several experiments of this type indicated that the level of tRNA nucleotidyltransferase in Xenopus liver nuclei was also 3% or less of that in the homogenate (data not shown). Thus, the difference between oocytes and liver was not due to the species under study, but either to an intrinsic difference between nuclei from oocytes and somatic cells, or to the differences in nuclear isolation procedures used for the two types of cells. Unfortunately, it was not possible to use non-aqueous procedures for isolation of liver nuclei because these methods led to inactivation of tRNA nucleotidyltransferase (data not shown).

Leakage of tRNA nucleotidyltransferase from oocyte nuclei

In order to test whether the presence of tRNA nucleotidyltransferase in oocyte nuclei might be due to a more stable association of the enzyme with a nuclear structure in this system compared to liver, the kinetics of leakage of the enzyme from germinal vesicles was determined. The data in Fig. 3 show that tRNA nucleotidyltransferase leaks out of oocyte nuclei quite rapidly, with 50% leakage in about 2 hours and close to 90% leakage in 5 hours. Microscopic examination of the germinal vesicles throughout the experiment indicated that the leakage was not due to rupture of the organelle. Inclusion of 2% polyvinylpyrrolidone in the storage buffer decreased leakage from about 70% to 30% over a period of 3 hours (Fig. 3). Thus, these data suggest that the ability to isolate oocyte nuclei containing tRNA nucleotidyltransferase is not due to an intrinsically stable association of the enzyme with this organelle.

DISCUSSION

The data presented in this paper directly demonstrate that tRNA nucleotidyltransferase is present in both the cytoplasmic and nuclear compartments of Xenopus oocytes, and is in agreement with the conclusion reached from micro-injection studies (8). tRNA nucleotidyltransferase is thought to be essential for eukaryotic tRNA biosynthesis, and presumably serves this function in the nucleus (2). Surprisingly, about three-fourths of the detectable activity was found in the cytoplasm. It is known that tRNA nucleotidyltransferase is involved in the 3' repair of tRNA in many systems (2), and this process would be expected to occur in the cytoplasm where the functioning tRNA is located. However, end-turnover of the 3' terminus is generally a slow process (2), and it is curious that such a high proportion of the total

Fig. 3. Leakage of tRNA nucleotidyltransferase from oocyte nuclei. Leakage from nuclei was determined as described in "Methods" in duplicate samples. Activity was determined as in Fig. 1. At each time point the percentage leakage is expressed relative to the total activity in the same sample of nuclei, and is the average of duplicate batches of germinal vesicles. The point designated (+ PVP) was in the presence of 2% polyvinylpyrrolidone.

tRNA nucleotidyltransferase activity is found in the cytoplasm. Perhaps, some fraction of tRNA molecules complete their maturation in the cytoplasm, rather than the nucleus, or tRNA end-turnover may be more rapid in this system.

On the other hand, the finding that about 25% of the detectable tRNA nucleotidyltransferase activity is found in the nucleus represents an enrichment of the enzyme in this organelle. Since the oocyte nucleus is only 4% of the cell volume (11), the enrichment may be as much as six-fold. If one takes into account the yolk platelet volume, which is thought to be inaccessible to protein, the enrichment based on volume is still two-to-three-fold (15). Likewise, the specific activity of tRNA nucleotidyltransferase in the nucleus was about twice that of the cytoplasm (Table I). One wonders what mechanism could account for this distribution of tRNA nucleotidyltransferase between nucleus and cytoplasm! The rapid leakage of the enzyme from isolated nuclei suggests that it is not firmly bound to a nuclear structure, as has been proposed for some nuclear proteins (12,15-18). One possibility, of course, is that the nuclear and cytoplasmic enzymes are distinct proteins with specificities for the different cell compartments. Purification of the two enzymes to test this possibility would be worthwhile.

These studies also help to clarify the apparent differences between oocytes and somatic cells with respect to tRNA nucleotidyltransferase distri-

bution, in particular, and tRNA processing, in general. We suspect that the difference between oocyte and liver nuclei simply reflects the time required to isolate nuclei from each source. Oocyte nuclei, which can be isolated without resorting to centrifugation procedures, can be obtained in minutes and retain tRNA nucleotidyltransferase, although the enzyme will leak out quite rapidly upon storage of the nuclei. In contrast, isolation procedures for liver nuclei require several hours, large volumes and changes of supporting medium which would promote leakage. In fact, assays of crude liver nuclear fractions, which are prepared relatively rapidly, revealed substantial levels of activity which could not be accounted for by equally high levels of cytoplasmic contamination. In addition, since the nuclear volume of somatic cells is proportionately much lower than that in oocytes, it is not clear what percentage of total nucleotidyltransferase would be expected in a nuclear preparation. Development of a suitable non-aqueous procedure which retains tRNA nucleotidyltransferase activity should resolve this issue conclusively.

Finally, it should be pointed out that the problem of nuclear leakage during preparation of somatic cell nuclei may make development of a complete transcription-processing system from these cells a difficult undertaking. Since many of the processing enzymes may not be tightly-bound to nuclear structures and may leak out of nuclei easily, it might not be possible to retain a complete processing pathway in the nucleus. In fact, just such a leakage of a tRNA processing nuclease has been observed upon washing of yeast nuclei (19). However, in the case of a largely cytoplasmic Hela cell transcription system, it has been suggested that the deficiency of tRNA processing in this system may be due to processing nucleases remaining in the nucleus (20). Obviously, further work on the intracellular location of tRNA processing enzymes would be of interest.

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