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Active Aminoacyl-tRNA Synthetases Are Present in Nuclei as a High Molecular Weight Multienzyme Complex*

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Recent studies suggest that aminoacylation of tRNA may play an important role in the transport of these molecules from the nucleus to the cytoplasm. However, there is almost no information regarding the status of active aminoacyl-tRNA synthetases within the nuclei of eukaryotic cells. Here we show that at least 13 active aminoacyl-tRNA synthetases are present in purified nuclei of both Chinese hamster ovary and rabbit kidney cells, although their steady-state levels represent only a small percentage of those found in the cytoplasm. Most interestingly, all the nuclear aminoacyl-tRNA synthetases examined can be isolated as part of a multienzyme complex that is more stable, and consequently larger, than the comparable complex isolated from the cytoplasm. These data directly demonstrate the presence of active aminoacyl-tRNA synthetases in mammalian cell nuclei. Moreover, their unexpected structural organization raises important questions about the functional significance of these multienzyme complexes and whether they might play a more direct role in nuclear to cytoplasmic transport of tRNAs.

Aminoacyl-tRNA synthetases catalyze the first step in protein biosynthesis, the attachment of an amino acid to its cognate tRNA (1). In higher eukaryotes the aminoacyl-tRNA synthetases are part of a highly organized translation system (2, 3) and can be isolated from cells as a high molecular weight multienzyme complex (4–7). The number of synthetases in this fragile complex varies in different laboratories, but a stable core of nine of these enzymes plus three non-synthetase proteins can be isolated reproducibly (5–7). Evidence has accumulated that the multienzyme synthetase complex reflects associations among these proteins that pre-exist *in vivo* (8–10).

Despite the fact that protein synthesis takes place in the cytoplasm of eukaryotic cells, several studies suggested that aminoacyl-tRNA synthetases might also be present in nuclei. Thus, in early work, aminoacyl-tRNA synthetase activities could be detected in crude nuclear fractions (11, 12); however, it could be argued that these activities were due to cytoplasmic adherence to the outer nuclear surface. Aminoacyl-tRNA synthetases, as well as elongation factor 1 (EF1),[1] also could be detected in nuclei by immunochemical methods (13–16). However, these studies provided no information as to whether the nuclear-localized synthetases were active. Nuclear localization was also suggested by the identification of possible nuclear localization signals in yeast aminoacyl-tRNA synthetases (17).

Recently, nuclear aminoacyl-tRNA synthetases have attracted considerable interest because of their potential involvement in nuclear to cytoplasmic transport of tRNA. Exclusion of defective tRNA from the cytoplasm was shown to be due to nuclear proofreading, and it was proposed that aminoacylation of tRNA serves as this proofreading step (18–20). While there is still some discussion whether aminoacylation or binding to the nuclear export receptor, exportin-t, is actually responsible for proofreading, there is agreement that nuclear aminoacylation increases tRNA export efficiency (21, 22).

In light of these findings, it was of interest to carefully examine nuclei for the presence of active aminoacyl-tRNA synthetases and to determine what percentage of total cellular synthetase activity might reside in the nucleus. The data reported here directly demonstrate that active aminoacyl-tRNA synthetases are present in the nuclei of two different mammalian cell lines. Most importantly, we find that the nuclear aminoacyl-tRNA synthetases are organized into a high molecular weight, multienzyme complex that is even more stable than the complex present in the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials—^{3}H-Labeled arginine, proline, leucine, glycine, histidine, valine, and ^{14}C-labeled isoleucine, phenylalanine, lysine, and threonine were purchased from NEN Life Science Products. ^{3}H-labeled aspartic acid, serine, and tryptophan were from Amersham Pharmacia Biotech. Complete™ Protease Inhibitor Cocktail Tablets were from Roche Molecular Biochemicals. Cell culture reagents were from Life Technologies, Inc. Rabbit kidney (LCC-RK1) cells and Chinese hamster ovary (CRL-1781) cells were obtained from the American Type Culture Collection. Rabbit liver tRNA was prepared as described previously (2).

Cell Culture—Rabbit kidney cells were cultured as described previously (8). CHO cells were maintained as monolayers in α minimum essential medium containing ribonucleosides and deoxyribonucleosides and supplemented with 10% fetal bovine serum. Cells were cultured in Nunc flasks at 37 °C in air containing 5% CO_2 and were transferred every 2–3 days. Cells were harvested at 80–90% of confluence by incubation with warm phosphate-buffered saline (PBS) supplemented with 0.53 mM EDTA. The cells were washed twice with ice-cold PBS, resuspended in sucrose buffer (10 mM Tris-HCl, pH 8.0, containing 0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, and protease inhibitors (1 tablet/50 ml of solution)) and counted in a hemacytometer. The cell suspension was immediately supplemented with an equal volume of the same buffer containing 1% Nonidet P-40 for aminoacyl-tRNA synthetase assays.

Preparation of S10 Supernatant (Cytoplasmic) Fractions—After harvesting and washing in PBS, the rabbit kidney cell pellet was quickly frozen in an ethanol-dry ice bath and thawed in Buffer A (20 mM HEPES-KOH buffer, pH 7.4, containing 0.5 mM spermine, 130 mM KCl, 1% thiodiglycol, 0.5 mM EDTA, 2 mM $CaCl_2$, and protease inhibitors). The cells in this buffer were incubated on ice for 15 min with occasional stirring. The crude lysate, prepared in this manner, was centrifuged at 10,000 × *g*, and the supernatant fraction was used for gel filtration. The cytoplasmic fraction of CHO cells was prepared in the same way except that the cells were thawed in the sucrose buffer.

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[1] The abbreviations used are: EF1, elongation factor 1; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

TABLE I

Activities of aminoacyl-tRNA synthetases in nuclear fractions of CHO and rabbit kidney cells

Cells were prepared as described under "Experimental Procedures." Nuclei after isolation were resuspended in sucrose buffer and counted in a hemacytometer. Approximately 4×10^4 cells or 2×10^6 to 8×10^6 nuclei were assayed for aminoacyl-tRNA activities for 2 min. A unit of activity corresponds to the formation of 1 nmol of aminoacyl-tRNA/min at 37 °C.

Synthetase	Aminoacyl-tRNA synthetase activity					
	CHO cells			Rabbit kidney cells		
	Total	Nuclear	Amount in nuclei	Total	Nuclear	Amount in nuclei
	units/10^9 cells or nuclei		%	*units/10^9 cells or nuclei*		%
Arginyl-	130	4.1	3.2	200	1.3	0.67
Isoleucyl-	260	3.5	1.3	220	0.71	0.32
Leucyl-	110	2.7	2.4			
Lysyl-	210	6.5	3.1	260	1.7	0.67
Prolyl-	98	2.8	2.9			
Threonyl-	84	0.33	0.40	160	0.24	0.15

Nuclear Isolation—After harvesting, the frozen pellet of rabbit kidney cells was thawed in ice-cold Buffer A containing 0.1% Nonidet P-40. Cells at a concentration of $\sim 2 \times 10^7$ cells/ml were disrupted in a Dounce-type tissue homogenizer (Wheaton) using five strokes of the B pestle. The cell lysate was spun down for 5 min at $1000 \times g$, and the same procedure (resuspension, homogenization and centrifugation) was repeated with the pellet. The final crude nuclear pellet was suspended in sucrose buffer containing 0.5% Nonidet P-40. This suspension was mixed with an equal volume of 10 mM Tris-HCl, pH 8.0, containing 2.2 M sucrose, 5 mM magnesium acetate, and 0.1 mM EDTA. Purified nuclei were collected by centrifugation through the sucrose solution using 2.2 M sucrose as a cushion (23).

The same procedure was used for isolation of CHO cell nuclei except that the cell pellet was thawed directly in sucrose buffer, and after five strokes with the Dounce homogenizer the procedure described in Ref. 23 was followed directly. Sucrose at 1.9 M was used for the cushion.

Aminoacylation Assay—Aminoacyl-tRNA synthetase activity assays were carried out at 37 °C in reaction mixtures containing: 250 mM Tris-HCl, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mg/ml bovine serum albumin, 1.5 mg/ml rabbit liver tRNA, 0.1 mM ^{3}H- or ^{14}C-labeled amino acid (~20–100 cpm/pmol), and sufficient cell extract, nuclear extract, or column fraction to measure significant synthetase activity within the linear range. Reactions were stopped by the addition of 10% trichloroacetic acid containing 0.5% casamino acids (Difco). Aminoacyl-tRNA precipitates were collected and counted as described previously (4). In this study we did not attempt to optimize the assay conditions for each amino acid in terms of pH, ionic strength, cation requirements, etc. Rather, the same assay conditions were used for measuring all of the aminoacyl-tRNA synthetase activities.

Immunobloting Procedure—After electrophoresis on 8% polyacrylamide gels (24, 25), proteins were transferred to a polyvinylidene difluoride membrane in Tris-glycine buffer (0.375 M Tris, 0.192 M glycine, 20% methanol). Blocking of the membrane was with a 5% solution of nonfat milk in TBS buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl) for 2 h at room temperature. The membrane was then treated with monoclonal antibody F7 directed against rabbit arginyl-tRNA synthetase (8) in TBS buffer supplemented with 0.2% Tween 20 (2 μg of antibody/10 ml of buffer) overnight at 4 °C with shaking. Visualization of the protein bands followed the ECL Western blotting protocol (Amersham Pharmacia Biotech) using, as the secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase.

RESULTS AND DISCUSSION

Purity of Nuclei—To ensure that the purified nuclei were free of contamination, nuclei at each step of the purification procedure were stained with azure C and examined by light microscopy (26). Cytoplasmic adherence was clearly visible in crude nuclei, but after purification, no cytoplasmic contamination was evident. Moreover, assay of the cytoplasmic marker enzyme, lactate dehydrogenase, confirmed that the purified nuclei were devoid of cytoplasm. Thus, CHO and rabbit kidney cell extracts contained approximately 600 and 300 units of lactate dehydrogenase activity/10^6 cells, respectively (1 unit = 1 μmol NADH formed per min). Lactate dehydrogenase activity in nuclei from each of these sources was below the level of detection (25 millinunits per 10^6 nuclei) or <0.01%.

Purified nuclei were also assayed for the possibility of mitochondrial contamination. The amounts of citrate synthase and cytochrome *c* oxidase in nuclei from each cell were again below the level of detection representing <0.2% of that present in detergent-treated cell extracts.

Most importantly, mammalian cell cytoplasmic extracts are known to contain two forms of arginyl-tRNA synthetase, one present in the multienzyme complex and one free form (27, 28). As will be shown below, the low molecular weight form is absent from the nuclear preparations. These data all support the conclusion that the nuclear preparations used for these studies were free of contamination.

Quantitation of Nuclear Aminoacyl-tRNA Synthetase Activity—Total cellular aminoacyl-tRNA synthetase activity, determined by assay of lysates prepared by Nonidet P-40 treatment, and total nuclear activity, determined in nuclear lysates, were compared for the two cell lines (Table I). Nuclear aminoacyl-tRNA synthetase activity clearly was present in both cells. However, in each case, it represented only a small percentage of the total cellular activity, amounting to about 2–3% for the CHO cells and less than 1% for the rabbit kidney cells.

There are several possible explanations for the higher percentage of nuclear activity in CHO cells compared with rabbit kidney cells. One reason may be the difference in growth rate of the two cells (10 h doubling time for CHO cells and 45 h for rabbit kidney cells). The higher growth rate for CHO cells would necessitate a greater flux of tRNA from nucleus to cytoplasm and consequently an increased requirement for nuclear aminoacyl-tRNA synthetases. Second, rabbit kidney cells are much more difficult to disrupt, and isolation of their nuclei also requires a greater period of time. This may result in increased nuclear breakage and/or leakage of nuclear synthetases during the isolation procedure. Nevertheless, these data show that active aminoacyl-tRNA synthetases are present in mammalian nuclei, although at a relatively low level.

Structural Organization of Nuclear Aminoacyl-tRNA Synthetases—Many cytoplasmic aminoacyl-tRNA synthetases are found in extracts as part of a multienzyme complex (4–7). It was of interest, therefore, to ascertain whether the corresponding nuclear enzymes might also be part of a complex or whether complex formation is only a cytoplasmic phenomenon. For this purpose, the size distribution of 13 aminoacyl-tRNA synthetases in cytoplasmic and nuclear extracts were examined by gel filtration on Sephacryl S-400. Since very similar patterns were obtained for rabbit kidney and CHO cell extracts, only those relating to CHO cells will be presented here. Prior to chromatography, the nuclear fraction was concentrated so that the amounts of nuclear and cytoplasmic synthetase activities loaded on the column would be more comparable. Assay conditions (amount of column fraction and time of assay) were also adjusted to ensure that the levels of nuclear and cytoplasmic activities would be similar. Treatment of the rabbit kidney

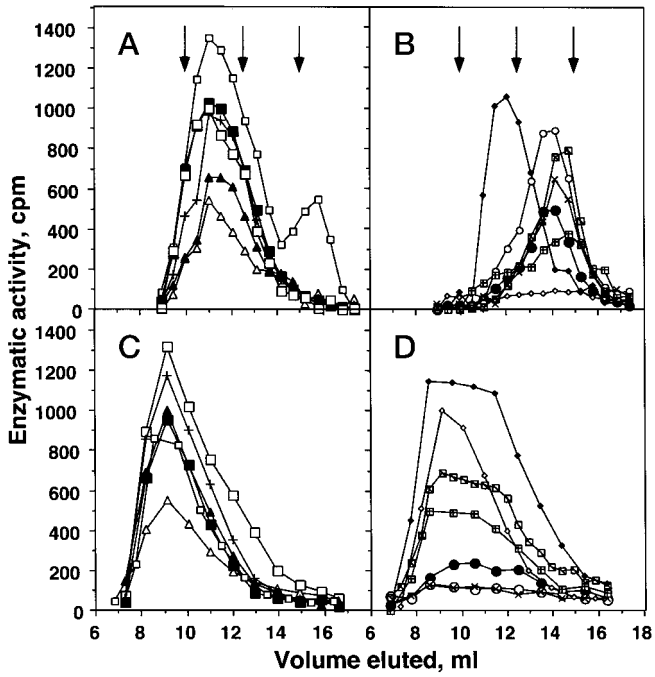

FIG. 1. **Gel filtration on Sephacryl S400 of the cytoplasmic and nuclear fractions of CHO cells.** Cytoplasmic fraction was prepared as described under "Experimental Procedures." The nuclear fraction was resuspended in the protein extraction buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol (v/v), 0.4 M NaCl, 1 mM dithiothreitol, and 1 unit/μl RNasin (Promega). After 1 h of shaking at 4 °C, the nuclear fraction was centrifuged at 18,000 × *g* for 15 min. The supernatant fraction, containing more than 90% of the nuclear aminoacyl-tRNA synthetase activities, was concentrated by ultrafiltration on a Diaflo YM30 membrane. The cytoplasmic fraction (1.3–1.5 mg) or the concentrated nuclear extract (5.5–6.0 mg), 170 μl of each, was applied to a column (0.7 × 50 cm) of Sephacryl S400 equilibrated with 50 mM Tris-HCl, pH 7.5, 10% glycerol (v/v), 0.2 mM dithiothreitol, 0.2 mM EDTA, and 100 mM NaCl and eluted at a flow rate of 2.5 ml/h. Fractions of 0.5 ml were collected. Cytoplasmic fractions (20 μl) were assayed for 10 min, and nuclear fractions (60 μl) were assayed for 20 min. *A* and *B*, cytoplasmic fraction; *C* and *D*, nuclear fraction. *A* and *C*, aminoacyl-tRNA synthetases specific for arginine (□), aspartic acid (▲), isoleucine (■), leucine (□), lysine (+), proline (△); *B* and *D*, aminoacyl-tRNA synthetases specific for glycine (○), histidine (×), phenylalanine (◇), serine (*open box with slash*), threonine (●), tryptophan (⊞), valine (◆). *Arrows* indicate the elution volumes of the size markers (blue dextran (2×10^3 kDa), thyroglobulin (650 kDa), and catalase (250 kDa) from *left* to *right*) determined in a separate run. According to the manufacturer, the exclusion limit of Sephacryl S400 is 8×10^6. Based on extrapolation from the other standards, V_o would be at 6.3 ml.

nuclear extract with DNase I (1200 units/ml for 90 min) did not alter the elution profiles (data not shown).

The size distribution of cytoplasmic synthetase activities is presented in Fig. 1, *A* and *B*. Of the 13 activities assayed, aminoacyl-tRNA synthetases specific for arginine, aspartic acid, isoleucine, leucine, lysine, and proline co-eluted as a high molecular weight peak with an apparent molecular mass of $\sim 10^6$ Da (Fig. 1*A*), in complete agreement with the known composition of the stable core of the multienzyme complex from CHO cells (13). Arginyl-tRNA synthetase also had a second peak of activity in the low molecular weight region, as expected (27, 28). Of the seven other synthetase activities measured, all except valyl-tRNA synthetase eluted primarily as free proteins, although in some cases shoulders of higher molecular weight forms were also observed (Fig. 1*B*). Valyl-tRNA synthetase is known to form a complex with EF-1H (29) resulting in a higher molecular weight entity, but one that is clearly smaller than the multienzyme synthetase complex (Fig. 1*B*).

The elution profiles of the nuclear aminoacyl-tRNA synthetases are presented in Fig. 1, *C* and *D*. Surprisingly, not

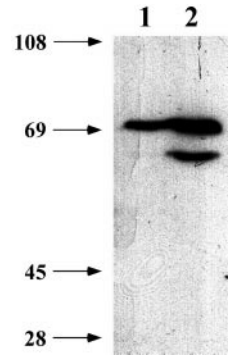

FIG. 2. **Low molecular weight arginyl-tRNA synthetase is not present in the nuclear fraction of rabbit kidney cells.** Cytoplasmic fraction was prepared as described under "Experimental Procedures." After isolation, nuclei were resuspended in sucrose buffer. Immunobloting was carried out with monoclonal antibody F7 (8). The film was overexposed to ascertain the absence of the band corresponding to the low molecular weight form of the arginyl-tRNA synthetase in the nuclear fraction. *Lane 1*, nuclear fraction, corresponding to 150 μg of total protein; *lane 2*, cytoplasmic fraction, corresponding to 20 μg of total protein. The values on the *left* (in kilodaltons) indicate the position of the size standards.

only do the nuclear activities elute as high molecular weight proteins, but more of them do so. The synthetases that normally are found in the cytoplasmic multienzyme complex (Fig. 1*A*), also co-elute in the nuclear fraction, strongly suggesting that they exist as a multienzyme complex in nuclei as well. Moreover, the enzymes that are predominantly found as free forms in the cytoplasm (Fig. 1*B*) elute as much larger entities in the nuclear extract (Fig. 1*D*), with at least a portion of all of them eluting as part of the complex. As a consequence, the nuclear complex is considerably larger than its cytoplasmic counterpart ($\sim 2.5 \times 10^6$ compared with $\sim 1 \times 10^6$ Da).

Additional evidence for the nuclear multienzyme complex comes from immunoprecipitation experiments. Using the monoclonal antibodies against glutamyl-tRNA synthetase (8), we were able to co-immunoprecipitate at least three other aminoacyl-tRNA synthetases (data not shown). These data support the conclusion that the nuclear aminoacyl-tRNA synthetases are physically associated.

Interestingly, the low molecular weight form of arginyl-tRNA synthetase, which is prevalent in the cytoplasmic extract (Fig. 1*A*), is not seen in the nuclear extract (Fig. 1*C*). Furthermore, Western immunoblot analysis of proteins from rabbit kidney nuclei failed to detect any low molecular weight arginyl-tRNA synthetase (Fig. 2). These data confirm the purity of the nuclear preparation and indicate that this form of arginyl-tRNA synthetase is exclusively a cytoplasmic component. It was postulated that the low molecular weight form of arginyl-tRNA synthetase provides arginyl-tRNA for the N-terminal arginylation of proteins targeted for degradation by the ubiquitin-dependent pathway (30). The present data suggest that this process is located only in the cytoplasm.

Multienzyme complexes of aminoacyl-tRNA synthetases have been isolated from a variety of higher eukaryotic cells (5–7). However, the composition of these complexes often varies. A relatively stable complex of nine aminoacyl-tRNA synthetases plus three non-synthetase proteins can be isolated reproducibly, but smaller and larger complexes have also been isolated. This has led to some uncertainty as to whether the multienzyme complex only contains nine synthetases or whether that form of the complex simply represents a more stable core from which the more easily dissociable components have already been removed during preparation. Our findings that nuclei contain a larger, apparently more stable complex suggest that the different sizes of complex obtained from dif-

ferent sources and by different laboratories may, in fact, be due to stability during isolation. It is particularly noteworthy that in the nuclear system the tightly bound core synthetases are still exclusively found in the complex, whereas those usually found as free forms in the cytoplasm are found in the nucleus both in the large complex and in intermediate-sized entities. However, almost no free forms are seen. This argues strongly for partial breakdown of the nuclear complex leading to partial removal of the more easily dissociable synthetases. If this explanation is correct, it suggests that the nuclear and cytoplasmic forms of the multienzyme complex may be assembled into a similar organized structure.

This, of course, raises the interesting question of what might be the function of a nuclear multienzyme aminoacyl-tRNA synthetase complex. The simplest explanation is that these enzymes always remain associated with each other *in vivo,* whether in the cytoplasm or the nucleus, to carry out their function of aminoacylation. Aminoacyl-tRNA is known to be channeled *in vivo* (3, 31), and the existence of aminoacyl-tRNA synthetases in a multienzyme complex may facilitate direct transfer of all aminoacyl-tRNAs to EF1.

Interestingly, EF1 is also present in the nucleus (15, 16), and very recent work indicates that it also participates in nuclear to cytoplasmic transport of tRNA (32). Thus, in this context, the aminoacylation of tRNA and its transfer to EF1 in the nucleus could be completely analogous to the process in the cytoplasm. Alternatively, the multienzyme complex may itself function as a high efficiency tRNA transport machine. In this case, newly synthesized aminoacyl-tRNA synthetases would enter the nucleus, either as free enzymes that assemble into a multienzyme complex or as a preformed complex. Synthetases would then associate with their cognate tRNAs, and multiple tRNAs and synthetases would be transported to the cytoplasm together, perhaps in cooperation with exportin-t (21, 22) or EF1 (32). One advantage of such a model would be to maintain a cytoplasmic ratio of tRNA to cognate synthetase of 1:1 and would result in partial assembly of the cytoplasmic translation apparatus already in the nucleus. If this model were correct, the channeling of tRNA, known to occur in the cytoplasm, would need to be extended to nuclear processes as well. Current work clearly shows a role for aminoacyl-tRNA synthetases in nuclear to cytoplasmic transport of tRNA (18–22). Whether this role is limited only to proofreading by aminoacylation or whether there might also be a role as an actual tRNA transporter remains to be determined.

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