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A channeled tRNA cycle during mammalian protein synthesis

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ABSTRACT **In earlier studies it was shown that the mammalian translation system is highly organized *in vivo* and that the intermediates in the process, aminoacyl-tRNAs, are channeled—i.e., they are directly transferred from the aminoacyl-tRNA synthetases to the elongation factor to the ribosomes without dissociating into the cellular fluid. Here, we examine whether spent tRNAs leaving the ribosome enter the fluid phase or are transferred directly to their cognate aminoacyl-tRNA synthetases to complete a channeled tRNA cycle. Using a permeabilized CHO cell system that closely mimics living cells, we find that there is no leakage of endogenous tRNA during many cycles of translation, and protein synthesis remains linear during this period, even though free aminoacyl-tRNA is known to rapidly equilibrate between the inside and outside of these cells. We also find that exogenous tRNA and periodate-oxidized tRNA have no effect on protein synthesis in this system, indicating that they do not enter the translation machinery, despite the fact that exogenous tRNA rapidly distributes throughout the cells. Furthermore, most of the cellular aminoacyl-tRNA synthetases function only with endogenous tRNAs, although a portion can use exogenous tRNA molecules. However, aminoacylation of these exogenous tRNAs is strongly inhibited by oxidized tRNA; this inhibitor has no effect on endogenous aminoacylation. On the basis of these and the earlier observations, we conclude that endogenous tRNA is never free of the protein synthetic machinery at any stage of the translation process and, consequently, that there is a channeled tRNA cycle during protein synthesis in mammalian cells.**

tRNA plays a central role in translation, acting as the carrier of both the monomeric units of proteins, the amino acids, and the growing polypeptide chains (1). As a consequence of this central role, tRNAs must interact, in turn, with most of the components of the protein-synthesizing machinery—including the aminoacyl-tRNA synthetases, the elongation factors, and the ribosomes. Understanding how tRNA is transferred from one component to the next during its cycling is essential for a complete description of the translation process at the molecular level.

Recent work, using a permeabilized Chinese hamster ovary (CHO) cell system, which closely mimics living cells, has provided strong evidence that the translation apparatus is highly organized *in vivo* (2). Thus, while large molecules can enter these cells, very little RNA or protein leaks out; moreover, only ions and other low-molecular-weight compounds need to be added back to sustain rates of protein synthesis comparable to those in living cells (2). Additional information supporting an organized translation system comes from the isolation of multienzyme complexes and other assemblies containing multiple translation components (3–7), from the interaction of these macromolecules with cytoskeletal elements (8–10), and from colocalization of these components in cells (11–13).

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The conclusion that the translation system is organized has been reinforced by functional studies indicating that protein biosynthesis is a channeled pathway in which aminoacyl-tRNAs are directly transferred from the aminoacyl-tRNA synthetases to the elongation factor to the ribosomes without dissociation into the cellular fluid (14, 15). In addition, endogenous aminoacyl-tRNA in permeable cells is sequestered and protected against RNase treatment, in contrast to the disposition of tRNA molecules added from the outside (16). These data, coupled with the loss of channeling and rapid protein synthesis upon cell disruption (15), support the idea that it is the organization within the cell that is responsible for the large difference in protein synthesis between living cells and cell-free systems.

The channeling studies presented to date have dealt only with tRNA up to its use in peptide bond formation (2, 15). However, tRNAs, upon leaving the ribosome, must reassociate with their cognate aminoacyl-tRNA synthetases to regenerate the aminoacyl-tRNAs necessary for maintenance of protein synthesis. In this paper we show that the uncharged tRNAs leaving the ribosome are directly transferred to their cognate synthetases without being released into the surrounding cytosol. Taken together with the earlier data showing channeling of aminoacyl-tRNA (2, 14, 15), these findings demonstrate that tRNA is part of a channeled cycle during protein synthesis in mammalian cells.

MATERIALS AND METHODS

Materials. A mixture of five ^{3}H-labeled amino acids (leucine, lysine, phenylalanine, proline, and tyrosine) was purchased from Amersham. Unlabeled amino acids, ATP, GTP, creatine phosphokinase, phosphocreatine, saponin, and trypan blue were obtained from Sigma. Cell culture reagents were from GIBCO. Rabbit liver tRNA was isolated as described (16). Oxidized tRNA was prepared by treatment with sodium periodate (17).

Cell Culture and Permeabilization. CHO cells were obtained and cultured as described (2) and were used 1 day after reaching confluency. Cells were washed once with Dulbecco's phosphate-buffered saline (PBS) and harvested from plates by incubation with trypsin for 4 min at 37°C. Approximately 10 ml of 10% (vol/vol) calf serum in PBS was added per 75-cm^2 flask, and released cells were incubated at 37°C for 20 min. This treatment allowed cells to recover from removal from the plates and led to 2- to 3-fold higher rates of protein synthesis. The cells were washed twice with ice-cold PBS, once with permeabilization buffer S (130 mM sucrose/50 mM potassium acetate/50 mM KCl/20 mM Hepes, pH 7.4), and were suspended in buffer S using ≈60 μl of buffer per 10^7 cells. The total volume of cell suspension was measured by pipetting, and an equal volume of saponin solution (150 μg/ml) in buffer S was added to give ≈1.2 μg of saponin per 10^6 cells. The suspension was mixed, incubated at 37°C for 6 min, cooled on ice, centrifuged for 1 min at 420 × *g* at 4°C, and washed once with PSW buffer (130 mM sucrose/50 mM potassium acetate/50 mM KCl/20 mM Hepes, pH 7.4/5 mM ATP/13 mM phosphocreatine/6.1 mM $MgCl_2$/2.6 mM $CaCl_2$/5.3 mM

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EGTA/5 mM glucose). Cells were suspended in 4–5 vol of PSW buffer and were immediately used. A small portion was taken to measure cell permeability by trypan blue exclusion and for cell counting.

Measurement of Protein Synthesis. Protein synthesis experiments in permeable cells were carried out in PSW buffer supplemented with the following components: 0.1 mM GTP; 250 μM each of 20 amino acids; ^{3}H-labeled leucine, lysine, phenylalanine, proline, and tyrosine (≈25 dpm/pmol each); and creatine phosphokinase at 30 μg/ml. The packed cell volume was one-fifth of the total volume of liquid added. The suspension was left on ice for 5 min to allow equilibration between inside and outside volumes. Permeabilized cells were incubated at 28°C, and at various times portions were taken, centrifuged to separate the cell and supernatant fractions, precipitated with 10% trichloroacetic acid, and boiled for 5 min; the remaining precipitate was collected on Whatman GF/C filters. The hot trichloroacetic acid-insoluble radioactivity was determined and was considered to represent newly synthesized protein.

Measurement of Aminoacyl-tRNA. Samples were taken as for protein synthesis, cooled on ice, and immediately centrifuged to separate the cell and supernatant fractions. Both fractions were extracted with phenol in 0.3 M sodium acetate (pH 6.8). The aqueous phases were divided into two equal portions. One portion was used to measure total trichloroacetic acid-precipitable radioactivity, and the second was first incubated with RNase A (2 μg/ml) for 10 min at 28°C before acid precipitation. The difference in radioactivity between these two portions was considered to represent aminoacyl-tRNA.

RESULTS

Retention of tRNA During Protein Synthesis. In earlier studies we showed that exogenous aminoacyl-tRNA added to permeabilized CHO cells immediately equilibrates between the inside and the outside compartments (15) and that fluorescent-labeled aminoacyl-tRNA added to these cells rapidly distributes throughout the cell interior (2). Thus, we would expect that if tRNA were ever free during its cycling in the translation process, some of it would diffuse out of the cell at each cycle and lead to a decrease in the intracellular level of aminoacyl-tRNA and in the rate of protein synthesis. The data in Fig. 1 show that this does not happen. Over a period of 30 min, during which the tRNA population undergoes at least 70 cycles of translation, protein synthesis remains linear (Fig. 1*A*), and there is no decrease in the level of aminoacyl-tRNA within the cell or an increase in the supernatant fraction (Fig. 1*B*). These findings suggest that tRNA never becomes free during the translation process, neither when it exists as aminoacyl-tRNA or peptidyl-tRNA nor when it is uncharged and must transfer from the ribosome back to the aminoacyl-tRNA synthetase. Thus, tRNA appears to be channeled throughout the translation cycle.

Exogenous tRNA Does Not Affect Protein Synthesis. A second prediction of a channeled tRNA cycle is that exogenous tRNA added to permeabilized cells would be unable to enter the translation system and, consequently, would have no effect on protein synthesis. As shown in Fig. 2, this prediction was fulfilled. Concentrations of tRNA equivalent to as much as three times that of endogenous tRNA does not increase the rate of protein synthesis, even though, as will be shown below, the exogenous tRNA can be aminoacylated. To eliminate the possibility that the lack of an effect of exogenous tRNA is because endogenous tRNA is already saturating for protein synthesis, the influence of periodate-oxidized tRNA was also examined (Fig. 2). Oxidized tRNA is a potent inhibitor of aminoacylation (see below) and would be expected to rapidly reduce the rate of protein synthesis. The data in Fig. 2 show

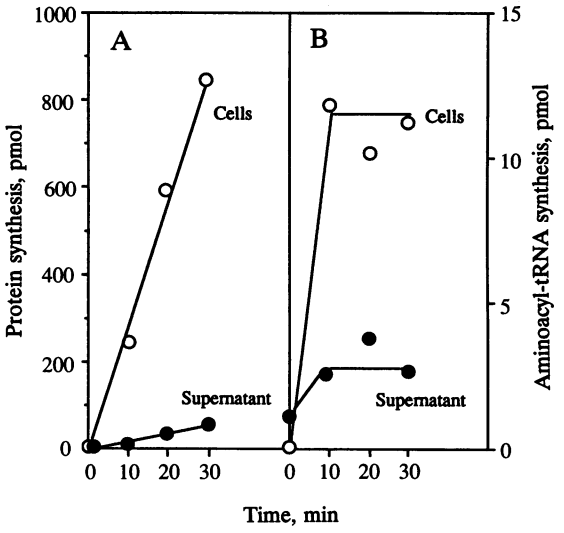

FIG. 1. Protein synthesis and aminoacyl-tRNA levels in permeabilized CHO cells. Protein was synthesized for the indicated times under the described conditions. At each time point two portions were taken, cooled on ice, and separated into cell and supernatant fractions. One portion was used to measure protein synthesis (*A*), and one was used to measure aminoacyl-tRNA (*B*). Each point represents 2.2×10^6 cells. Note the different scales for *A* and *B*.

that this is not the case. Oxidized tRNA also has no effect on protein synthesis in this system. These data show that during translation endogenous tRNAs are never in a state in which they can mix with and be competed against by exogenous tRNA molecules.

Exogenous tRNA Can Be Aminoacylated But by Only a Fraction of the Aminoacyl-tRNA Synthetases. On the basis of the inability of either exogenous tRNA or oxidized tRNA to affect protein synthesis and presumably being unable to gain entry to the translation apparatus, we expected that exogenous tRNA also would not be aminoacylated in permeabilized cells. However, Table 1 shows that tRNA added to permeable cells can be converted to aminoacyl-tRNA. The aminoacyl-tRNA synthetases responsible for this activity are tightly associated with the permeabilized cells. In the course of permeabilization and washing the cells, ≈30% of the total aminoacyl-tRNA synthetase activity leaks out and is discarded; the remaining 70% remains with the cells and is not removed by repeated washing (data not shown); it is this population of synthetases that is examined here.

Although exogenous tRNA can be aminoacylated by permeabilized cells, only a fraction of the aminoacyl-tRNA synthetases remaining with the cells is responsible for this activity

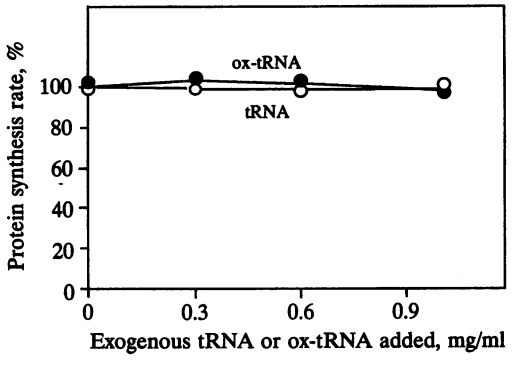

FIG. 2. Effect of tRNA and periodate-oxidized (ox) tRNA on the rate of protein synthesis in permeabilized cells. Protein was synthesized for 10 min and 20 min, as described, with the indicated concentrations of rabbit liver or oxidized rabbit liver tRNA.

Table 1. Aminoacylation of exogenous tRNA by preparations of permeabilized CHO cells

Permeabilized CHO cell preparation	Aminoacyl-tRNA synthetase activity: pmol aa-tRNA/min per 10^6 cells	Aminoacyl-tRNA synthetase activity: %	Inhibition by ox-tRNA, %
Control	124	100	58
Sonicated	295	238	70
Treated with 1% Nonidet P-40	351	283	69
Treated with 0.2% Triton X-100	348	281	61

Reaction mixtures of 100 μl contained the same components as for protein synthesis and the indicated cell preparation. For each preparation, samples were incubated with or without rabbit liver tRNA at 1 mg/ml for 1.5 min at 28°C and precipitated with 10% trichloroacetic acid. The difference between incorporation in the presence of added tRNA and in its absence was considered to represent incorporation into exogenous tRNA. For the inhibition studies oxidized tRNA was present at 2 mg/ml. aa, Aminoacyl.

(Table 1). Thus, sonication of the cells or treatment with the nonionic detergents, Nonidet P-40 or Triton X-100, revealed an additional population of synthetases that could now act on exogenous tRNA. This latter population, amounting to 60–65% of the cell-associated aminoacyl-tRNA synthetase activity, is initially inaccessible to the exogenous tRNA and only becomes available upon disruptive treatment of the cells. We do not know whether the synthetase population that is accessible to exogenous tRNA is normally in that state or whether it represents a portion of the translation machinery that has been damaged by the permeabilization procedure. However, it is unlikely that the two populations of synthetases come from different cells that are more or less damaged because based on microautoradiography essentially all of the cells are equally capable of protein synthesis (data not shown). Nevertheless, these data show that a major portion of the cellular aminoacyl-tRNA synthetases are in a state in which they cannot interact with exogenous tRNA molecules.

Aminoacylation of Exogenous tRNA Is Sensitive to Oxidized tRNA. The aminoacylation of exogenous tRNAs by the accessible population of aminoacyl-tRNA synthetases provided a direct means to differentiate these reactions from those with the endogenous tRNAs that are postulated to be sequestered within the translation apparatus. As presented in Fig. 3, aminoacylation of endogenous tRNAs, under conditions of protein synthesis, is unaffected by the addition of periodate-oxidized tRNA, whereas aminoacylation of exogenous tRNAs is dramatically inhibited. Moreover, as shown in Table 1, disruption of the cells by sonication or detergents, which renders the sequestered aminoacyl-tRNA synthetase population accessible to exogenous tRNA, also makes them sensitive to inhibition by oxidized tRNA. Likewise, sonication renders aminoacylation of endogenous tRNA sensitive to periodate (data not shown). These data are entirely consistent with the inability of oxidized tRNA to inhibit protein synthesis (Fig. 2) and with the conclusion that endogenous tRNA never is in a state in which it can be competed against by oxidized tRNA. Thus, both the endogenous tRNAs and a major portion of the aminoacyl-tRNA synthetases are continually sequestered within the translation machinery.

It is known that at least some of the aminoacyl-tRNA synthetases can be isolated as a multienzyme complex (3), and recent evidence suggests that this complex exists *in vivo* (18). To determine whether there is any relation between synthetases in the complex and resistance to periodate-oxidized tRNA, we examined lysyl-tRNA synthetase activity, which is known to be present in the multienzyme complex (3). However, just as

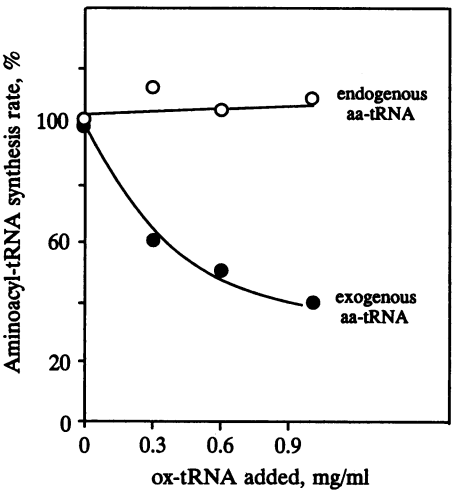

FIG. 3. Effect of periodate-oxidized (ox) tRNA on aminoacylation of endogenous or exogenous tRNA. Cells were incubated under the conditions for protein synthesis, except that the amino acid concentration was 50 μM, and rabbit liver tRNA at 0.3 mg/ml was added (exogenous) or absent (endogenous). Incubation was for 1.5 min at 28°C in the presence of the indicated concentration of oxidized tRNA. Aminoacyl (aa)-tRNA formation was determined after phenol extraction. For aminoacyl-tRNA formation from exogenous tRNA, the contribution due to endogenous aminoacyl-tRNA synthesis (≈15% of exogenous) was subtracted.

was found for the mixture of amino acids (Fig. 3), lysine attachment to endogenous tRNA is resistant to oxidized tRNA, whereas addition to exogenous tRNA is sensitive (data not shown), indicating the existence of two populations of lysyl-tRNA synthetase. Therefore, regardless of their tendency toward multienzyme complex formation, synthetases in the permeabilized cells can be found in two states differentiated by their ability to function with exogenous tRNA and to be inhibited by oxidized tRNA.

DISCUSSION

The data presented here provide strong evidence that a spent tRNA leaving the ribosome during its cycling in translation is directly transferred to its cognate aminoacyl-tRNA synthetase and does not mix with the surrounding cellular fluid. This conclusion is based on several findings. (*i*) There is no leakage of endogenous aminoacyl-tRNA from permeabilized cells during many cycles of protein synthesis, even though free aminoacyl-tRNA is known to rapidly equilibrate between the inside and outside compartments (16). Moreover, protein synthesis remains linear during this period. (*ii*) Exogenous tRNA and periodate-oxidized tRNA have no effect on protein synthesis in permeabilized cells, indicating that they cannot enter the translation machinery despite the fact that exogenous tRNA can rapidly distribute throughout the cell (2). (*iii*) A major portion of the aminoacyl-tRNA synthetases in permeabilized cells is inaccessible to exogenously supplied tRNA but still functions with endogenous tRNA. (*iv*) Aminoacylation of endogenous tRNA is unaffected by the presence of oxidized tRNA, a potent inhibitor of aminoacylation of exogenous tRNA molecules. Taken together, these data demonstrate that tRNA, whether in the form of aminoacyl-tRNA, peptidyl-tRNA, or as the uncharged molecule, is never free of the translation machinery as protein synthesis proceeds.

Several studies in the literature lend support to the conclusion that tRNA is directly transferred from the ribosome to an aminoacyl-tRNA synthetase. In one study (19), tRNA injected into *Xenopus* oocytes was a poor substrate for aminoacylation, whereas 10-fold higher activity was found in extracts from

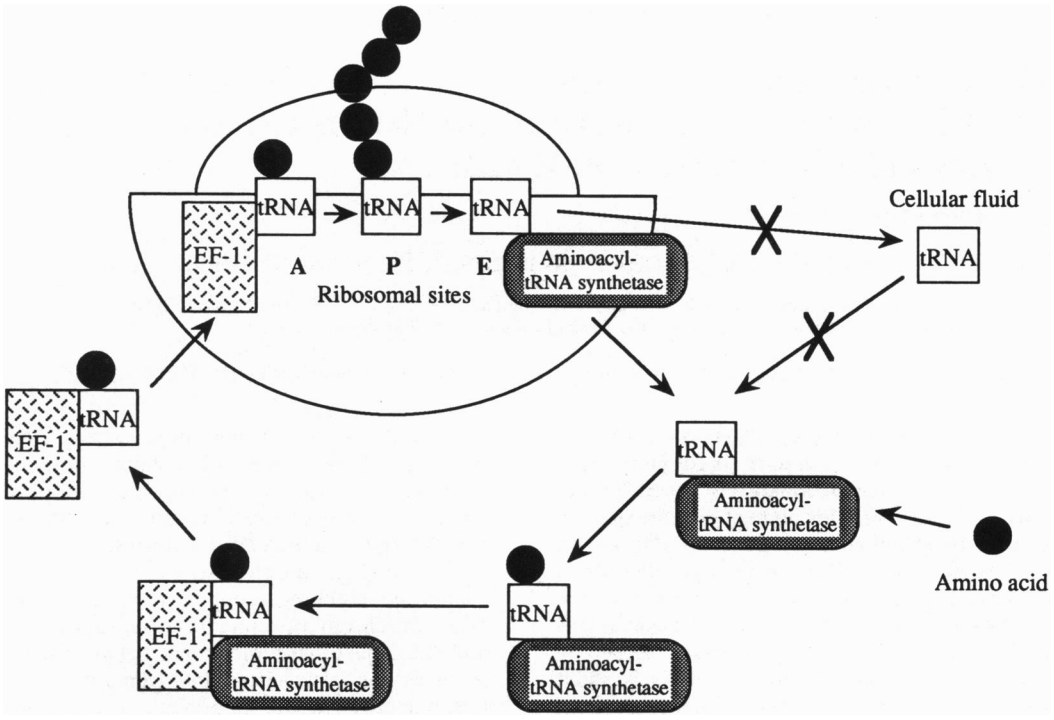

FIG. 4. Model for a channeled tRNA cycle. Diagrammed are the various states of tRNA during translation and the components to which they are bound. The arrows indicate tRNA movement during translation within the protein-synthesizing machinery. Dissociation of tRNA into the surrounding medium or entry of exogenous tRNA into the cycle does not occur, and this fact is shown by crossed-out arrows. The implications of this model are that components of the translation apparatus associate as tRNA is transferred from one component to the next.

these cells using the same tRNA. In a second study (20), it was found that dissociation of tRNA from the E site of the ribosome *in vitro* was stimulated by the presence of aminoacyl-tRNA synthetases. In a third study (17), addition of oxidized tRNA to a cell-free system did not inhibit protein synthesis. Although these earlier studies were carried out for different purposes than those reported here, they are all consistent with the idea that a highly organized translation apparatus results in the direct transfer of tRNA from the ribosomal E site to the synthetase and that this step is insensitive to tRNA from outside the system.

On the basis of the findings presented here and our earlier studies showing that aminoacyl-tRNA is channeled into protein (2, 15), we propose that there is a completely channeled tRNA cycle during mammalian protein synthesis and that direct transfer of tRNA between the translation components occurs at each step of the process (Fig. 4). How the translation system is organized and how it is assembled to accomplish this task remains to be determined, but some hints are becoming evident. Many components of the translation apparatus associate with the cytoskeleton (8–10), and addition of drugs that affect the cytoskeleton can affect protein synthesis (ref. 9 and unpublished observations). These data suggest an involvement of the cytoskeleton, particularly the microfilament network. In addition, the finding that treatment with neutral detergents renders more of the aminoacyl-tRNA synthetases accessible to exogenous tRNA (Table 1) suggests that membranous structures, or at least hydrophobic interactions, might also be important. Earlier studies with the aminoacyl-tRNA synthetase complex led to similar conclusions (21). Finally, the fact that free tRNA is unable to enter the translation system has important ramifications for how newly synthesized tRNA leaving the nucleus can enter. One likely possibility is that in its egress from the nucleus, tRNA is already associated with one or more of the other translation components that facilitates its entry into the protein-synthesizing system. Further studies will be necessary to examine how the translation system is assembled.

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