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Uptake of Transfer Ribonucleic Acid by Normal and Leukemic Cells*

Flor Herrera, Richard H. Adamson, and Robert C. Gallo

SECTION ON CELLULAR CONTROL MECHANISMS, HUMAN TUMOR CELL BIOLOGY BRANCH, AND SECTION ON PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, LABORATORY OF CHEMICAL PHARMACOLOGY, NATIONAL CANCER INSTITUTE, BETHESDA, MARYLAND 20014

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Abstract. Uptake of tRNA (*Escherichia coli*) was demonstrated in the murine leukemia, L1210 and in a human lymphoblast (NC-37) cell lines. In both cell lines, uptake of tRNA was rapid, reaching a maximum within 45 sec, and was linear with concentration up to about 50 μg/ml. This uptake of exogenous tRNA apparently was not due to altered membrane permeability or impaired cell viability, nor to ribonuclease degradation of the macromolecule. Furthermore, about 20% of the tRNA taken up by the cells remains functional and apparently intact. This was demonstrated by: (*a*) acylation with *E. coli* aminoacyl-tRNA synthetases; (*b*) methylation with leukemic cell tRNA-methylases, and (*c*) demonstrating ^{14}C-labeled 4S RNA in the cytoplasmic fraction of the leukemic cells after the addition of *E. coli* [^{14}C]tRNA. The results demonstrate that tRNA can enter mammalian cells and suggest that an energy independent, carrier-mediated, mechanism may be operative.

Our laboratory has been interested in the concept that tRNA plays an important role in the control of protein synthesis and, therefore, the control of differentiation and growth.[1–5] As part of this study we have explored whether isolated tRNA can enter mammalian cells.

Materials and Methods. Preparation of tRNA: [^{14}C]tRNA was isolated from log phase *Escherichia coli* grown with 0.05 mM [^{14}C]uridine for 3 hr, followed by a period of 45 min with 5 mM unlabeled uridine. The cells were ground with alumina and suspended in 2 volumes of buffer (10 mM Tris·HCl, pH 7.4–1 mM EDTA–10 mM $MgCl_2$) and centrifuged at 105,000 $\times$ *g* for 1 hr. The supernatant was aspirated, deproteinized twice with phenol, and the tRNA precipitated with ethanol. tRNA was further purified by extraction with 1 M NaCl, treatment with DNase, and removal of the DNase with phenol. tRNA was again precipitated with ethanol and applied to a Sephadex G-100 column. Fractions containing the tRNA, which eluted as a sharp peak, were combined. The tRNA was precipitated with ethanol and stored at −170°C. This tRNA was free of measurable DNA, protein, and ^{14}C-labeled acid-soluble material. It had excellent amino acid acceptor activity. tRNA from normal and leukemic leukocytes was prepared as previously described.[2] For calculating the weight of tRNA, a conversion factor of 20 A_{260} per mg tRNA in the buffer described above was used.

Cells, media, and uptake studies: L1210 cells were grown in spinner cultures using RPMI 1630 medium, containing 20% fetal calf serum.[6] Normal human lymphoblasts (NC-37)[2] were grown in static culture in RPMI 1630 medium supplemented with 15% fetal calf serum. The cells were harvested in early log phase, washed three times in medium without serum, and resuspended to a concentration of 1×10^6 cells/ml.

E. coli [^{14}C]tRNA was added at the start of incubation (at 37°C). Aliquots were taken at intervals and deposited on HA Millipore filters (0.45 μm pore size). The filters were immediately washed with 30 ml of 0.15 M saline, and macromolecules were precipitated with cold 10% trichloroacetic acid (TCA). The filters were then washed with 5% TCA, dried, and the radioactivity of the cells and filtrate counted. Control experiments showed that [^{14}C]tRNA added to cells placed on filters did not result in adherence of the [^{14}C]tRNA to the cells.

Presence of ribonucleases in the medium was ruled out by the following experiment: Aliquots of incubated L1210 cells were removed at intervals over a 30-min period, centrifuged, and the cell-free medium aspirated and assayed for ribonuclease activity as described previously.[7] No ribonuclease activity was detected throughout incubation.

For concentration uptake studies, aliquots were taken 10 sec after the addition of increasing concentrations of tRNA.

Determination of cell viability and membrane integrity: To ascertain whether cell membranes were intact and cell viability was maintained after addition of exogenous tRNA:

(*a*) Nigrosine dye, at a concentration of 0.5%, was added to L1210 cells in 1630 medium after 5 min of incubation with *E. coli* tRNA; the percentage of cells that took up dye was determined.

(*b*) The uptake of [^{14}C]inulin (0.83 μCi/μg), average molecular weight 3000–4000, by L1210 cells was determined as described above for uptake of *E. coli* [^{14}C]tRNA.

(*c*) Cell viability was also examined by determining the rate of induction of leukemia in recipient animals. 5 ml of L1210 cells (10^6 cells/ml) were incubated with unlabeled *E. coli* tRNA (10 μg/ml, 0.4 μM). After incubation of control cells and tRNA-treated cells for 2 min, the volume was diluted from 5 ml to 15 ml and 0.5-ml aliquots were injected intraperitoneally to 10 CD_2F_1 female mice. The median survival time was determined.

Electron microscopy: L1210 cells were incubated with [^{14}C]tRNA for 2 min. The cells were harvested and radioautographed by electron microscopy.[8]

Subcellular fractionation: To determine the intracellular localization of the [^{14}C]tRNA, leukemic cells were subjected to subcellular fractionation. After incubation for 5 min with *E. coli* [^{14}C]tRNA, the cells were washed with fresh medium, resuspended in cold hypotonic buffer (10 mM Tris·HCl–10 mM KCl–1.5 mM $MgCl_2$, final pH 7.4), and homogenized with a Dounce homogenizer. Sucrose was added to a concentration of 0.2 M. The cells were examined microscopically to determine if they were adequately disrupted, and then centrifuged at 1200 $\times$ *g* for 15 min, to obtain a nuclei–membrane pellet and supernatant no. 1. The nuclei and membranes were separated as described by Boone *et al.*[9]

Supernatant no. 1 was centrifuged at 2000 $\times$ *g* for 10 min to obtain a mitochondrial pellet and supernatant no. 2. The latter was centrifuged at 38,500 $\times$ *g* for 12 min to obtain a lysosomal pellet and supernatant no. 3. Supernatant no. 3 was centrifuged for 60 min at 100,000 $\times$ *g* to give a microsomal fraction and "high-speed supernatant." The entire procedure was done at 0°C. Each fraction was examined by electron microscopy for homogeneity and aliquots were taken for counting radioactivity.

Recovery of intact tRNA: To determine whether the labeled material within L1210 cells was *intact E. coli* tRNA, the elution of the intracellular tRNA on a Sephadex G-100 column was compared to a standard of unlabeled *E. coli* tRNA. It would be expected that some of the labeled tRNA within the cell might be metabolized and converted to low molecular weight compounds (and, possibly, by reutilization of the low molecular weight products to high molecular weight RNA) which could be distinguished from tRNA by Sephadex G-100 chromatography. This procedure might also provide further evidence for cellular uptake of tRNA. L1210 cells were incubated with *E. coli* [^{14}C]tRNA (500 μg/ml) for 30 min. The incubation was terminated by washing with cold medium; nuclear and cytoplasmic tRNA were isolated after removal of the membranes. Samples and standard were applied to the column, fractions collected, and radioactivity and A_{260} were determined.

Recovery of functional tRNA: L1210 cells were incubated with unlabeled *E. coli* tRNA (500 μg/ml) for 30 min. Then, the cells were washed, homogenized, and the membranes, nuclei, and cytoplasm were separated. The membrane fraction was discarded, and tRNA was purified separately from nuclei and cytoplasm. These tRNA preparations were shown to be entirely 4S by Sephadex chromatography. They were used for acylation and methylation assays as described below.

Assays for amino acid acceptance by the various tRNAs were performed in a cell-free system as previously described.[2] Reaction mixtures, in a final volume of 50 μl, contained: 50 μg of the "experimental tRNA" isolated from L1210 cells after incubation with *E. coli* tRNA; 0.5 μCi of [^{14}C]leucine; 0.1 M Tris·HCl, pH 7.5; 10 mM KCl; 20 mM Mg acetate; 5 mM ATP; 5 mM CTP; 10 mM β-mercaptoethanol; and partially purified *E. coli* aminoacyl-tRNA synthetases. The *E. coli* aminoacyl-tRNA synthetases were prepared as previously described,[2] except for additional purification by Sephadex G-100 chromatography.

Determination of tRNA methylation was performed with 50 μg of the "experimental tRNA" and 20 μg of protein from a high-speed supernatant of homogenized L1210 cells as the source of tRNA methylases. The details of tRNA methylase preparation and assay have been described.[7] "Standard curves" (control curves) for aminoacyl-tRNA synthesis and tRNA methylation were obtained from experiments performed with reaction mixtures containing a constant amount of L1210 tRNA (50 μg) and increasing concentrations of *E. coli* tRNA for both acylation and methylation. In both cases, control experiments showed no reaction with L1210 tRNA alone (50 or 100 μg) but a linear reaction with increasing amounts of *E. coli* tRNA and 50 μg (or 100 μg) of L1210 tRNA. These curves were used to estimate the amount of functional *E. coli* tRNA recovered from the L1210 cells.

Results and Discussion. tRNA uptake is rapid and the uptake can be saturated: Kinetics of uptake of [^{14}C]tRNA, at a concentration of 1 μg/ml, is shown in Fig. 1 for L1210 leukemic cells (*upper panel*) and normal human lymphoblasts in tissue culture (NC-37) (*lower panel*). For L1210 cells, each solid line is the average value obtained from six experiments. The variation for two standard-errors is given for each value (broken lines). The rate of uptake of *E. coli* tRNA is extremely rapid; maximum uptake is observed by 45 sec. Similar kinetics were also found when fresh, human, peripheral blood lymphocytes were used. When the concentration of tRNA was increased to 500 μg/ml, the kinetics were identical.

Cells were incubated with increasing concentrations of tRNA for 10 sec to determine if tRNA uptake could be saturated (Fig. 1, *middle panel*). This is a point in the linear portion of the uptake curve for each of the concentrations employed. As can be seen, uptake is proportional to tRNA concentration up to 50 μg/ml. A plateau is reached by 500 μg/ml, indicating saturation of uptake.

Endogenous L1210 tRNA was found to be 0.5–1.0 μg/10^6 cells. The maximum amount of *E. coli* tRNA taken up by L1210 cells was approximately 30% of the endogenous tRNA.

The rapid uptake, combined with evidence for saturation, suggested the possibility of a transport mechanism. The rate and total uptake of tRNA, at a concentration of 10 μg/ml, was about doubled at 4°C compared to 37°C. Neither addition of 10 mM sodium azide or 1 mM dinitrophenol, nor omission of glucose inhibited tRNA uptake. These results indicate that the transport mechanism may not require metabolic energy. These results are compatible with a energy-independent carrier mechanism, a highly energy-dependent efflux mechanism, or an intracellular "binding" of tRNA.

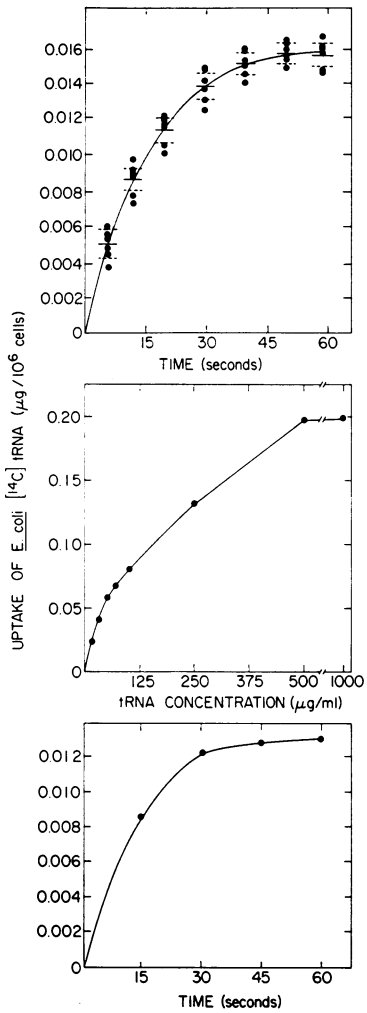

FIG. 1. *Upper panel:* Uptake of *E. coli* [^{14}C]tRNA (1 μg/ml; 0.01 μCi/μg) by L1210 cells. Similar kinetics were obtained at concentrations of 500 μg/ml. *Middle panel:* Saturation of rate of uptake of *E. coli* [^{14}]CtRNA (0.01 μCi/μg). L1210 cells were incubated with increasing concentrations of *E. coli* [^{14}C]tRNA, and aliquots were taken 10 sec after addition of tRNA. *Lower panel:* Uptake of *E. coli* [^{14}C]tRNA by normal lymphoblasts (NC-37). *E. coli* [^{14}C]tRNA (1 μg/ml; 0.01 μCi/μg) was added at the start of incubation.

Cell viability and membrane integrity were normal: The percentage of L1210 cells stained with nigrosine dye was 2% in both the untreated L1210 cells and in cells incubated with *E. coli* tRNA. The median survival time of mice inoculated with control and tRNA-treated L1210 leukemic cells did not differ. Both results indicate that viability of L1210 cells was not impaired by *E. coli* tRNA.

[^{14}C]inulin uptake by L1210 cells was not significant (less than 0.05% uptake of inulin occurred under the usual incubation conditions compared to 2% uptake of tRNA). Thus, uptake does not appear to be due to impaired cell boundaries or to the presence of nonviable cells. This is also indicated by the observation that another cell line derived from human lymphoblasts (NC-37) also takes up tRNA, and the kinetics of uptake are similar. Furthermore, if cells were incubated at pH 5.0 for different times, resulting in diminished viability (10% stained with nigrosine dye), the kinetics of uptake were identical to untreated cells.

tRNA uptake is not due to adherence to the cell membrane: Electron microscopic autoradiographs of cells incubated with [^{14}C]tRNA show grains within the cell, distributed in the cytoplasm and nuclei (Fig. 2). 30–70% of the cells were labeled during 2 min of incubation, and there was no evidence of vesicle formation characteristic of pinocytosis. This same experiment, repeated at several time intervals, consistently revealed intracellular grains, in over 50 autoradiographs, without membrane adherence.

After uptake, tRNA is distributed in each subcellular fraction: The radioactivity was present in all subcellular fractions after 5 min of incubation. The distribution of the label was similar in the different subcellular fractions, with the exception of the nucleus which con-

tained 25–60% of the counts. The disproportionate recovery of the label in the nuclear fraction suggests some tRNA catabolism and reutilization of the products for RNA synthesis and is discussed in more detail below.

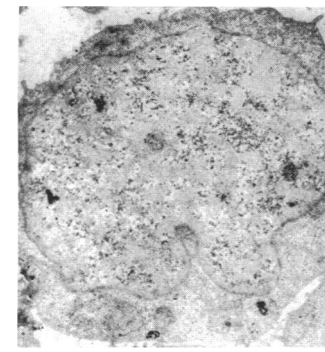

FIG. 2. Electron-microscope autoradiograph of L1210 cells incubated with *E. coli* [^{14}C]-tRNA (1 μg/ml).

Uptake of tRNA is not due to its extracellular degradation: The possibility of extracellular ribonuclease degradation of tRNA was eliminated by: (*a*) analysis of medium showed no ribonucleases liberated during the course of incubation. (*b*) As shown in Table 1, after incubation and uptake of *E. coli* [^{14}C]tRNA, no significant amounts of acid-soluble products appear in the medium. (*c*) If some, or all, of the radioactivity taken up by L1210 cells was due to a breakdown product of *E. coli* [^{14}C]tRNA (labeled in the uracil moiety), inhibition of uptake of these uracil-labeled fragments would be predicted in the presence of an excess of unlabeled, low molecular weight tRNA products. The nucleoside, uridine, is efficiently taken up by these cells and should be an eventual product of tRNA breakdown. As illustrated in Table 1, addition of excess unlabeled uridine did not alter the uptake of tRNA. (*d*) Most significantly, we recovered functional *E. coli* tRNA from L1210 cells after a 30- or a 60-min incubation.

Some of the *E. coli* tRNA remains functional within L1210 cells: *E. coli* tRNA can be functionally distinguished from L1210 tRNA by: (*a*) the relative speci-

TABLE 1. *Evidence that the uptake of ^{14}C is not from low molecular weight breakdown products of [^{14}C]tRNA in the medium.**

Time of incubation (min)	Acid-precipitable material in medium (dpm/ml)	Acid-soluble material in medium (dpm/ml)
1	11,718	36
5	11,540	30
15	12,103	36
30	11,781	26
60	11,670	36
120	11,730	40

tRNA uptakes†

Time of incubation (min)	No uridine (dpm/10^6 cells)	20 μM uridine (dpm/10^6 cells)
0.25	215	208
0.5	240	236
1	279	288
2	300	294
5	296	301
15	304	294

* TCA-precipitable and soluble radioactivity in the medium was measured after incubation of L1210 cells (10^6 cells/ml) with *E. coli* [^{14}C]tRNA. No significant amount of low molecular weight degradation products appear in the medium even after 2 hr of incubation.

† Effect of 20 μM uridine on uptake of *E. coli* [^{14}C]tRNA by L1210 cells. The uptake of the [^{14}C]tRNA is not altered by the presence of unlabeled uridine at a concentration 200 times the molar concentration of the [^{14}C]tRNA.

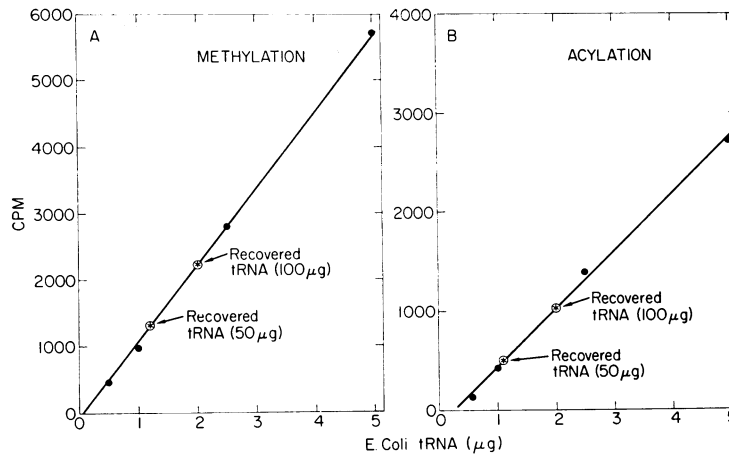

FIG. 3. (*A*) Standard curve for tRNA methylation (○——○). Methylation of tRNA (50 or 100 μg) recovered from L1210 cells treated with *E. coli* tRNA is indicated by the crossed circles (⊗——⊗). (*B*) Standard curve for aminoacyl-tRNA synthesis (●——●). Leucyl-tRNA formation, utilizing the "experimental" tRNA (either 50 or 100 μg) recovered from L1210 cells treated with *E. coli* tRNA is indicated by the crossed circles (⊗——⊗).

ficity of *E. coli* leucine-activating enzymes for *E. coli* tRNA; (b) by *in vitro* methylation of *E. coli* tRNA with L1210 methylases. Since L1210 tRNA is fully methylated *in vivo*, it will not serve as an *in vitro* substrate for methylation. On the other hand, *E. coli* tRNA is an excellent substrate for *in vitro* methylation by L1210 methylases. Advantage was taken of these observations to determine whether functional *E. coli* tRNA could be recovered from L1210 cells.

As shown in Fig. 3, tRNA recovered from L1210 cells after a 30-min incubation with *E. coli* tRNA consisted of both L1210 tRNA and *E. coli* tRNA. This was demonstrated by showing that recovered tRNA had methyl acceptance with L1210 tRNA methylases (3*A*), and acceptor activity for leucine with *E. coli* leucine-activating enzyme (3*B*), while L1210 tRNA is not a substrate for either reaction. Similar results were obtained with a 60-min incubation. Thus, recovery of functional tRNA from the L1210 cells provides additional evidence for uptake of this tRNA, and demonstrates that some tRNA (approximately 20% of the total *E. coli* tRNA taken up) remains functional after 30 or 60 min of incubation.

Part of *E. coli* tRNA remains intact but intracellular degradation does occur: Data showing that *E. coli* tRNA recovered from L1210 cells accepts leucine and can still be methylated suggests that some tRNA may remain intact. To analyze this further, the tRNA recovered from nuclei and cytoplasm of L1210 cells after incubation with *E. coli* [^{14}C]tRNA was subjected to Sephadex chromatography. Results for the cytoplasm are shown in Fig. 4 (*top panel*). Three major peaks of radioactivity corresponding to A_{260} peaks are seen. The first peak, representing high molecular weight material, may form from intracellular degradation of *E. coli* tRNA and reutilization of breakdown products for RNA

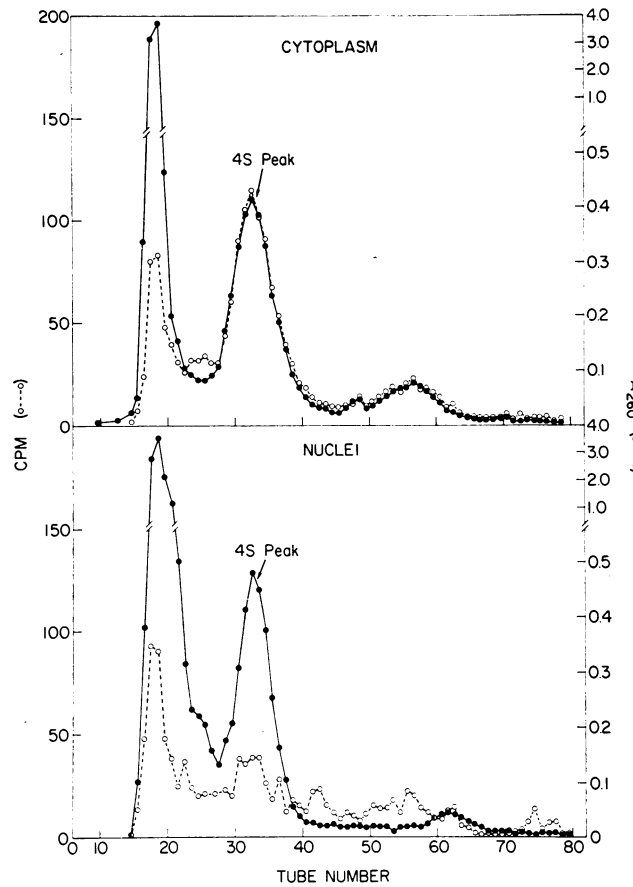

FIG. 4. Sephadex G-100 chromatography of RNA isolated from cytoplasm (*top panel*) and nuclei (*bottom panel*) of L1210 cells after incubation for 30 min with *E. coli* [^{14}C]tRNA.

synthesis, since no high molecular weight contaminant was found prior to incubation. This is supported by the appearance of the third (small) peak eluting late from the column (tube numbers 50–65). However, a portion of the cytoplasmic material remains as 4 S. The labeled material isolated from the nucleus (*lower panel*) is predominantly high molecular weight material. Unlike the cytoplasmic 4S material, the labeled 4S material from the nucleus had no acceptor activity and was not methylated by L1210 tRNA methylases.

Demonstration of significant intracellular degradation of *E. coli* tRNA over the short period of incubation suggests that tRNA turnover in L1210 is more rapid than the 80 hr reported for liver cells,[10] or that heterologous tRNA (or any exogenous tRNA) may be recognized as foreign and subjected to more rapid degradation.

In cell-free experiments, it has been shown that a limitation in the amount of a tRNA species can regulate mRNA translation.[11] However, a direct demonstra-

tion of control of protein synthesis by tRNA (or any other regulatory processes in intact cells) *in vivo* has been an elusive problem. The results reported in this communication demonstrate that mammalian cells can take up exogenous tRNA and that an appreciable percentage of the ingested tRNA may remain functional for at least 1 hr. Biological effects of foreign nucleic acids and proteins have been described.[12,13] Our observations open the possibility of testing the biological effects of various foreign tRNAs in mammalian cells. It has been shown that the addition of aminoacyl-tRNAs to chick blastodiscs largely reversed the inhibition of hemoglobin formation produced by actinomycin or 8-azaguanine.[14] Previous findings led to the suggestion that tRNA may exert regulatory functions in cell differentiation,[3,4] that changes in tRNA may lead to neoplastic transformation,[1,2,5,15] and that specific aminoacyl-tRNAs may be involved in transcriptional regulation.[16-18] It is conceivable that experiments based on a nonphysiological input of foreign tRNA might help clarify any regulatory role of tRNA.

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