UPTAKE AND AMINOACYLATION OF EXOGENOUS ESCHERICHIA COLI tRNA BY MOUSE FIBROBLASTS

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Summary: Mouse fibroblasts (L-cells) in suspension culture take up exogenous Escherichia coli tRNA in the presence of DEAE-Dextran. Tritium-labeled formylmethionine tRNA and valine tRNA are both taken up at very low levels. Tritium activity is associated solely with 4S material as judged by chromatography of cell extracts on Sephadex G-100. Further analysis of this material on a dihydroxyboryl-substituted cellulose indicates that a small portion of the tRNA taken up is acylated by the L-cells.

Studies on the infectivity of viral RNAs and on the mechanism of interferon have stimulated work on RNA uptake by mammalian cells (1-4). Macromolecules or segments of macromolecules may become useful in chemotherapy (5,6) or in the treatment of genetic diseases (7). As an extension of work from this laboratory on the uptake and utilization of exogenous nucleotides by mouse fibroblasts (L-cells) (8,9), we have examined the uptake of tRNAs by these cells. We have chosen to study tRNA because of its well-defined structure and function. In addition, the recent reports that a 4S RNA serves as a primer in DNA transcription in RNA viruses (10-12) and that E. coli tRNAs inhibit reverse transcriptase in vitro (13) suggested that studies on uptake of tRNA may be of interest in the control of pathology.

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Previous studies have indicated that animal cells take up exogenous tRNAs in homologous (14,15) and heterologous (16) systems. Homologous tRNAs have been shown to be aminoacylated (14) by cells after uptake. Although it has been reported that yeast $tRNA^{Phe}$ after microinjection into oöcytes of Xenopus laevis is aminoacylated (17), prokaryotic tRNAs have not been shown to be so utilized by cells. Our studies indicate that mouse fibroblasts take up exogenous E. coli tRNAs and aminoacylate these tRNAs to a small extent.

The specific tRNAs used in these studies were made radioactive by NaB^3H_4 reduction of pure iso-accepting tRNAs which had been photochemically cross-linked between 4-thiouridine (residue 8) and cytosine (residue 13) (18-21). This reaction is a common feature of all E. coli tRNAs which contain 4-thiouridine. These reduced tRNAs, in addition to being radioactively labeled, are highly fluorescent and retain their capacity to accept amino acids (18). Furthermore, if these tRNAs are degraded, it is unlikely that the label will be reutilized for the biosynthesis of cellular tRNAs.

Materials and Methods

$tRNA^{fMet}$ from Escherichia coli B was the gift of Dr. A.D. Kelmers of the Oak Ridge National Laboratory; $tRNA^{Val}$ from E. coli MRE 600 was purchased from Boehringer-Mannheim; Macaloid was from the Baroid Division, National Lead Co. DEAE-Dextran (mol. wt. = 2×10^6), poly-L-ornithine·HBr (mol. wt. = 1.2×10^5) and pancreatic RNase were obtained from Sigma. Tritiated sodium borohydride (sp. act. 293 Ci/mole) was purchased from Amersham, [2-^{14}C]uracil and uniformly-labeled L-[^{14}C]methionine from Calbiochem, and uniformly-labeled L-[^{14}C]valine from New England Nuclear Corp.

Mouse fibroblasts were maintained in suspension using Joklik-modified minimal essential medium supplemented with 10% fetal calf serum, as described previously (8). Cells in exponential growth ($2\text{-}5 \times 10^5$ cells/ml) were used for uptake experiments. Mixed [^{14}C]

labeled tRNAs were extracted from E. coli 15 TAU (relA$^+$) grown in the presence of [^{14}C]uracil (22).

[^{3}H]tRNA^{fMet} and [^{3}H]tRNA^{Val}. The tRNA (500 µg/ml) in 0.01 M K^+-cacodylate buffer (pH 7.0) - 5 mM $MgCl_2$ was irradiated at 335 nm in a Farrand Mk I spectrofluorometer until the 4-thiouridine absorption peak at 335 nm was lost (approx. 8 hr) (18). The cross-linked tRNA was treated in the cuvette with 2 mCi of NaB^3H_4 in 0.5 mCi portions over 30 minutes; released $^3H_2$ gas was trapped on activated PtO_2. The fluorescence of the solution (excitation at 385 nm, emission at 435 nm) was measured, after which an additional 0.5 mCi of NaB^3H_4 was added. The fluorescence was again measured and found to be unchanged. Approximately 2 mg of unlabeled $NaBH_4$ was added to ensure complete reaction and then 0.2 ml of 2 M KOAc (pH 4) was added to destroy unreacted $NaBH_4$. The solution was neutralized with dilute KOH and chromatographed on a column of Sephadex G-100 (1.0 x 35 cm) equilibrated in 0.01 M KOAc, pH 7.0. The material eluting in the tRNA region was pooled and concentrated by ultrafiltration using an Amicon concentrator. Yields of radioactive tRNA were 50% and 65% for tRNA^{fMet} and tRNA^{Val}, respectively, based on the expected incorporation of one tritium atom per tRNA molecule. Specific activities were of the order of 1.5 to 2.0 x 10^6 cpm/mg tRNA.

Aminoacylation of [^{3}H]tRNAs. Using aminoacyl-tRNA synthetases from E. coli purified through the DEAE-cellulose step (23), unlabeled tRNA^{fMet} accepted 0.60 moles [^{14}C]methionine/mole nucleic acid; unlabeled tRNA^{Val} accepted 0.65 moles [^{14}C]valine/mole nucleic acid. The corresponding [^{3}H]-labeled tRNAs accepted 0.41 mole [^{14}C]methionine and 0.45 mole valine/mole tRNA. Both tRNAs were also charged by an L-cell synthetase preparation purified through the DEAE-cellulose step (24). Unlabeled tRNA^{fMet} accepted 0.05 mole methionine/mole tRNA and unlabeled tRNA^{Val} accepted 0.07 mole valine/mole using the L-cell preparation. Both [^{3}H]tRNA^{fMet} and [^{3}H]tRNA^{Val} accepted about 0.05 mole of the respective

amino acids per mole nucleic acid using the L-cell enzyme preparation. It is possible that the L-cell enzymes are acylating a small amount of unmodified tRNAs in the latter experiments, however, this should make no difference if [^{3}H]activity can be shown to be associated with aminoacyl-tRNAs in the uptake experiments.

Uptake Kinetics. Cells growing in suspension culture (2-5 x 10^{5}/ml) were washed twice at 37^{o} with serum-free medium and resuspended in serum-free medium containing E. coli mixed [^{14}C]tRNAs (50 µg/ml) and DEAE-Dextran (100 µg/ml) (15). Cell-associated activity was measured by centrifuging an aliquot of cells and washing three times with phosphate-buffered saline (PBS) (8). After resuspension in PBS, an aliquot was counted in 3a70 scintillation fluid (Research Products International) using a Packard liquid scintillation spectrometer. To determine the amount of tRNA taken up, the washed cells were treated with pancreatic RNase (10 µg/ml) at 37^{o} for 5 minutes. Cells were then centrifuged, washed three times with cold PBS, resuspended and an aliquot counted as described above.

Uptake of [^{3}H]tRNAs. L-cells (5 x 10^{5}/ml, 25 ml) were treated with the appropriate tRNA (10 µg/ml) for one hour in the presence and absence of DEAE-Dextran (100 µg/ml) (15) as described for the kinetic experiments, with the exception that RNase treatment was eliminated. Cells were washed three times with PBS at 4^{o} and lysed in 1 ml of 0.14 M NaCl - 0.05 M NaOAc (pH 5.1) - 0.3% sodium dodecyl sulfate (5). An equal volume of 90% phenol was added and the mixture shaken at room temperature for 10 minutes. The aqueous fraction containing all cell-associated tRNA was separated by centrifugation at 5000 rpm for 15 min and chromatographed on a column (1.0 x 35 cm) of Sephadex G-100 packed in 0.05 M NaOAc, pH 5.0. The tRNA peak was pooled and concentrated by ultrafiltration on an Amicon apparatus.

Analysis of tRNA on DBAE Cellulose. The radioactive tRNA isolated

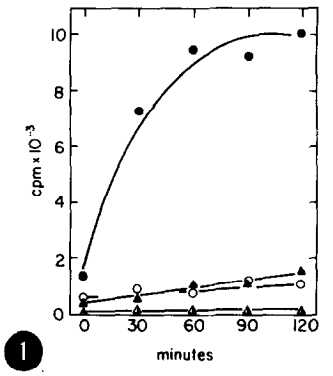
after Sephadex G-100 chromatography was divided into two portions. One portion was carefully taken to pH 7.7 and chromatographed on a column (0.5 x 4 cm) of acetylated N-(m-dihydroxyborylphenyl)succinamidoethyl cellulose (DBAE cellulose, Collaborative Research) packed in 0.6 M KCl - 0.05 M 4-methylmorpholine·HCl (pH 7.7) - 20% (v/v) ethanol (25). Aminoacylated tRNA does not complex with the boryl groups under these conditions and elutes with this buffer. To elute uncharged tRNAs the buffer is changed to 0.2 M NaCl - 0.05 M NaOAc, pH 5.0. Fractions of 0.5 ml were collected and analyzed for tritium activity. The second portion of tRNA was treated with 1.8 M Tris, pH 8.1 for 0.5 hr at 37° to strip acylated tRNAs. The solution was carefully adjusted to pH 7.7 and chromatographed as above.

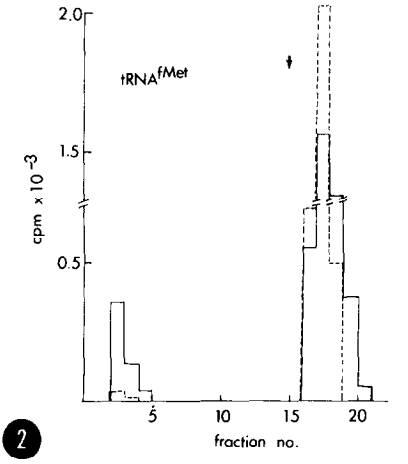
Results and Discussion

Because of the presence of RNA-degrading enzymes in the fetal calf serum used in the growth medium, serum-free medium was used for uptake experiments. No loss of cell viability was observed under these conditions for the incubation periods used. The substitution of horse serum or heated fetal calf serum for the normal serum did not reduce the RNase activity. Attempts to inhibit the RNase activity by the addition of Macaloid, DEAE-Dextran or poly-L-ornithine were not successful.

Uptake experiments with mixed *E. coli* [^{14}C]tRNAs (Fig. 1) show that about 20% of the input tRNA is cell associated after 1 - 1.5 hr; approximately one-fifth of this cell-associated tRNA (3-4% of the input) becomes RNase resistant. This RNase resistant fraction corresponds to about 2×10^6 molecules of tRNA per cell. This quantity is similar to those reported by others using other cell systems (15,16). DEAE-Dextran enhances the association of tRNAs with the cell as well as the uptake of tRNAs by the cell (Fig. 1), as demonstrated previously (15).

Experiments with the [^{3}H]tRNAs indicated that all cell-associated radioactivity chromatographs on Sephadex G-100 as tRNA (not shown).





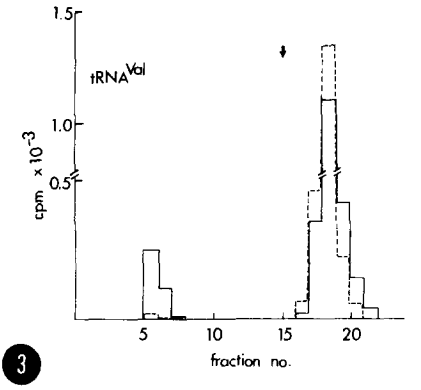


Figure 1. Kinetics of cell association (circles) and uptake (triangles) of E. coli mixed [^{14}C]tRNAs by L-cells in the absence (open symbols) and presence (closed symbols) of DEAE-Dextran. Details in text.

Figure 2. Chromatography of cell-associated [^{3}H]tRNA^{fMet} on DBAE cellulose before (solid line) and after (dotted line) stripping. Arrow denotes change of buffer as described in the text.

Figure 3. Chromatography of cell-associated [^{3}H]tRNA^{Val} on DBAE cellulose before (solid line) and after (dotted line) stripping. Arrow denotes change of buffer as described in the text.

There is no evidence of degradation or reutilization of radioactivity which would show up either as more slowly eluting activity or more rapidly eluting activity on Sephadex chromatography. If this total cell-associated activity is analyzed by chromatography on DBAE cellulose, a small amount of isotope (about 10 to 20% of the cell-associated label) from both tRNA^{fMet}- and tRNA^{Val}-treated cells (Fig. 2 and 3, respectively)

is not retained on DBAE cellulose. This suggests that the 3'-terminus of some of the tRNAs is blocked and cannot form stable adducts with the boronic acid residues of the cellulose. Treatment of the tRNAs with Tris at pH 8.1, which removes amino acids esterified to tRNA, causes most of the activity to be retained on the cellulose (Fig. 2 and 3, broken lines), although a much smaller amount of isotope (<5% of cell-associated label) still is not retained. This residual isotope is probably due to incomplete stripping of the tRNAs under the conditions used.

Chromatography of the tRNAs on DBAE cellulose before exposure to cells showed that the tRNAs were not acylated (less than 0.1%) prior to incubation; control experiments in the absence of cells did not show acylation of tRNA by the medium. The data indicate that a small portion of exogenous E. coli tRNA is incorporated within the L-cells and amino-acylated and that this tRNA which is incorporated is not degraded. Thus, exogenous E. coli tRNA, which is more readily available than tRNAs from animal cells, may be useful in studying the feasibility of the alteration of gene expression in animal cells.

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