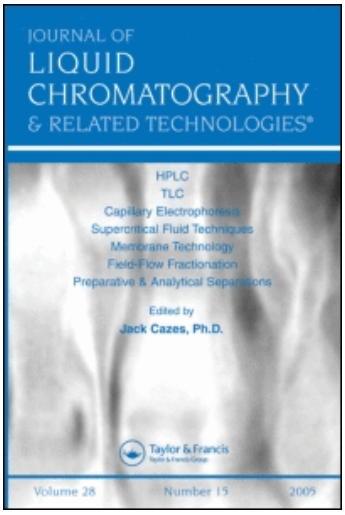

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SEPARATION OF RAT LIVER CYTOPLASMIC tRNAs BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Experiments aimed at developing a chromatographic map of cytoplasmic tRNA population from rat liver are described. HPLC tRNA fractionation by a linear gradient of acetonitrile/ammonium acetate, results in a chromatogram with more than 60 significant peaks, having area peak percentages ranging from 0.001 to 5.0. This paper presents evidence that each peak corresponds to a specific tRNA species. Specificity of tRNA-peak assignment was established by charging total tRNA with a mixture of 19 unlabeled aminoacids plus a radioactive aminoacid, and isolating the radioactive aminoacylated iso-tRNAs by gel electrophoresis, followed by HPLC acetonitrile gradient. The radioactive aminoacyl-tRNA species were eluted as well-behaved peaks. Here we describe the analysis of $tRNA^{Trp}$, which gave 2 well separated radioactive peaks, most likely corresponding to $tRNA^{Trp}$ isoacceptor species postulated by the wobble hypothesis. Taken together, the present data validate this procedure for monitoring quantitatively and qualitatively tRNA cell population.

INTRODUCTION

New quantitative and qualitative tRNA patterns have often been observed in different tissues, in cancer cells, under different growth conditions, and during cell differentiation or transformation (1-5).

Because of these observations, it has been suggested that changes in tRNA population might be mechanistically involved in the regulation of gene expression (6-8). On the other hand, at present, only laborious and time-consuming procedures are available for fractionating the complex molecular mixture represented by a normal tRNA cell population. This represents a noteworthy obstacle to the understanding of the role of tRNA in the translational regulation of gene expression.

Taking advantage of the highly resolving power of HPLC, recently we succeeded in establishing a simple and highly reproducible procedure for fractionating the numerous cellular tRNA species (9,10). Rat liver tRNA population could be resolved into as many as 60 peaks using a linear gradient of acetonitrile/ammonium acetate. In order to validate this procedure as a reliable means of studying the correlation between tRNA cell composition and translational efficiency, and, in general, tRNA biology (11-13), qualitative characterization of the chromatographic peaks was carried out in the present study.

MATERIALS AND METHODS

Materials. L-[3,4-^{3}H]proline, L-[3,4(n)-^{3}H]valine (26 Ci/mmol), L-[3-^{3}H]threonine (18 Ci/mmol), L-[5-^{3}H]tryptophan (29 Ci/mmol), L-[4,5-^{3}H]lysine (75 Ci/mmol), L-[3,5-^{3}H]tyrosine (54 Ci/mmol), L-[4-^{3}H]phenylalanine (28 Ci/mmol), L-[methyl-^{3}H]methionine (15 Ci/mmol) were from Amersham, UK. Enzymes were from Boehringer, Mannheim, except where differently stated.

tRNA Isolation. Hepatic cytoplasmic tRNA was extracted and purified from Wistar male albino rats as already described in detail (14, 15).

In vitro tRNA Aminoacylation. Purified rat liver tRNA was deacylated by incubation in 0.1 M Tris-HCl, pH 9.5, at 37°C for 1 hr (16). Then the tRNA solution was brought to pH 7.5 and reacylated. The conditions for aminoacylation were: 0.3 M Tris-HCl, pH 7.6, 0.1 M KCl, 0.02 M $MgSO_4$, 0.01 M dithiothreitol, 0.01 M ATP, 0.5 mM each of 19 unlabeled aminoacids, 200 μCi labeled aminoacid, and 7.5 mg/ml aminoacyl-tRNA synthetases as crude enzyme extract (17). The final volume was 3.0 ml. After incubation for 45 min at 37°C, the reaction was terminated by adding an equal volume of phenol, and phases were separated by centrifugation for 10 min at 10,000 rpm. [^{3}H]aminoacyl-tRNA was ethanol-precipitated from the aqueous phase, collected on a GF/C filter (2.5 cm, Whatman), freed from residual unreacted radioactive aminoacid by thorough washing with ethanol and eluted from the filter.

Gel Electrophoretic Separation of tRNA. After *in vitro* aminoacylation, [^{3}H]aminoacyl-tRNA was purified by 2 dimensional polyacrylamide gel electrophoresis. Electrophoretic conditions were identical to those described by Kuchino (18). At the end of the run, the gel was assayed for [^{3}H]-radioactivity. The [^{3}H]aminoacyl-tRNA was eluted and concentrated by repeated extraction with sec-butyl alcohol, precipitated with 3 vols ethanol, centrifuged, liophylized, dissolved in 0.05 M ammonium acetate, pH 6.6, and analyzed by using the HPLC gradient of acetonitrile/ammonium acetate described in the next section.

HPLC Fractionation of tRNA. Separation of tRNA species was carried out on a Perkin Elmer liquid chromatograph equipped with an LC-100 column oven, an LC-55B spectrophotometric detector with a 254nm filter and a Shimadzu C-R64 Chromatopac. The column was a RP-18 LiChrosorb (250mm x 4mm I.D.; particle size 5μm) pre-fitted with a 7μm guard column, packed with the same material as the analytical column. Column and precolumn were from Merck (Darmstadt, Germany). Elution was performed by a linear gradient using buffer A (0.05 M ammonium acetate, pH 6.6) and solution B (50% acetonitrile) which increased B by 1% every 10 min. The chromatographic run was carried out at 37°C at a flow rate of 0.5 ml/min (5,9,10).

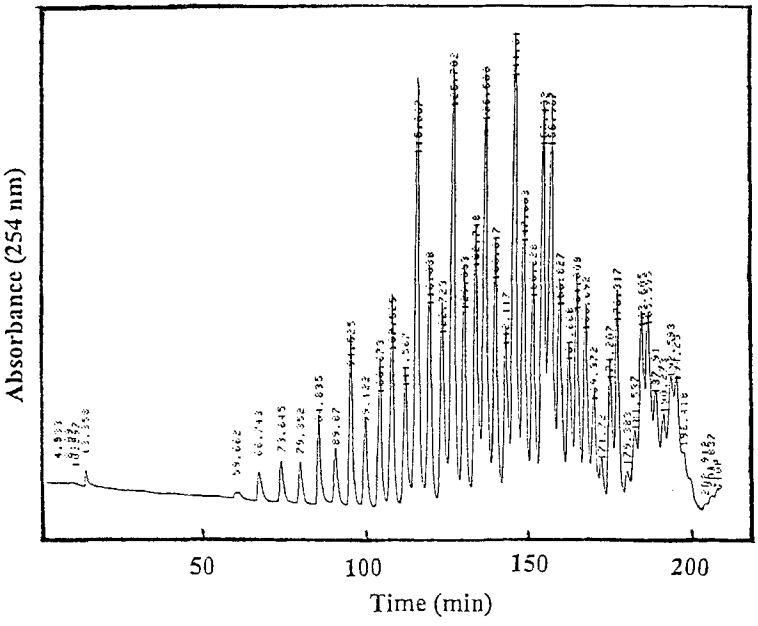

FIGURE 1 - Chromatogram of rat liver cytoplasmic tRNA obtained by acetonitrile/ammonium acetate gradient. Ninty $A_{260\ nm}$ tRNA dissolved in ammonium acetate 0.05 M, pH 6.6, were applied to a C_{18} column.

RESULTS

Fig. 1 illustrates a typical tRNA fractionation that was obtained by a linear gradient of acetonitrile/ammonium acetate. In this experiment tRNA was resolved into more than 60 peaks of which 37 had an area peak percentage higher than 0.5%. In the validation of this methodology, the qualitative identification of the numerous peaks of Fig. 1 was undertaken as the first step, in order to obtain a precise chromatographic map of the tRNA species. The possibility of using *in vitro* or *in vivo* aminoacylation to characterize the HPLC gradient procedure has already been examined. A series of experiments where radioactive proline, methionine, valine, threonine, tryptophan, lysine, tyrosine, phenylalanine were singly used as labeled aminoacid in the tRNA aminoacylation reaction *in vitro* (or

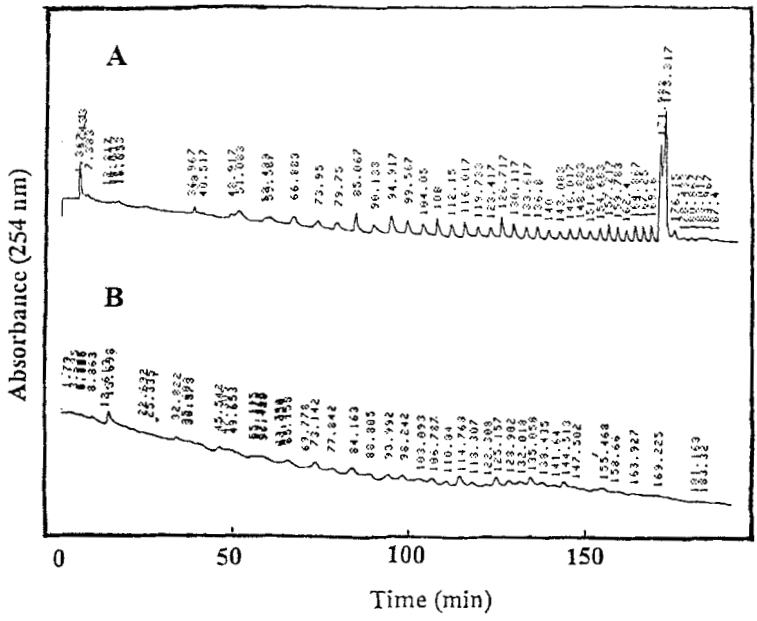

FIGURE 2 - A) Chromatogram by acetonitrile/ammonium acetate gradient of L-[5-^{3}H]tryptophan-tRNA aminoacylated in vitro and isolated by 2-dimensional gel electrophoresis. L-[5-^{3}H]tryptophan tRNA (2.6 $A_{260\ nm}$) was applied to a C_{18} column.
B) Background chromatogram of the acetonitrile/ammonium acetate gradient. At zero time 100 µl ammonium acetate 0.05 M, pH 6.6, were applied to a C_{18} column.

in vivo) was carried out, but results were unsatisfactory (10; Kanduc and Bracalello, unpublished). In fact, the major part of radioactivity eluted fast at the beginning of the chromatographic run and did not coincide with any UV-absorbing peak. That was interpreted as indicative of a possible detachment of the radioactive aminoacid from charged tRNAs during chromatographic separation by the acetonitrile/ammonium acetate gradient (10). However, these results dictated that peaks be assigned by a different qualitative procedure. Consequently, the following experimental design was undertaken. Total rat liver tRNA was deacylated and aminoacylated with a mixture of 19 unlabeled aminoacid plus a

TABLE 1

Comparative Analysis of L-[5-^{3}H]Tryptophan-tRNA Retention Times with Respect to Chromatogram of Rat Liver tRNA Population.

	1st peak	2nd peak
Retention times of *in vitro* amino acylated L-[5-^{3}H]tryptophan-tRNA	171.98-171,20[a] (2)	173.32-174.21 (2)
Retention times of corresponding peaks from chromatograms of rat liver tRNA population[b]	171.66±0.38 (5)	174.05±0.59 (5)

[a]Data are reported as mean ± S.D. with the number of determinations given in parentheses. Where only two experiments were carried out, the figures indicate the range.
[b]Validation of the determination of the two $tRNA^{Trp}$ peaks was carried out by comparing the retention times values obtained when pure L-[5-^{3}H]tryptophan-$tRNAs^{Trp}$ were chromatographed (see Fig. 2), to those relative to chromatograms of rat liver tRNA population (see Fig. 1).

radioactive one. Then the [^{3}H]aminoacyl-tRNAs were separated by two-dimensional gel electrophoresis, eluted from the gel and chromatographed by the HPLC acetonitrile/ ammonium acetate gradient. If each peak corresponded to single tRNA species, by applying specific [^{3}H]aminoacyl-tRNAs on the column, a limited number of peaks should be found. More exactly, one should recover a number of peaks corresponding to the number of the isoaccepting tRNA species relative to the examined radioactive aminoacid. Fig. 2 demonstrates that is the case: when L-[5-^{3}H]-tryptophan-tRNA was isolated by gel electrophoresis and chromatographed, only two UV-absorbing peaks with retention time 172 and 173 were detected. The precision of the separation was determined by comparing the retention times values of the two peaks obtained by chromatography of L-[5-^{3}H]tryptophan-tRNA to the equivalent peaks (i.e. peaks having the same retention time) from chromatograms of entire hepatic tRNA population: the relative data are reported in Table 1. Taken together, the data from Fig. 2 and Table 1 demonstrate unequivocally that the single peaks derive from single tRNA species and clearly show the high accuracy of the method. Similar specific resolutions

were also obtained for iso-tRNAs relative to other aminoacids (manuscript in preparation). We are currently in the process of deriving a complete high performance map of rat liver cytoplasmic tRNAs.

DISCUSSION

Transfer RNAs form a large family of 80-90 molecules with physico-chemically related properties. The multiplicity of tRNA species for a single aminoacid is explained by the degeneracy of the genetic code and the wobble in the third anticodon site. In the present work tryptophan-tRNA was resolved into two peaks: possibly that might indicate the two isoaccepting species caused by the wobble in the third anticodon site. The significance of the great number of isoaccepting species might consist in the speeding up protein synthesis by the use of alternative tRNAs. This would mean a regulatory role of tRNA in gene expression and modulation at translational level. On the other hand, central to any hypothesis or experimental design on tRNA role is the possibility of quantitatively and qualitatively monitoring the complex mixture of tRNAs during the various phases of cell cycle as well as under different growth and development conditions. So far, this possibility has remained beyond the reach of experimental probing, mainly because of the difficulty of efficiently separating the various tRNA species.

This study represents the completion of the first phase of a program directed toward the development of HPLC tRNA mapping as a method of studying the role of tRNA changes in gene regulation. The experiments here reported validate HPLC acetonitrile/ammonium acetate gradient as methodology having the requirements of reproducibility and specificity, and able to generate a complete tRNA chromatographic map. Data of Table 1 are an example of the high specificity by which this procedure resolves tRNAs into chromatographic peaks corresponding to specific tRNA species. Another important feature of this technique, inherent to the determination of a possible regulatory role to tRNA, is its ability to monitor the minor tRNA species that appear to play an important role

in modulating the rate of protein synthesis (19). Thus tRNA fractionation by this HPLC procedure may add a dimension to comparative analyses of the relative distribution patterns of tRNA species in order to assess eventual tRNA involvement in modulation and regulation of gene expression at translational level.

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