Enrichment and characterization of the mRNAs of four aminoacyl-tRNA synthetases from yeast

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ABSTRACT

We have partially purified the messenger RNAs for yeast arginyl-, aspartyl-, valyl-, α and β subunits of phenylalanyl-tRNA synthetases in order to study their biosynthesis and ultimately, to isolate their genes. Sucrose gradient fractionation of poly U-Sepharose selected mRNAs resulted in a ten fold enrichment of the in vitro translation activity of these mRNAs. The translation products of messenger RNAs for arginyl- and valyl-tRNA synthetases have the same molecular weight as the purified enzymes; translation of aspartyl-tRNA synthetase messenger RNA yielded a 68 kD molecular weight polypeptide (while the purified crystallisable enzyme appears as a 64-66 kD doublet, which, as we showed is a proteolysis product). The translation of the mRNAs for α and β phenylalanyl-tRNA synthetase gave polypeptides having the same molecular weight as those obtained from the purified enzyme, but the major translation products are slightly heavier, indicating that they may be translated as precursors. As estimated from centrifugation experiments mRNAs of arginyl-, aspartyl-, α and β subunits of phenylalanyl-tRNA synthetase were 1700-2000 nucleotides long, indicating that α and β are translated from two different mRNAs.

INTRODUCTION

Aminoacyl-tRNA synthetases play a key role in the cell. The most obvious one is to ensure the correct attachment of each aminoacid to its corresponding tRNA (1). But together with tRNAs they are also directly or indirectly involved in multiple other processes (for a review see (2)). For instance tRNAs or aminoacyl-tRNAs are involved in the regulation of many bacterial operons (for a review see (3)) or in the pleiotropic stringent response (for a review see (4)). A regulatory role has also been postulated for aminoacid transport (5,6) and for initiation of replication in mammalian cells (7,8).

Although they must be constitutive enzymes, their synthesis can be affected by variations of the level of the cognate aminoacid or the growth rate of the cell (9,10,11) and the level of each of aminoacyl-tRNA synthetases seems to be adapted to the composition of the proteins being synthesized (12). Except for some E.Coli aminoacyl-tRNA synthetases (13,14,15) very few
informations are available concerning the mechanisms of the regulation of these enzymes.

Aminoacyl-tRNA synthetases are also part of extensively studied enzymologic or protein-nucleic acid interaction models (for review see (14,15)). Further progresses in such studies rely for a large part on the knowledge of the primary structure of these enzymes. These structural informations can be rapidly obtained by sequencing the corresponding cloned genes, the isolation of which being also a necessity for studying the regulation of the biosynthesis of these enzymes.

Some aminoacyl-tRNA synthetases genes from bacterial or yeast have already been cloned and sequenced: E.Coli alanyl- (14), threonyl-, phenylalanyl- (16), tryptophanyl- (17), methionyl- (18); Bacillus stearothermophilus tyrosyl-tRNA synthetase (D. Barker personal communication) and yeast methionyl-tRNA synthetase (19). Some data about their regulation have been obtained (14, 15).

We are interested in cloning the genes of several aminoacyl-tRNA synthetases from yeast for structure and regulation studies. These enzymes have been extensively studied in our laboratory: arginyl-tRNA synthetases (ArgRS) (20) a 70 kD monomer; aspartyl-tRNA synthetase (AspRS) (21) demonstrated here to be an $\alpha_2$ (68 kD) dimer, phenylalanyl-tRNA synthetase (PheRS) an $\alpha_2\beta_2$ (a:73 kD, $\beta$:63 kD) tetramer (22) and valyl-tRNA synthetase (ValRS) a 120 kD monomer (23).

No yeast mutant being available for these enzymes, we chose to use the classical technique of first cloning the cDNAs of the corresponding messenger RNAs. This report describes the enrichment, the in vitro translation, the partial characterization of these messenger RNAs and immuno-assays for the in vitro or in vivo synthesized aminoacyl-tRNA synthetases.

MATERIALS AND METHODS

Protein A-Sepharose and Poly U-Sepharose were from Pharmacia (Uppsal, Sweden). Tris, bovine serum albumine and SDS were from Sigma (St.Louis, USA). Dowex AG50I X 8 was from BioRad (Touzart et Matignon, Vitry s/Seine, France). Rivanol (2'-ethoxy-6-9-diaminoacridine lactate) was from ICN Pharmaceuticals Inc. PAGE blue 83, acrylamide and NN'Methylenebisacrylamide were from BDH; TEMED was from Kodak; Radiolabeled aminoacids were from C.E.A.. 2-mercaptoethanol and Triton X 100 were from ROTH.

All other chemicals or reagents were analysis grade from Merck or Prolabo.
Aminoacyl-tRNA synthetases and tRNAs

The enzymes have been purified from commercial yeast (a gift from Société Industrielle de Levure FALA, Strasbourg) according to previously described procedures (20-23) unfractionated yeast tRNA was from Boehringer (Mannheim, Germany).

Antibodies

Antibodies were raised in rabbits in a similar way for each of the purified synthetases. For each rabbit 1 ml of 10 mM phosphate buffer pH 7.0, 140 mM NaCl containing 1 mg of protein was emulsified with incomplete Freund's adjuvant and injected intramuscularly. Three injections were given to the rabbit, one every two weeks. The rabbits were first bled six weeks after the first injection and then every three weeks. IgG were occasionally purified by selective precipitation with rivanol according to (24).

Immunoadsorption of in vivo or in vitro synthesized aminoacyl-tRNA synthetases

For in vitro synthesized enzymes (see below), immunoadsorption was generally conducted in 200 μl of 10 mM phosphate buffer pH 7.0, 140 mM NaCl, 1 mg/ml bovine serum albumine. PheRS, for instance was first allowed to react 15 min with 1 μl anti PheRS serum, then 600 μg protein A-Sepharose were added in 20 μl of a stock solution containing 50% glycerol, 10 mM phosphate buffer pH 7.0, 140 mM NaCl. Binding of the conjugate on protein A-Sepharose was obtained by gentle agitation in a stoppered 1.5 ml Eppendorf conical tube during 45 min. In order to reduce non specific binding, a first adsorption was done in the same way in the presence of non immune serum. Protein A-Sepharose was then centrifuged and washed three times by successive resuspension in 10 mM phosphate buffer pH 7.0, 140 mM NaCl, 0.05% (v/v) 2-mercaptoethanol and centrifugation. Before the last washing, protein A-Sepharose was transferred in a new centrifuge conical tube to avoid the contamination of the proteins adsorbed on the tube. The immunoadsorbed proteins were finally liberated by heating 2 min at 100°C in the presence of 30 μl 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 100 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue (dissociation buffer).

The first supernatant was saved for the immunoadsorptions of the other synthetases which were conducted in a similar way except for PheRS which was sometimes immunoadsorbed overnight.

Immunoadsorption of non radioactive proteins either pure or in a crude extract was conducted in the same way with the single exception that ten times more antibodies were used.
**SDS gel-electrophoresis**

Immunoadsorbed proteins were analysed on SDS gels according to Laemmli (25). After electrophoresis the gel was immersed two times 30 min in a 5% (w/v) TCA bath then stained with PAGE blue.

**Preparation of total yeast RNA**

Two techniques were used. In the first one 400 g of yeast (harvested in exponential growth phase) were mixed with 400 ml of phenol saturated with a 100 mM acetate buffer pH 5.0, 1 mM EDTA, 0.5% (w/v) SDS, then ground in a Dyno-mill grinder with glass beads of 0.45 mm diameter. The mixture was extracted four times by an equal volume of phenol-chloroform (0.5, 0.5 v/v), rendered 0.2 M NaCl and precipitated during 2 hours at -20°C after addition of two volumes of absolute ethanol. The pellet was resuspended in 3 M potassium acetate, 5 mM EDTA and centrifuged; this was repeated three times. The final pellet was dissolved in 200 ml water and ethanol precipitated. The second technique was according to SCHATZ (personal communication): as follows little chunks of frozen yeast were ground in a Virtis 45 homogenizer in liquid nitrogen. 120 g of powder were dumped in 400 ml of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4 and 0.5% SDS and homogenized; then 500 ml of water-saturated phenol, chloroform, isoamylic alcohol (25:24:1 v/v) was added. The mixture was agitated 5 min and centrifuged; the supernatant was saved and the bottom phase and the interphase were reextracted with 500 ml of a 10 mM Tris-HCl pH 7.4, 1 mM EDTA solution. After centrifugation, the supernatant was pooled with the first one.

These pooled solutions were reextracted with 200 ml of water saturated phenol and chloroform (1:1 v/v) two or three times until the bottom phase was clear. The solution was made 300 mM sodium acetate and nucleic acids were precipitated by two volumes of pure ethanol. The precipitate was redissolved in 100 ml water, this solution was made 3 M LiCl and left to precipitate 4h at 0°C. The precipitate was redissolved in water and then ethanol precipitated and washed with 70% ethanol.

The first procedure allows the preparation of very large amounts of RNA but the second seems to give a better RNA in terms of translation.

**Cautions against RNA degradation**

All the glassware was baked overnight at 180°C. Tubings, flow cells or non heatable containers were treated 24 hours with 100 mM NaOH, then rinsed thoroughly with sterile water: solutions were autoclaved whenever it was possible and treated for 12 hours at 37°C with a solution of 0.1% diethylpyrocarbonate. Diethylpyrocarbonate was destroyed by heating 2 hours at 70°C. All solutions were made with bidistilled sterile water.
Selection of polyadenylated RNAs on oligo dT-cellulose

The selection of polyadenylated RNAs on oligo dT-cellulose was done under the conditions described by Aviv and Leder (26). The RNA was dissolved (0.5 -5 mg/ml) in bidistilled water, then maintained 5 min in a 95°-100°C water bath then chilled. The solution was made 500 mM NaCl, 0.2% SDS, 1 mM EDTA, 10 mM Tris-HCl and loaded on an oligo dT-cellulose column (1 g of oligo dT-cellulose for 10-30 mg of total RNA) at a 0.5-2 ml per min flow rate. After extensive washing by the same solution, the polyadenylated (poly A+) enriched RNA was eluted with a 10 mM-Tris HCl pH 7.5, 1 mM EDTA, 0.2% SDS solution; the eluted product was heated again and made 500 mM NaCl and the same cycle was done again on the same oligo dT-cellulose column.

Selection on polyadenylated RNAs on poly U-Sepharose

The RNA solution was first heated then chilled as above, made 700 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA and 25% (v/v) formamide previously deionized by a 3-5 hours agitation with the ion exchanger Dowex AG 50 X 8 (5 g/100 ml). The final concentration of RNA was adjusted to about 1 mg/ml. The solution was loaded on a poly U-Sepharose column (3-4 mg of total RNA per ml of gel) at 1 ml/min flow rate. After extensive washing with the same solution the poly A+ RNA was eluted with a solution 90% formamide, 50 mM Tris-HCl pH 7.5. The solution was then made 150 mM NaCl and the RNA was finally precipitated by two volumes of ethanol. A second cycle was usually done.

RNA fractionation by centrifugation in isokinetic sucrose gradients

These gradients were formed according to the tables calculated in (27). The top solution was 10% sucrose, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.2% SDS; the heavy solution was 32% sucrose, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.2% SDS. Before loading, 50 to 150 µg RNA were heated 2 min at 100°C in 100-200 µl water, then immediately chilled. The run was made in a SW 41 Beckman rotor during 12 hours at 34000 rpm at 20°C. The gradients were collected by pumping the solution from the bottom of the tube. Optical density was continuously monitored and 0.5 ml fractions were collected. The fractions were rendered 150 mM NaCl and ethanol precipitated with 20 µg yeast tRNA as carrier. In order to eliminate the SDS, two additional dissolutions and reprecipitations were done, the pellets were then washed twice with 70% ethanol 100 mM NaCl and once with absolute ethanol, dried under vacuum and finally redissolved in 15 µl water. 2 µl of these solutions were generally used for in vitro translation.

In vitro translation and quantitation of translated aminoacyl-tRNA synthetases

For in vitro translation we used rabbit reticulocyte lysates, either
prepared according to (28) or bought from NEN and used as specified by the manufacturer. We used generally a 25 μl final volume with optimal concentrations of mRNA, Mg$^{++}$, K$^+$ and yeast tRNA.

After a 90 min incubation, the reaction was stopped by chilling and 1 μl aliquots were spotted on Whatman 3 MM paper discs which were then processed as described under figure 1, except that the last TCA wash was made at 100°C. This allowed the determination of the total [$^{35}$S] radioactivity incorporated in the proteins. The immunoadsorption of the synthetases was then conducted as described above. The immunoadsorbed proteins were liberated in 30 μl of dissociation buffer and 5 μl aliquots were taken and processed as above to determine the radioactivity immunoadsorbed. The bulk of the suspension was

![Diagram of immunoadsorption of aminoacyl-tRNA synthetases](image)

**Figure 1: Immunoadsorption of the aminoacyl-tRNA synthetases**
The translations were done in 25 μl final volume with partially purified mRNAs. Immunoadsorption, SDS electrophoresis, autoradiography and scanning were conducted as described in "Methods". PheRS was immunoadsorbed overnight. The immunoadsorption was done in the presence of anti-ArgRS (1)(2); anti-ArgRS + anti-ValRS (3)(4); anti-AspRS (5)(6); anti-PheRS (7)(8) and of 100 μg competitor protein ArgRS (2); ValRS (4); AspRS (6) and PheRS (8). The arrows indicate the migration of the purified enzymes.
loaded on a 1 mm thick 10% polyacrylamide SDS slab gel. Electrophoresis was conducted as above. The gel was then dried and autoradiographed with Kodak film XR1 during various times. When only the aminoacyl-tRNA synthetases bands were visible, the proportion of them in the radioactive proteins was deduced immediately from the measures given by the aliquots. When contaminant bands were visible, we scanned the film with a Transidyne 2955 gel densitometer and determined the proportions of synthetases in the immunoprecipitate from the areas of the different peaks. In that case we verified that in our conditions, the darkening of the film for a given band was proportional to time.

Figure 2: Immunoadsorption of AspRS and PheRS on fresh yeast crude extracts. Yeast, harvested in logarithmic phase was resuspended in 50 mM Tris-HCl pH 7.5, 30 mM KCl, 5 mM glutathione. 1 ml of this suspension was added to 2 ml of glass beads (0.45 mm diameter) in a 15 ml conical centrifuge tube. Yeast was ground by agitation of this tube with a table top vortex during 2-5 min (B. Winsor: personal communication) 2 ml of the above buffer was added and the preparation was centrifuged 20 min at 5000 rpm. Protein concentration was determined according to B. Ehresmann (29) and 1.5 mg proteins were taken and immunoadsorption was performed in 0.5 ml final volume. Immunoadsorption done in the presence of non immune serum: lines 2, 4; anti AspRS: lines 5, 6, 7, 8; Anti PheRS: lines 9, 10, 11, 12. An excess of 200 µg AspRS: lines 6, 8 or 200 µg PheRS: lines 10, 12. Some samples of crude extract were preincubated 6 hours at 37°C before immunoadsorption: lines 3, 4, 7, 8, 11, 12. No serum was added in lanes 1 and 3. Some slots were loaded with molecular weight markers (31, 43, 67, 94 kD) or purified AspRS respectively 0.5, 1 and 2 µg of purified enzyme: lines 13, 14, 15 or PheRS: lines 16, 17, 18.
RESULTS AND DISCUSSION

Immunodetection of in vitro translated aminoacyl-tRNA synthetases

After in vitro translation, aminoacyl-tRNA synthetases were immuno-adsorbed either sequentially or ValRS and ArgRS simultaneously and then AspRS and finally PheRS. The immunoadsorbed products were analysed by SDS gel electrophoresis and autoradiography. Immunoadsorption of ValRS and ArgRS gave major bands co-migrating with the purified enzymes (figure 1); we verified that the addition of a large amount of purified enzyme led to the disappearance of the corresponding radioactive band. If ArgRS and ValRS are immunoadsorbed simultaneously only the radioactive ValRS band was competed out when 100 μg of purified ValRS was added in the immunoadsorption mixture. This shows that we can specifically and unambiguously detect and quantitate ArgRS and ValRS after in vitro translation.

There is a single polypeptide (about 68 kD) immunoadsorbed in the presence of anti AspRS and specifically displaced by purified AspRS while purified AspRS appears as a doublet (about 64 and 66 kD).

Immunoadsorption of PheRS yielded generally two polypeptides co-migrating with the α and β subunits of the purified enzyme. But there were also other bands, heavier than α and β. All these polypeptides are displaced by purified PheRS. Quantitation from these data shown that each of the four antibodies allowed the specific adsorption of 0.02-0.05% of the total counts when the translation system was programmed with total poly A+RNA.

Immunoadsorption of AspRS and PheRS from a crude extract

The above observation could be explained if the mRNAs for AspRS and at least the α subunit of PheRS were in vivo translated as precursor proteins.

Figure 3 : Sucrose gradient fractionation of poly A+ RNA

A. Sucrose gradient fractionation of RNA selected on oligo dT-cellulose.
   Selected RNA heated 3 min at 100°C (---) or not heated (...), non selected RNA (——).
   The arrows indicate the position of 18 and 28S RNAs.

B. Sucrose gradient centrifugation of RNA selected on poly U-Sepharose. Optical density profile.

C. The RNA in fractions 5 to 16 was translated aliquot was submitted to SDS gel electrophoresis.
   The gel was dried and autoradiographed.

D,E,F The remaining translation mixture was submitted to 3 immunoadsorptions in the presence of
   D : anti-ArgRS + anti-ValRS
   E : anti-AspRS
   F : anti-PheRS

The immunoadsorbed proteins were then analysed in the same way as the total proteins.
Another more trivial explanation could be that these two enzymes underwent limited proteolysis during purification. To test this hypothesis, we conducted immunoadsorptions on fresh yeast crude extracts. Figure 2 shows that actually in the crude extract AspRS immunoadsorption yielded a single band, slightly heavier than the doublet of purified AspRS. These results indicate that probably, the purified AspRS is a proteolysed form. Moreover, in the crude extract, the heavy form of AspRS seemed quite protected from proteolysis since a 7 hours incubation at 37°C did not result in any visible degradation.

On the contrary for PheRS, figure 2 shows that in the crude extract, in the conditions used, the immunoadsorbed polypeptides are identical to α and β subunits of PheRS. This indicates that proteolysis must occur very soon after grinding. An alternative explanation is that α and β subunits could be synthetized as precursors.

This type of experiments was also used to estimate the proportion of ArgRS, AspRS, PheRS, and ValRS in the crude extract. The amount of specifically immunoadsorbed proteins was estimated from this type of gel by comparison to a scale of standards. This allowed us to estimate to 0.02% the proportion of each of the four aminoacyl-tRNA synthetases in the crude extract. This is quite comparable to the proportion found for the specific mRNAs from in vitro translation. (figure 2).

Selection of polyadenylated mRNA (poly A⁺ RNA)

We used both oligo dT-cellulose and poly U-Sepharose. The yield in RNA for both systems was 2-5% of input RNA, but poly U-Sepharose was found more effective for selecting yeast polyadenylated RNAs than oligo dT-cellulose. Indeed, in spite of repeated cycles of heating and chromatography on oligo dT-cellulose it can be seen in fig. 3A that the contamination by ribosomal RNA is very important. Contamination after two cycles of selection on poly U-Sepharose, though lower, is still quite visible. Further cycles did not improve the purity of the poly A⁺ enriched mRNAs.

This contamination can be explained by aggregation of RNAs. This aggregation was clearly seen when we centrifuged on sucrose gradient an oligo dT-cellulose selected RNA without previous heating (figure 3A). Aggregation was also a problem when fractionating the poly A⁺ enriched RNA. This is probably the reason of the existence of a heavy form of ArgRS mRNA (figure 4B) which was sometimes observed.

Sucrose gradient fractionation of poly A⁺ RNAs

Figures 3 and 4 show the results of typical mRNA fractionation. Each fraction was translated, analysed by immunoadsorption and gel electrophoresis,
Figure 4: Localisation of mRNA for different aminoacyl-tRNA synthetases after sucrose gradient centrifugation

Quantitation of mRNA activities after centrifugation of poly U-sepharose selected RNA (A,B) or after recentrifugation of pool I (C) or pool II (D,E). Arrows indicate the positions of 18S and 28S.

- [35S]methionine incorporated into total proteins (---), in ArgRS (●●●) in AspRS (▲▲▲) in PheRS (●●●) in ValRS (●●●).

as described under methods. mRNA for ValRS on one side and mRNAs for AspRS, ArgRS and PheRS on the other side were separated. Enriched fractions were pooled, ethanol precipitated, redissolved in about 100 µl of water and recentrifuged in the same conditions.

When in vitro translation system was programmed with these purified mRNAs, anti ValRS and anti ArgRS allowed the specific adsorption of 0.4% of total counts, showing a ten fold enrichment of these messenger RNAs. Figure 4 shows that the enrichment for PheRS and AspRS mRNAs should be close to the one of ArgRS.
Estimation of the molecular weights of the mRNAs for ArgRS, AspRS, PheRS and ValRS

Centrifugation in isokinetic gradients can be used to estimate the size of nucleic acid molecules. We used as standards the RNA species constituting the genome of Alpha Alpha mosaic virus (AMV) kindly provided by Dr. L. PINCK. These RNAs were centrifuged in the same conditions as the poly U-Sepharose selected RNAs (figure 5). According to the scale given by AMV RNAs we could estimate the size of the considered mRNAs. The length of these molecules is 1700-2000 nucleotides for mRNAs of ArgRS, AspRS and α and β PheRS subunits; this shows that these subunits are translated from two different mRNAs; mRNA ValRS is much longer (3700 nucleotides). The comparison of these data with the length of corresponding translation products also shows that the non translated region of these mRNAs must be short.
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ABBREVIATIONS

IgG (Immunoglobulines), Tris (Tris(hydroxymethyl)amino-methane), ATP (Adenosine triphosphate), SDS (Sodium dodecyl sulfate), EDTA (Ethylene diaminotetraacetic acid), poly A RNA (polyadenylated RNA), BSA (Bovine serum albumin), TEMED (NNN'N' Tetramethyl-ethylenediamine). PAGE, polyacrylamide gel electrophoresis.

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