A Specific Hepatic Transfer RNA for Phosphoserine*

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Abstract. Radioactive O-phosphoryl-L-serine was detected after alkaline deacylation of rat and rooster liver [3H]seryl-tRNA acylated in vitro with homologous synthetases. Ribonuclease treatment of this tRNA yielded a compound with the properties of phosphoseryl-adenosine. Benzoylated DEAE-cellulose chromatography of seryl-tRNA yielded four distinct peaks, only one of which contained phosphoserine. A unique fraction for phosphoserine was also found on chromatography of nonacylated tRNA. In ribosome binding studies, this fraction responded very slightly with poly(U,C), but not with any of the known serine trinucleotide codons. Substantial incorporation of [3H]-serine into protein from this tRNA species was observed in an aminoacyl-tRNA dependent polysomal system derived from chick oviducts. No phosphoserine was found in Escherichia coli or yeast seryl-tRNA acylated with homologous enzymes, nor in E. coli seryl-tRNA acylated with liver synthetase. In the absence of tRNA, free phosphoserine was not formed in reaction mixtures, which suggests that phosphoseryl-tRNA arises by phosphorylation of the unique seryl-tRNA species. These results demonstrate a discrete tRNA species in rat and rooster liver containing phosphoserine and suggest that this tRNA is involved in ribosomal polypeptide synthesis.

There are several species of tRNA for most amino acids (so-called isoaccepting tRNAs). In a few instances, the amino acid on one of these isoaccepting species is modified for a specific cellular function. The most extensively studied example is that of the formylation of a discrete methionyl-tRNA species to yield formylmethionyl-tRNA,2 which serves as an initiator of polypeptide synthesis. In addition, in certain bacteria, glutaminyl-tRNA is synthesized by the amidation of a specific fraction of the tRNA species which accept glutamic acid.3,4 A unique isoaccepting rat liver glutaminyl-tRNA is specifically cyclized to pyrrolidone carboxylic acid-tRNA.5 During studies on estrogen-induced changes in rooster liver tRNA, a specific fraction of the isoaccepting tRNAs for serine was found which contained phosphoserine. This communication reports the isolation and characterization of this phosphoseryl-tRNA.

Materials and Methods. Preparation of tRNA, aminoacyl-tRNA synthetases, and aminoacyl-tRNA from rat and rooster liver was carried out as previously described.6 E. coli (strain W) and baker's yeast tRNA were obtained from General Biochemicals, [U-3H]l-serine (specific activity 3.79 Ci/nmol) from New England Nuclear, GTP and O-phosphoryl-L-serine from Sigma, and RNase A, crystalline trypsin, and E. coli alkaline

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phosphatase from Worthington. Alkaline phosphatase was purified free from nuclease activity.\textsuperscript{7} $\gamma$-$^{32}$P$^{32}$P$^3$ATP was either obtained from International Chemical & Nuclear (specific activity >10 Ci/mMg) or prepared according to Glynn and Chappell.\textsuperscript{8} Enzymes and substrates for this preparation were from Boehringer-Mannheim and $^{32}$P was from New England Nuclear.

**Benzoylated DEAE-cellulose (BD-cellulose) chromatography of tRNA:** The details of this procedure are published elsewhere.\textsuperscript{6} The amount of tRNA applied to the column (0.9 × 20 cm) was adjusted to 2 mg by adding unacylated homologous tRNA. Elution was performed at room temperature with a NaCl gradient (400 ml; 0.6 to 1.0 M NaCl containing 0.01 M MgCl$_2$ and 5 mM sodium acetate, pH 4.43), followed by a NaCl plus ethanol gradient (80 ml; 1.0 M NaCl to 1.5 M NaCl–14% ethanol containing 5 mM sodium acetate, pH 4.43). Fractions were prepared for liquid scintillation counting as previously described.\textsuperscript{4}

**Ribosomal binding of seryl-tRNAs** was measured according to Leder\textsuperscript{9} using *E. coli* K 12 ribosomes prepared by the method of Nirenberg.\textsuperscript{10} Trinucleoside diphosphates were a gift from Dr. M. Nirenberg and poly(U,C) was from Miles Laboratories.

**Aminoacyl-tRNA dependent polysomal system:** Polysomes and a partially purified preparation of aminoacyl transfer factor from chick liver were a gift from Dr. R. Palmiter. Polysomes were prepared by sucrose density gradient centrifugation of chick oviduct extracts from animals receiving estrogen.\textsuperscript{11} The transfer factor preparation was made according to Moldave,\textsuperscript{12} omitting the gel filtration step.

**Paper chromatography and electrophoresis:** Descending chromatography on Whatman 3 MM paper was performed with solvents A (butanol–acetic acid–water, 12:3:5 by volume), B (ethanol–0.02 M citrate pH 4.8, 7:3), and C (ethanol–ammonia 90:5). High-voltage electrophoresis on Whatman 3 MM paper was at pH 3.5 (pyridine–acetic acid–water 1:10:89, 30 V/cm) or pH 1.9 (4% formic acid, 39 V/cm).

**Results. Identification of phosphoserine and phosphoseryl-adenosine from seryl-tRNA:** Paper electrophoresis at pH 3.5 of alkaline deacylation products of rat and rooster liver $[^3]H$ seryl-tRNA acylated *in vitro* with homologous enzymes showed, in addition to serine, another compound which was less cationic than serine. To determine whether the compound was derived from a specific tRNA$^{\text{Ser}}$ species, rooster liver $[^3]H$ seryl-tRNA was chromatographed on BD-cellulose. Four distinct peaks were observed, three eluting during the NaCl gradient and the fourth after addition of the ethanol–NaCl gradient to the column (Fig. 1). Alkaline deacylation and paper chromatography (solvent A) of peaks I, II, and III revealed radioactive serine exclusively. However, peak IV yielded, in addition to serine, a labeled compound migrating identically as O-phosphoryl-l-serine with solvent A (Fig. 2). The compound also migrated as phosphoserine with solvents B and C, as well as upon paper electrophoresis at pH 1.9 and 3.5. Treatment of the material with alkaline phosphatase changed its electrophoretic mobility (pH 3.5) to that of serine. The compound was labeled with $^{33}$P when prepared from seryl-tRNA acylated in the presence of $\gamma$-$^{32}$P$^3$ATP. Essentially identical results were obtained upon chromatography of rat liver seryl-tRNA.

To establish that the phosphoserine derived from peak IV was bound to tRNA in aminoacyl-ester linkage to the 3' terminal adenosine, doubly labeled peak IV was isolated by BD-cellulose chromatography of rooster liver $[^3]H$seryl-tRNA acylated in the presence of $\gamma$-$^{32}$P$^3$ATP. Electrophoresis of RNase A digestion products of peak IV revealed two spots of tritium radioactivity (Fig. 3b). One corresponded to seryl-adenosine (yielding $[^3]H$serine upon alkaline deacylation),
while the other migrated identically as the single cationic spot of $^{32}$P-radioactivity (Fig. 3a). Treatment with phosphatase at pH 5 of this doubly labeled material yielded a $^{3}$H-labeled compound migrating identically as seryl-adenosine (Fig. 3c), demonstrating the existence of phosphoseryl-adenosine and proving that peak IV contained phosphoseryl-tRNA.

Although phosphoseryl-tRNA was found in approximately equivalent amounts in rat and rooster liver seryl-tRNA, no phosphoserine was detected in deacylation products of E. coli or yeast seryl-tRNA acylated in vitro with homologous synthetases, or in E. coli seryl-tRNA acylated with rooster liver enzyme. In the absence of tRNA, no free $[^{3}$H]phosphoserine was detected by chromatography (solvents A and B) of liver acylation reaction mixtures containing $[^{3}$H]serine.

Specificity of tRNA$^{^{3}$Ser} species for phosphoserine: Unacylated rooster liver tRNA was chromatographed on BD-cellulose to determine whether the tRNA that contains phosphoserine is a unique tRNA or whether it elutes in the ethanol fraction because of the esterified phosphate. Acylation of the eluted tRNA fractions revealed the four tRNA$^{^{3}$Ser} species (Fig. 4). Deacylation of seryl-tRNAs prepared from the tRNA in peaks I, II, and III yielded only radioactive serine, while peak IV contained both $[^{3}$H]serine and $[^{3}$H]phosphoserine (Fig. 5), demonstrating that the acylated and phosphorylated tRNA$^{^{3}$Ser} species chromatographs in exactly the same way as the phosphoserine-containing tRNA.

Codon specificity of seryl-tRNA fractions: The acylated rooster liver tRNA$^{^{3}$Ser} peaks (Fig. 4) were examined for their binding to E. coli ribosomes at 0.02 M Mg$^{2+}$ (Table 1). Seryl-tRNA peak I bound with AGU and AGC, peak II with UCG, and peak III with UCU and UCA, but peak IV did not bind with
any of the known serine trinucleotide codons. Very slight binding of seryl-tRNA peak IV was observed with poly (U,C).

**Participation of seryl-tRNA fractions in polypeptide synthesis:** To determine whether phosphoseryl-tRNA participates in polypeptide synthesis, the incorporation of $[^3H]$-serine into protein was studied in a chick oviduct polysome system that was fully dependent upon added aminoaeryl-tRNA. The polysomes were derived from chick oviducts following a secondary estrogen stimulation in vivo known to induce a marked synthesis of ovalbumin by oviduct cells. This system was chosen because ovalbumin contains two phosphoserine residues per polypeptide chain. The incorporation from unfractionated $[^3H]$seryl-tRNA was dependent on GTP, polysomes, and aminoaeryl transfer factors, and the product was susceptible to trypsic hydrolysis (Table 2). Control reactions in which $[^3H]$seryl-tRNA was replaced with free $[^3H]$serine plus either 19 $[^14C]$amino acids or 20 $[^14C]$aminoacyl-tRNAs did not incorporate $[^3H]$serine into protein. $[^3H]$serine was incorporated into protein from all of the rooster liver $[^3H]$seryl-tRNA species. There was substantial incorporation with peak IV (Table 3). This incorporation showed characteristics identical with those of the unfractionated $[^3H]$seryl-tRNA, indicating that the phosphoserine-containing tRNA fraction participates in polypeptide synthesis.

**Discussion.** A new tRNA specific for phosphoserine has been found in rat and rooster liver. The mechanism of formation of phosphoseryl-tRNA remains to be clarified. It appears to arise by phosphorylation of a unique species of tRNA$_{56}$, with the phosphate group derived from the γ-phosphate of ATP. The specificity of the phosphorylation is quite high, since only one of four tRNA$_{56}$ fractions formed phosphoseryl-tRNA. However, this fraction was incompletely phosphorylated under the acylation conditions used. It is probable that the entire fraction constitutes a tRNA for phosphoserine, but...
that the optimal conditions (or possible cofactors) for the phosphorylation are unknown. The fraction containing phosphoserine does not appear to be heterogeneous since it eluted as a single peak in an ethanol–NaCl gradient, regardless whether it was acylated (and partially phosphorylated) or nonacylated. Furthermore, no evidence for heterogeneity of the acylated, but incompletely phosphorylated, fraction was observed in ribosome binding or polysome incorporation experiments.

**Table 1.** Ribosomal binding with [\(^3\)H]seryl-tRNA peaks.

<table>
<thead>
<tr>
<th>Addition (pmol)</th>
<th>[(^3)H]seryl-tRNA peaks (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>None*</td>
<td>0.05</td>
</tr>
<tr>
<td>UCU†</td>
<td>0.04</td>
</tr>
<tr>
<td>UCC</td>
<td>0.03</td>
</tr>
<tr>
<td>UCA</td>
<td>0.03</td>
</tr>
<tr>
<td>UCG</td>
<td>0.04</td>
</tr>
<tr>
<td>AGU</td>
<td>0.69</td>
</tr>
<tr>
<td>AGC</td>
<td>0.92</td>
</tr>
<tr>
<td>Poly(U,C)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Amount bound to ribosomes in the absence of trinucleotide.
† Amount bound to ribosomes in response to trinucleotide or poly(U,C). The amount bound to ribosomes in the absence of trinucleotide or poly(U,C) has not been subtracted from these values.
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Fig. 4. BD-cellulose chromatography of unacylated rooster liver tRNA. 30 mg of tRNA was chromatographed as in Fig. 1. The tRNA was recovered by combining every two consecutive fractions, precipitating with 2.5 vol of 95% ethanol at −20° C, and collecting the precipitates on Millipore filters. The tRNA was eluted from the filters with water; the eluate was analyzed for [3H]serine acceptance. The peaks of serine-accepting activity (shaded areas) were pooled, precipitated with ethanol, and lyophilized.

The significance of the failure of the phosphoserine-containing tRNA to bind to ribosomes in the presence of the known serine trinucleotide codons is unclear. Moreover, peak IV showed a consistently meager response to poly (U,C) (compare peak III binding), suggesting that it is not the UCU and UCC-specific

Fig. 5. Electrophoresis at pH 3.5 of deacylation products of [3H]seryl-tRNA peaks isolated as in Fig. 4. [3H]seryl-tRNA was prepared from the isolated peaks and aliquots of peaks I, II, and III were pooled. Deacylation was as in Fig. 2, and electrophoresis as in Fig. 3.
TABLE 2. Polysomal incorporation from unfractionated [3H]seryl-tRNA.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Incorporation (pmol)</th>
<th>% Incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>0.360</td>
<td>54.0</td>
</tr>
<tr>
<td>- GTP</td>
<td>0.024</td>
<td>1.2</td>
</tr>
<tr>
<td>- transfer factors</td>
<td>0.014</td>
<td>2.1</td>
</tr>
<tr>
<td>- polysomes</td>
<td>0.009</td>
<td>1.4</td>
</tr>
<tr>
<td>+ trypsin</td>
<td>0.024</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Reaction mixtures (0.5 ml) contained 0.09 M KCl, 0.01 M MgCl₂, 0.12 mM GTP, 1 mM DTT, and 25 mM TES pH 7.5; 95 μg of chick oviduct polyribosomes, 0.2 A₅₅₀ unit of rooster liver 20 [₁⁴C]-aminoacyl-tRNAs, 0.8 mg of transfer factor preparation, and 0.665 pmol of unfractionated rooster liver [₃H]seryl-tRNA. Incubation for 20 min at 37°C was followed by an additional 5 min incubation in the presence of RNase A (15 μg). Where indicated, crystalline trypsin (1 mg) was added for 30 min at 37°C. Two volumes of ice-cold 10% trichloroacetic acid were added, and after 30 min at 0°C, the precipitates were collected on glass fiber filters. The precipitates were solubilized for counting by incubation of the filters with NCS-solubiliser (30 min at 37°C). Counting efficiency was about 40% in a toluene based fluor.

TABLE 3. Polysomal incorporation from [3H]seryl-tRNA peaks.

<table>
<thead>
<tr>
<th>Addition</th>
<th>[³H]seryl-tRNA peaks (pmol)</th>
<th>Incorporation (pmol)</th>
<th>% Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Complete system</td>
<td>5.72</td>
<td>1.59</td>
<td>7.54</td>
</tr>
<tr>
<td>- transfer factors</td>
<td>2.07</td>
<td>0.30</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>% Incorporation</td>
<td>35</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

Reaction mixtures were prepared and incubated as in Table 2. tRNA₅₅₀ fractions employed are described in Table 1.
residues) in roosters correlates with specific increases in two of four seryl-tRNA species. However, the amount of the phosphoserine-containing peak remained constant and was identical in every case with the control.

The incorporation of serine from the phosphoserine-containing tRNA peak by a polysomal system suggests that phosphoseretyl-tRNA is directly involved in ribosomal peptide bond synthesis. This incorporation was dependent on GTP and transfer factors and the product was digested with trypsin. The incorporation was not due to deacylation of the tRNA and subsequent amino acid incorporation since substitution of free [3H]serine for seryl-tRNA did not yield a radioactive product. Attempts to demonstrate the incorporation of $^{32}$P into protein from $^{32}$Pphosphoseryl-tRNA were not successful, because of the impurity of the liver synthetase preparation. Substantial $^{32}$P labeling of tRNA chains was observed when [γ-$^{32}$P]ATP was used in acylations and this radioactivity obscured any incorporation of $^{32}$Pphosphoserine.

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Abbreviations: BD-cellulose, benzoylated DEAE-cellulose; DTT, dithiothreitol; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

* A preliminary report of this work has appeared.†
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5 Bernfield, M. R., manuscript in preparation.