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In vivo overexpression and purification of *Escherichia coli* tRNA^{ser}

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DNA fragments corresponding to the sequences of *Escherichia coli* tRNA$_2^{ser}$ and amber suppressor tRNA^{ser}, were synthesized from overlapping oligonucleotides These were interposed between a strong promotor and a synthetic transcriptional terminator to ensure the production of a transcript of the correct size The genes of promotor, fragment and terminator were cloned into a conditional runaway replication plasmid. At temperatures below 37°C this vector has a low copy number but, following a temperature shift to 42°C, the copy number is no longer regulated. Using these constructs an overexpression of tRNA^{ser} of about 20 times the level of the wild-type pool could be obtained (corresponding e g. to 200 times the expression tRNA$_2^{ser}$). From these systems 10 mg quantities of tRNA^{ser}s could be isolated with a serine acceptance of 1,100 pmol/A_{280} unit.

Serine; tRNA; Aminoacyl-tRNA synthetase; Overexpression

1. INTRODUCTION

The specific recognition and interaction of aminoacyl-tRNA synthetases with their cognate tRNAs are fundamental steps in the protein biosynthetic pathway and in maintaining the fidelity of translation. One route to the study of this at the molecular level is the analysis by X-ray diffraction of crystals of the appropriate complex. Recently this has led to the description of these interactions for the glutaminyl complex of *Escherichia coli* [1] and the aspartyl system of yeast [2]. The enzyme component of the former is an example of a class 1 synthetase and that of the latter a class 2b synthetase [3,4]; in neither instance is a structure of the enzyme alone available. We have described the structure of seryl-tRNA synthetase from *E. coli* which is a class 2a synthetase [4,5] and wish to proceed to a structural analysis of the nucleoprotein complex.

Since the determination of the crystallization conditions of a synthetase:tRNA complex might require relatively large quantities of pure tRNA species we have undertaken the cloning and overexpression of synthetic genes coding for *E. coli* serine-specific tRNAs, in order to obtain the complex of tRNA^{ser} with seryl-tRNA synthetase. We report here the results obtained for the tRNA$_2^{ser}$ (*serU* gene product) and the amber suppressor tRNA^{ser}(*supD* gene product) [6].

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Abbreviations. BAPPS, *N,N'*-bis(3-aminopropyl)-piperazine substituted Sepharose; BSA, bovine serum albumin; TBE, tris borate EDTA buffer; TCA, trichloroacetic acid.

2. MATERIALS AND METHODS

2.1. *Bacteria, plasmids and reagents*

E. coli strain TG1 used is an EcoK-derivative of JM101 [7] with the genotype *supE hsdD5 thi Δ(lac-proAB)* F' {*traD* 36, *proAB*$^+$, *lacI*^{q} *lacZ ΔM15*}. The vector pUC19 was from Boehringer. Plasmid pGFIB-1 [8] was kindly provided by Jennifer Normanly (California Institute of Technology) and plasmid pOU71 [9] by Colin Hughes (Cambridge University) All radioactive chemicals were from Amersham. The sequencing enzymes (RNases U2, Phy M, B c. and T1) and T4 RNA ligase were from Pharmacia and 'Stains All' from Sigma

N,N'-Bis(3-aminopropyl)-piperazine substituted Sepharose (BAPPS) was prepared as previously described [10].

2.2. *Oligonucleotides synthesis and purification*

Oligonucleotides were synthesized on an Applied Biosystem 380 DNA synthesizer. These oligonucleotides were purified by electrophoresis on a 20% polyacrylamide/7 M urea gel, visualised by UV shadowing after ethidium bromide staining, excised and eluted in sterile water overnight at 4°C. After phenol/chloroform extractions the DNA was precipitated with 100% ethanol, washed with 70% ethanol, dried and resuspended in sterile water

2.3 *Gene synthesis*

Internal oligonucleotides (100 pmol) were phosphorylated in 50 mM Tris-HCl pH 7 6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 0.1 mM ATP, with 10 U of T4 polynucleotide kinase for 1 h at 37°C. The kinase was then inactivated by incubation at 65°C for 10 min. Oligonucleotides of the 5' ends of the fragment were kept unphosphorylated to avoid the concatemerization of the assembled fragment during ligation A mixture of 25 pmol of each oligonucleotide was heated to 95°C for 5 min, then cooled slowly to room temperature over a period of 12 h. Ligation of hybridized oligonucleotides was carried out in 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, 10 mM spermidine, 0 1 mg/ml BSA with 2 5 U of T4 DNA ligase at 15°C for 15–18 h. Electrophoresis on a 12% non-denaturing TBE-buffered polyacrylamide gel showed that the major ligation product corresponded to that expected for the tRNA gene coding fragment and was also used to purify this fragment.

2.4 *Construction of tRNA^{ser} overexpressing strains*

Different vectors were used to obtain overexpression of tRNA.

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Plasmids were cleaved by *Eco*RI and *Pst*I and were combined with the insert to give a weight ratio of 10:1 (insert/vector). Ligation was carried out under the conditions described above. The ligation mixture was used to transform competent *E. coli* TG1 cells. Screening of the transformants was done by plasmid DNA minipreparation [11] and restriction analysis. The plasmid DNA of the clone with an insert of the correct size were then sequenced by the chain termination method of Sanger et al. [12].

2.5. *Overproduction and purification of tRNA^{ser}*

Overproduction of $tRNA_2^{ser}$ and amber suppressor $tRNA^{ser}$ was carried out by growing 10 l culture of overexpressing clone at 37°C in LB medium supplemented with 50 mg/ml ampicillin to an A_{540} of 1.5. Then 8 l of LB Amp at 50°C were added. The culture was incubated further for 1.5 h at 44°C, followed by 30 min at 37°C. The cells were harvested by laminar-flow filtration and centrifugation.

To prepare unfractionated tRNA, wet cell paste (40–50 g) was lysed by incubation for 30 min in 125 ml of a buffer containing 63 mM Tris, 50 mM HCl pH 7.6, 1 mM EDTA and 50 mg lysozyme. After addition of 4 ml of 4% sodium deoxycholate and adjusting to 10 mM $MgCl_2$, DNA was digested with 50 U of RNase-free DNase I. The lysate was made 30 mM in NH_4Cl and then centrifuged for 40 min at $20,000 \times g$. The supernatant was centrifuged for 2 h at $100,000 \times g$ to eliminate ribosomes. The supernatant from this was extracted twice with an equal volume of phenol saturated with buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and finally with an equal volume of chloroform. The solution was made 0.3 M in sodium acetate pH 5.2 and precipitated overnight with one volume of ethanol. The recovered tRNA was resuspended in 10 ml 0.3 M sodium acetate pH 7. High molecular weight RNA was then precipitated by addition of 1/2 volume of isopropanol and incubation at room temperature for 1 h. After centrifugation a further 1/2 volume of isopropanol was added to the supernatant and incubated 1 h at 0°C to precipitate tRNA.

The precipitated tRNA was recovered by centrifugation, dissolved in 10 ml sterile water and applied to a BAPPS anion-exchanger column equilibrated with 1 M NaCl in 50 mM sodium acetate pH 5. The column was developed with a linear gradient from 1 M to 1.2 M NaCl, and finally by a step of 2 M NaCl. Fractions with $tRNA^{ser}$ acceptance activity were pooled and precipitated with ethanol. The $tRNA^{ser}$ pool from the BAPPS column was made 1.5 M in ammonium sulfate and applied to an FPLC phenyl-Superose HR 10/10 column (Pharmacia). This was developed with a negative gradient from 1.8 M to 0.8 M ammonium sulfate in 10 mM ammonium acetate pH 6.5. Fractions with $tRNA^{ser}$ acceptance activity were concentrated and desalted on a small column of DEAE-Sepharose CL-6B (Pharmacia) and precipitated with ethanol.

2.6. *Aminoacylation assays*

The reaction mixture (50 μl) contained 63 mM Tris, 50 mM HCl, 10 mM $MgCl_2$, 0.5 mM DTT, 2.25 mM ATP, pH 7.6, 20 μg/ml SRSEC, 0.1 mM [^{14}C]serine (50 mCi/mmol) and tRNA fraction. Incubation was 15 min at 37°C, and the reaction was stopped by the addition of 40 μl mixture to a Whatman GF/A filter prewetted with 20 μl TCA. Filters were washed 3 times with 5% TCA, once with ethanol, once with ethanol/ether and finally ether, then dried and counted.

2.7. *Sequencing of tRNA*

Purified tRNA (3 μg) was 3′-end labelled with [^{32}P]pCp using T4 RNA ligase [13]. The radioactive tRNA was separated from non-incorporated nucleotide with Nuctrap push columns (Stratagene). The tRNA was subjected to enzymatic sequencing as described previously [14].

3. RESULTS AND DISCUSSION

3.1. *Gene construction and overexpression*

The synthetic genes for $tRNA_2^{ser}$ and amber suppressor $tRNA^{ser}$ were each constructed from 7 overlapping oligonucleotides. Each tRNA gene sequence was flanked by an *Eco*RI cohesive-end at the 5′-end and a *Pst*I cohesive-end at the 3′-end. The oligonucleotides were annealed and ligated into the *Eco*RI/*Pst*I site of the plasmid pGFIB-I. This vector contains a synthetic promoter and a synthetic transcriptional terminator that provides the tRNA with functional 3′- and 5′-ends to avoid problems of extremity maturation. Since modifying enzymes can recognize both the sequence surrounding the nucleotide to be modified and the three dimensional structure [15], it was anticipated that these constructs would produce fully modified functional tRNA.

After ligation the reaction mixture was digested by *Sma*I to eliminate reconstructed vector molecules and used to transform competent TG1 cells. With the expression vector pGFIB-I (used for suppressor $tRNA^{ser}$ only) no positive clones were obtained. We considered that this might be due to a lethal effect of the overexpression of the suppressor tRNA. To verify this hypothesis we cloned the gene into the *Eco*RI/*Pst*I site of pUC19 (opposite to the direction of transcription from the lac promotor) to produce a non-functional transcript and to avoid instability due to gene expression. Plasmid DNA sequencing showed that, in this instance, we obtained an insert with the correct sequence. This was then cloned into a modified expression vector obtained by deleting the *lpp* promotor of pGFIB-I and replacing it by a synthetic weaker promotor based on the *trc* promotor (Tac 17) sequence [16] which had been constructed from four overlapping oligonucleotides. This promotor has, in contrast to Tac 16 only one possible transcriptional initiation site [17].

At the 5′-end of the fragment a *Nar*I restriction site was created and the last 4 bases at the 3′-end were modified to create an *Eco*RI site in order to keep +1 of the tRNA transcript in the correct position. This gave the plasmid pGTRC (Fig. 1). Overexpression of these constructs was tested by comparing the serine-specific acceptance activity of crude tRNA extracts, with an internal standard (valylation of $tRNA^{val}$). Only a low level of overexpression was observed for $tRNA_2^{ser}$ and suppressor $tRNA^{ser}$. The plasmid was very unstable, less than 1% plasmid-containing cells in an overnight culture. To avoid these stability problems, probably due to the overexpression from a multi-copy plasmid, we recloned the insert containing promoter-gene-terminator in pOU71; a runaway replication plasmid. The cloning of this DNA fragment was done in two steps. First plasmid pGTRC was digested by *Pvu*II, generating a 300-bp blunt-ends fragment. This fragment was then cloned into the *Sma*I site of pUC19. The resulting plasmid was digested by *Bam*HI and then partially digested by *Eco*RI. The longest fragment was isolated by electrophoresis on agarose gel and cloned in the unique *Eco*RI/*Bam*HI sites of pOU71 to give pOTSer2 and pOTSupD (Fig. 1). The orientation of the insert in pUC19, and

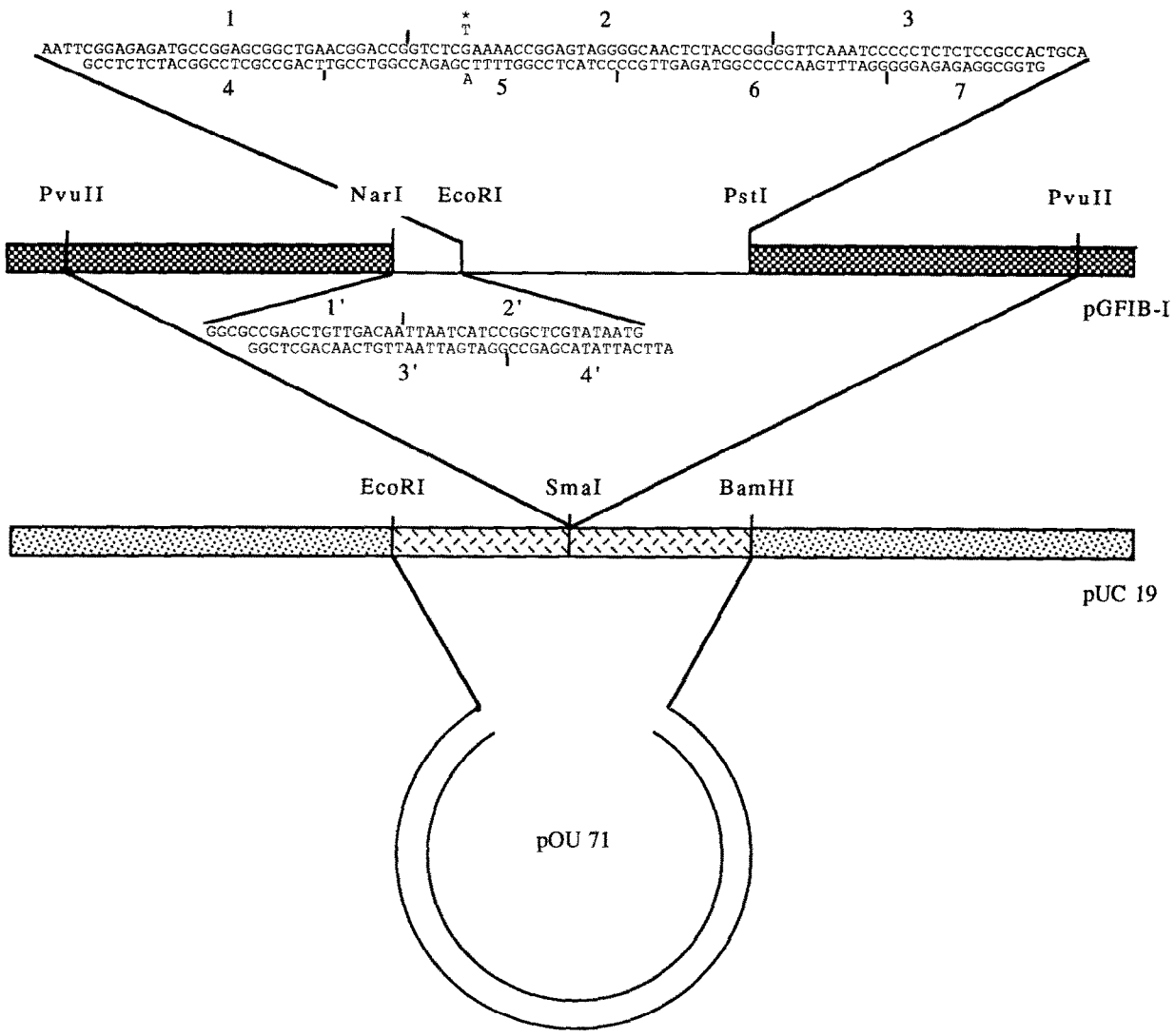

Fig. 1. Construction of a $tRNA^{ser}$ overexpressing plasmid. The lpp promotor is replaced by the *Trc* promotor construct from 4 oligonucleotides (1′, 2′, 3′, 4′), cloned in *NarI*/*Eco*RI site of pGFIB-I, to give pGTrc plasmid. The tRNA gene is cloned *Eco*RI/*Pst*I between the *Trc* promotor and the *rrnc* terminator. A *Pvu*II 300-bp fragment from pGTrc was recloned into the *Sma*I site of pUC19. The vector pUC19 is then digested by *Eco*RI/*Bam*HI and the new fragment is inserted into *Eco*RI/*Bam*HI cloning sites of pOU71 to give pOTSer2 or pOTSupD.
(*Difference between the two tRNAs: the central base of the anticodon in the amber suppressor is a U.)

after in pOU71, was not determined since the fragment contains a complete transcriptional unit producing a tRNA transcript whatever the orientation.

The plasmid pOU71 contains the λP_R promotor controlling expression of the replication control genes. In addition the plasmid contains the $\lambda cI857$ gene with its P_M promotor coding for the temperature sensitive λ repressor. The product of the *cI* gene controls the activity of the λP_R promotor. By increasing the temperature the λP_R promotor can be easily derepressed, allowing high expression of the replication control genes. With this construct below 37°C, the plasmid is present at one copy per chromosome, whereas at 42°C the replication of plasmid DNA becomes uncontrolled. After 1–2 h induction, 50% of the total DNA in the cell is plasmid, corresponding to more than 1,000 plasmid molecules/cell. With the plasmids pOTSer2 and pOTSupD expression of the cloned tRNA gene was inhibited at 37°C but was very high at 44°C. This tRNA expression system is stable at 37°C and allows at 44°C the production of total tRNA containing 20 times more $tRNA^{ser}$ than non-induced cells.

3.2. *Purification of tRNA*

Using the constructs described above 15 mg of tRNA could be obtained from 45 g of bacterial paste with a specific activity for $tRNA^{ser}$ of about 1,100 pmol/A_{260} unit as measured by aminoacylation assays (Table I). PAGE/urea analysis of tRNA from induced and non-induced cultures (Fig. 2) showed the appearance of a band in the induced sample with an M_r corresponding to the size of $tRNA_2^{ser}$ or amber suppressor $tRNA^{ser}$.

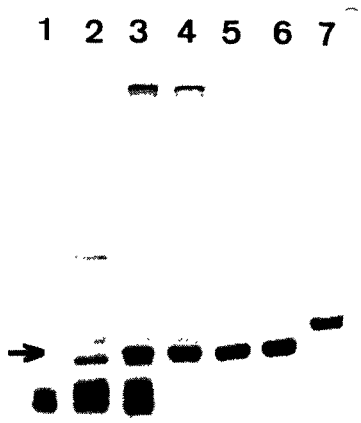

Fig. 2. Purification of *E. coli* $tRNA_2^{ser}$. 10% urea/polyacrylamide gel electrophoresis of purification fractions stained with 'Stains All'. Lane 1: *E. coli* $tRNA^{val}$ from Boehringer (activity: 1,100 pmol valine/A_{260} unit); lane 2, unfractionated tRNA from an overproducing construct without induction (37°C); lane 3, unfractionated tRNA prepared from induced culture (2 h at 43°C); lane 4, pool from BAPPS fractionation; lane 5 and 6, pool from FPLC phenyl-Sepharose by hydrophobic interaction chromatography (peaks 1 and 2 respectively); lane 7, *E. coli* $tRNA^{SelC}$ [20].

The first step in the fractionation by anion-exchange chromatography on BAPPS is very efficient due to the high affinity of $tRNA_2^{ser}$ for this matrix. Typically about 80 mg of $tRNA_2^{ser}$ with an acceptor activity of 700 pmol/ A_{260} unit can be obtained from 330 mg of unfractionated tRNA. The second chromatography step of hydrophobic interaction on phenyl-Superose

Table I

Purification of *E. coli* $tRNA_2^{ser}$

		Yield	Acceptor activity pmol/OD_{260}	% Purity
Crude extract		330 mg	250	19
BAPPS		79 mg	714	55
FPLC	Peak 1	8 mg	1,105	86
	Peak 2	9 mg	1,190	92

The crude extract was obtained from 45 g of induced cell paste. The acceptor activity was measured by the formation of [^{14}C]Ser-tRNA. The purity was estimated by comparing the measured acceptance activity of each pool to the theoretical maximum activity of the $tRNA_2^{ser}$. This maximal activity is determined by using a value of 30,900 as MW for the $tRNA_2^{ser}$ and an A_{260} of 1 for 40 μg of tRNA.

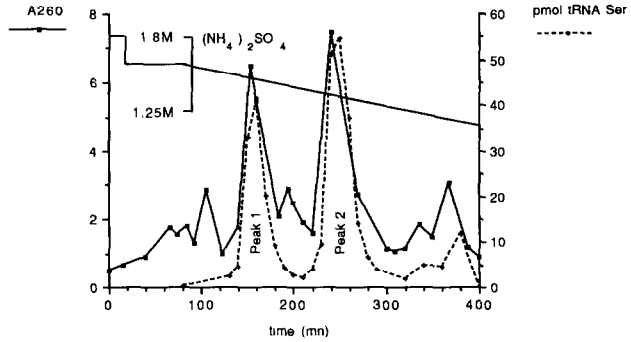

Fig. 3. Preparative scale separation of 20 mg tRNA from BAPPS by hydrophobic interaction chromatography on a phenyl-Superose HR 10/10 FPLC column. Reverse ammonium sulfate (in 10 mM ammonium acetate, pH 6.5) gradient from 1.8 M to 1.25 M at a flow rate of 0.8 ml/min.

produced two major peaks with $tRNA^{ser}$ acceptance activity (Fig. 3). Material from these two peaks has approximately the same specific activity of about 1,100 pmol/A_{260} units and analysis by PAGE showed that the species present in each peak had the same M_r. Since the relative area of the second peak increased when the rate of expression was lowered (induction at 40°C) we considered the possibility that the difference in chromatographic behaviour was due to variation in the modification of bases in the $tRNA^{ser}$ transcripts. (For the amber suppressor the same purification procedure was used and we obtained the same results.)

Consideration of the modified bases of $tRNA_2^{ser}$ indicated that the probable major effect on the chromatographic behaviour might be due to the modification of A37 by 2 methylthio-*N*6-isopentenyl-adenosine (ms2i6A). We think that this modification is sufficient to affect the recognition of A37 by RNase U2 or Phy M whereas other modifications such as 2-*O*-methylation of guanosine does not interfere with the recognition by RNase T1 [18]. Sequence studies of the fractions from the phenyl-Superose column indicated that tRNA in the first peak was cleaved at residue A37 and tRNA from the second was not. We assume that the differences in the $tRNA_2^{ser}$ and in the amber suppressor separated by this chromatographic step is due to modification of A37. These results seem to indicate that tRNA overexpressed in vivo from a synthetic gene can be modified, but in this process modifying enzymes are limiting. The accompanying report describes the crystallization of $tRNA_2^{ser}$ in a complex with *E. coli* seryl-tRNA synthetase [19].

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