CROATICA CHEMICA ACTA CCACAA **68** (3) 451–465 (1995)

ISSN 0011-1643
CCA-2245

Original Scientific Paper

A System for Studying the Specificity of Serylation with Yeast Seryl-tRNA Synthetase

Ivana Weygand-Đurašević

Department of Biochemistry, Faculty of Science, University of Zagreb, HR-10 000 Zagreb, Croatia

Department of Molecular Genetics, Ruđer Bošković Institute, HR-10 000 Zagreb, Croatia

Received November 30, 1994; revised January 30, 1995; accepted February 10,1995

Serylation is the covalent attachment of serine to a serine specific tRNA. It is catalyzed by the seryl-tRNA synthetase (SerRS). There are presumably two seryl-tRNA synthetases, encoded by different nuclear genes, that perform the serylation task in *Saccharomyces cerevisiae*. One works in the cytoplasm and the other in mitochondria. The gene for the cytoplasmic enzyme has been cloned, sequenced and can be functionally expressed both in yeast and in *Escherichia coli*. Its protein product is a 106 kD homodimer, which can be easily purified from bacterial and yeast overproducing strains. The enzyme recognizes six $tRNA^{Ser}$ isoacceptors and selenocysteine tRNA in yeast, as well as several non homologous tRNAs from prokaryotic and eukaryotic sources. By combining genetic and biochemical methods, a system for studying the recognition between yeast seryl-tRNA synthetase and tRNA substrates, both *in vivo* and *in vitro*, has been designed.

INTRODUCTION

A major element that ensures the accuracy of the genetic code translation is the recognition of tRNA by its cognate aminoacyl tRNA synthetases. A prokaryotic cell contains at least twenty aminoacyl tRNA synthetases,[1] each specific for one amino acid, and about sixty tRNA isoacceptors.[2] These numbers are somewhat higher in eukaryotic cells because the organelles contain some of their own synthetases as well as tRNAs. Thus each ami-

noacyl tRNA synthetase must distinguish its own set of isoacceptor tRNAs and efficiently catalyze the covalent attachment of the correct amino acid to the 3'end of only these species. The reaction is ATP-dependent:

$$AA + ATP + tRNA \rightarrow AA\text{-}tRNA + AMP + PPi$$

It has been found that aminoacyl-tRNA synthetases recognize a rather small set of nucleotides of their cognate tRNAs, called recognition elements. These determinants most frequently include the discriminator base[3] and often the anticodon,[4,5,6] or a part of acceptor stem[7,8] or both.[9,10] Irrespective of their location in tRNA, the determinants either interact directly with the enzyme or give the tRNA a conformation for a complementary fit with enzyme.[11–13] The second component of »tRNA identity« are nucleotides, referred to as »negative elements«, which protect tRNA from aminoacylation by a non-cognate synthetase.

Although the tertiary structure of tRNAs is conserved to a large extent, aminoacyl-tRNA synthetases are remarkable for their diversity in the sequence and oligomeric structure.[14] Based on the conserved sequence and structural motifs, the 20 synthetases are divided into two classes of 10 enzymes each.[15] Class I enzymes have a well-conserved *N*-terminal nucleotide binding (or Rossmann) fold responsible for amino acid binding, aminoacyl-adenylate formation and the interaction with tRNA acceptor helix, joined to a less conserved *C*-terminal domain responsible for anticodon loop binding. The second class of synthetases have been grouped according to two or three extended motifs which are incorporated into parts of the eight-stranded antiparallel β-structure and three α–helices that make up the core of the conserved structure characteristic of this group of synthetases. In the last five years important progress has been made in the determination of the crystal structures of four synthetase: tRNA complexes, those of the *Escherichia coli* glutaminyl-,[16] yeast aspartyl-[17] and seryl-system from both *E. coli*[18] and *Thermus thermophilus.*[13] Comparison of the structures of these two synthetase-tRNA complexes reveals quite different modes of interaction of tRNA with different catalytic domains of the two classes.

MATERIALS AND METHODS

Strains and plasmids

These are described in Table I. *SES1* gene was cloned in these vectors either as approx. 3-kb *SalI*/*Bam*HI fragment taken out of pUC19*SES1*,[19] which includes *SES1* promoter region within 350 bp of yeast *SES1* upstream sequence, *SES1* structural gene, approx. 900 bp of yeast DNA flanking *SES1* on the 3'side, and 375 bp *Eco*RI/*Bam*HI fragment from pBR322,

TABLE I

Strains and plasmids.

Strain	Genotype, description, source and/or reference
S. cerevisiae	
3A84	*ade1-UGA his4-260 leu2-2 ura3-52*[55]
S2088	MATa *ura3-52 trp1 lys2-801 leu2Δ1 his3-Δ200 pep4-HIS3 prb-Δ1.6R can1 GAL* (gift from B. Rockmill, Yale University)
E. coli	
XAC-A24	F' *ara argE(UAG) rpoB gyrA Δlac pro*/F' *lacI-Z proAB*$^+$ [41]
JR104	F' *trpA(UAG)211/glyV55 Δ(tonB-trpAB) argE(UAG) rpoB*[25]
K28	HfrC *serSC13 serS14 phoA4 supD32 rel-1 tonA22* T2^r
K229	F$^-$ *thy-35 str-120 serS15*
yeast plasmids	
pVTU-102 and 103	multicopy, *ADH* promoter, *URA3* selectable marker[58]
pUN70	*CEN*, *URA3* selectable marker[57]
pUN100	*CEN*, *LEU2* selectable marker[57]
YEp13	episomal, *LEU2* selectable marker[58]
pCJ11	multicopy, *GAL* promoter, *LEU2* selectable marker (gift of D. Pridmore, Nestle Research Centre)
pVTU-P*SES1*	pVTU-103 carrying SES1 gene on 3-kb *SalI*/*Bam*HI fragment[19] including *SES1* promoter, cloned into *Xho*I and *Bam*HI sites of the vector, this work
pVTU-103*SES1*	1.4-kb BamHI fragment, containing the yeast *SES1* structural gene,[19] cloned into pVTU-103 distal to the *ADH* promoter, this work
pVTU-102*SES1*	same as above, but in pVTU-102
pUN70*SES1*	pUN70 containing approx. 3-kb SalI/*Bam*HI fragment,[19] with the entire *SES1*structural gene and its promoter region[24]
pUN100*SES1*	same as above, but in pUN100
YEp13*SES1*	YEp13 carrying the SES1 structural gene and its promoter region[23]
pCJ11*SES1*	1.4-kb *Bam*HI fragment, containing the *SES1* structural gene, cloned distal to the *GAL* promoter of pCJ11[24]
E. coli plasmids	
pET3*SES1*	1.4-kb BamHI fragment containing the yeast *SES1* structural gene cloned into pET3 distal to T7 promoter[19]
pACYC*supSI*	190-bp RsaI-*Sal*I fragment carrying the *S. pombe supSI* gene cloned between *Eco*RV and *Sal*I sites of pACYC, distal to *tet* promoter[25]
pACYC*supSH*	520-bp DraI-*Sal*I fragment carrying the gene for human amber suppressor tRNA^{Ser} cloned between *Eco*RV and *Sal*I sites of pACYC, distal to *tet* promoter[25]
pUC119Ser2	pUC119 containing 114-bp *Eco*RI-*Bam*HI fragment carrying T7 RNA polymerase promoter followed by tRNA$_2^{Ser}$ synthetic gene construct for *in vitro* transcription (I. Weygand-Đurašević and D. Söll, in preparation)

or as 1.4-kb *Bam*HI fragment containing only the structural gene. This cassette was made by introducing two *Bam*HI sites by *in vitro* mutagenesis, one preceding the ATG initiation codon and the other following the TAA termination codon of *SES1* gene.[19] pET*SES1* carries 1.4-kb *Bam*HI cassette with the *SES1* structural gene cloned into pET3 transcription vector, distal to T7 promoter. For *in vivo* experiments, tRNA genes were carried on plasmid pACYC184,[20] which has a p15A origin of replication and is compatible with pBR-derived plasmids carrying the gene for seryl-tRNA synthetase.

Preparation of protein extracts and Western blot analysis

Crude protein extracts were prepared from *S. cerevisiae* strains 3A84 and S2088, transformed with shuttle plasmids carrying the *SES1* gene. Yeast transformations were performed by the lithium acetate procedure of Ito et al.[21] or by electroporation.[22] Whenever *SES1* was constitutively expressed, cells were grown at 30 °C under appropriate selection until the late exponential phase, harvested and lysed by vortexing with glass beads.[23] When *SES1* expression was governed by GAL promoter, cells were grown in a glucose selective medium until OD600 of 0.23, and then shifted to the galactose containing medium for promoter induction. Cells were harvested at OD of 2.5. For the expression of yeast SerRS in *E. coli*, strain BL21(DE3) carrying the T7 RNA polymerase promoter was transformed with plasmid pET3*SES1*.[19] The production of T7 RNA polymerase, and thus the transcription of *SES1* gene, was induced by addition of ß-D-thiogalactopyranoside to 1mM final concentration. The growth was continued for additional two hours, when the cells were harvested and disrupted by sonication. After electrophoresis on SDS-polyacrylamide gel and electrotransfer to the nitrocellulose membrane, Western blots were probed with anti-SerRS antibodies.[23] SerRS expression levels were deduced from the scanning of the bands of the blot.

Purification of yeast SerRS

Protein extracts were prepared from *E. coli* and *S. cerevisiae* overproducing strains, BL21(DE3)/pET3*SES1* and S2088/pCJ11*SES1*, respectively. Crude cellular extracts were clarified by ultracentrifugation at 100 000 x g. The purification of SerRS was performed either on FPLC MonoQ and MonoS columns,[19] or by conventional chromatographies on diethyleaminoethyl cellulose and phosphocellulose columns.[24]

Determination of kinetic parameters

SerRS activity was determined in a standard aminoacylation assay at 37 °C.[23] The reaction mixtures (62 µl) contained 25 µM [^{14}C]serine (New England Nuclear), 50 mM Tris/HCl pH 7.5, 15 mM MgCl2, 5 mM ATP, 4 mM

ditiothreitol and 250 μg of unfractionated brewer's yeast tRNA (Boehringer Mannheim). Determination of kinetic parameters for various tRNA substrates was described.[19,25]

Construction of synthetic $tRNA_2^{Ser}$ gene and in vitro transcription

According to the known nucleotide sequence of $tRNA^{Ser}$,[26] three pairs of complementary and overlapping oligonucleotides were designed (I. Weygand-Đurašević and D. Söll, in preparation). The $tRNA_2^{Ser}$ gene sequence is preceded by the T7 RNA polymerase promoter.[27] Transcription was as described,[27] using 0.5 mg of template DNA and 0.8 mg T7 RNA polymerase per mg of template DNA. The full-length transcript was purified by electrophoresis on a 12% polyacrylamide gel containing 7M urea. Transcript bands were visualized by toluidine blue staining and eluted from the gel.

Complementation of temperature-sensitive E. coli-strains with yeast SES1

To test the complementation, *E. coli* strains K28 and K229 were transformed with pET3*SES1* and plated on M9 glucose minimal plates supplemented with ampicillin (100 μg/ml) and serine (40 μg/ml) in the case of K28 or with thymine (200 μg/ml) for KL229, and grown at permissive and non-permissive temperatures.[19]

Suppression assay

The suppression of amber mutations in strains JR104 and XAC-A24 was tested by plating *E. coli* transformed with the plasmids carrying the genes for eukaryotic amber suppressor tRNAs and, where indicated, with the genes for synthetases on a compatible plasmid, on selective plates. M9 glucose minimal plates were used for testing *argE(UAG)* or *trpA(UAG)* suppression, while M9 lactose minimal plates were used for *lacI(UAG)-lacZ*. Plates were supplemented with ampicillin (100 μg/ml), chloramphenicol (50 μg/ml), arginine (40 μg/ml), and tryptophan (40 μg/ml) as required, and were incubated at 30 °C.

RESULTS AND DISCUSSION

Rationale and system design

The aim of this work was to develop a system for studying the specific recognition between *S. cerevisiae* seryl-tRNA synthetase and its cognate tRNA isoacceptors, as well as the mechanism of discrimination against non cognate tRNAs. Since *in vivo* and *in vitro* studies give complementary con-

tributions to the understanding of the recognition problem, both approaches have been exploited. An *in vivo* study allows evaluation of the effect of competition among synthetases, on the accuracy of the tRNA:synthetase complex formation. A kinetic analysis of the *in vitro* aminoacylation process typically gives an estimation of the catalytic parameter k_{cat} and Michaelis constant K_m. Both k_{cat} and K_m may determine the selectivity of aminoacylation.[28] However, due to the limited range of substrate concentrations that obey Michaelis-Menten conditionsas well as the sensitivity of the *in vivo* suppression assay, the interpretation of *in vivo* and *in vitro* experiments may differ. Both types of studies are aided by the analysis of tRNA or enzyme mutants. Although the *in vitro* approach provides a quantitative analysis of the effect of mutations on substrate recognition, the ultimate test of these effects is in a competitive environment *in vivo*.

Genetic experiments in E. coli

Complementation of temperature-sensitive E. coli strains with the yeast SES1 gene. A low level of crosscharging between yeast and bacteria was shown for the serine system by *in vitro* aminoacylation assays.[29] *S. cerevisiae* and *E. coli* seryl-tRNA synthetases share significant homology in their primary structures.[23,30] The biochemical properties of purified enzymes have been extensively analyzed.[31,32] Even though there are substantial sequence differences between the identity elements of yeast and bacterial $tRNA^{Ser}$, especially in the amino acceptor and anticodon stem,[33,34] we wanted to address the possibility of specific heterologous charging *in vivo* by complementation of the *E. coli* temperature sensitive mutations with yeast *SES1*. *E. coli* strains K28 and K229 are temperature sensitive for growth as a result of mutated seryl-tRNA synthetase.[35,36] Different plasmids carrying the yeast *SES1* gene show complementations of the temperature sensitive mutations, demonstrating that the yeast seryl-tRNA synthetase is actively expressed in *E. coli*, recognizes bacterial serine-specific tRNAs and substitutes for the *E. coli* enzyme *in vivo*.[19] This is a prerequisite for the development of a suppression assay based on the interaction between various eukaryotic suppressor tRNAs and yeast SerRS expressed in *E. coli*. Additionally, it allows a study of the yeast SerRS mischarging properties in the bacterial cell.

Development of an E. coli suppression system which depends on heterologous serylation. The suppression assay is based on the recognition between tRNA anticodon and a nonsense mutation in the reporter gene. In order to function as an efficient suppressor, tRNA must be aminoacylated (Figure 1). Thus, the efficiency of suppression, which can be quantitated by the ß-galactosidase assay,[37] reflects the efficiency of aminoacylation. We wanted to test *in vivo* the interaction between the eukaryotic $tRNA^{Ser}$ species and yeast SerRS, *i.e.* the recognition of the heterologous partners in an environment

TABLE II

Suppression of *E. coli* amber mutations by *S. pombe* and human $tRNA^{Ser}$.

strain	XAC-A24		JR104	
marker tested	$argE_{am}$	$lacI_{am}/lacZ$	$argE_{am}$	$trpA_{am}$
plasmids				
pACYC*supSI*	–	–	–	–
pACYC*supSH*	–	–	–	–
pACYC*supSI* +pET3*SES1*	+++	+++	+++	+++
pACYC*supSH* +pET3*SES1*	+	+	+	+

E. coli strains were transformed with vectors carrying the genes for *S. pombe* and human amber suppressor $tRNA^{Ser}$ and, as indicated, with the *S. cerevisiae SES1* gene on a compatible plasmid. The degree of suppression is indicated by very good (+++) or good (++) growth at 30 °C on selective plates.

sensitive to the competition effects. An easy way to monitor the proper utilization of eukaryotic tRNAs in the prokaryotic protein synthesis is by suppression of nonsense mutations. Serine-specific tRNAs are specially suitable to be used in such experiments since, in contrast to many other tRNAs,[34,38] the anticodon is not presumed to be a recognition element for interaction with the cognate synthetase and, consequently, the requirement for its alteration does not change the tRNA identity.[8,39,40] Various eukaryotic suppressor tRNAs and yeast SerRS were introduced into the bacterial cell by transformation with plasmids carrying different origins of replication , different resistance markers and the genes for the tRNA or the synthetase behind the bacterial or T7 RNA polymerase promoter. These are described in Table I. and in the Materials and Methods. The suppression of amber mutations in strains JR104 and XAC-A24 was tested by plating *E. coli*, transformed with the plasmids carrying the genes for eukaryotic amber suppressor tRNAs, on selective plates, as described in Materials and Methods. The suppression of *argE(UAG)* and *lacI(UAG)-lacZ* is not dependent on the nature of the inserted amino acid,[41] while the amber mutation at position 211 in *trpA* can be suppressed only by a limited set of amino acids, including serine.[42] Although we could show by the Northern analysis[25] that two serine specific tRNAs converted to amber suppressors, *S. pombe supSI*[43] and human $tRNA^{Ser}$,[44] can be transcribed and processed in bacteria, they do not function as active suppressors, presumably because of insufficient recogni-

tion and charging by the bacterial SerRS. However, co-expression of yeast seryl-tRNA synthetase led to functional amber suppression in *E. coli* (Table II). Interestingly, the best results of suppression were obtained with the pET3*SES1* construct, which expresses a very low amount of yeast SerRS in all *E. coli* strains not containing the gene for T7 RNA polymerase. Overproduction of the yeast enzyme in bacteria presumably – interferes with normal cell metabolism, as also shown previously for other systems.[45]

The system for studying mischarging properties of SerRS. The selectivity of suppression of *trpA* at position 211 in strain JR104 is based on the production of the nonfunctional *trpA* gene product after insertion of tyrosine at the position of the nonsense codon. However, an active tryptophan synthase subunit is made if the mutation in the gene is suppressed by serine (Figure 1.). Thus, if non cognate prokaryotic or eukaryotic amber suppressor tRNA is mischarged with serine, strain JR104 will grow on M9 glucose minimal plates lacking arginine and tryptophan. *E. coli* $tRNA^{Tyr}$ was chosen as a sub-

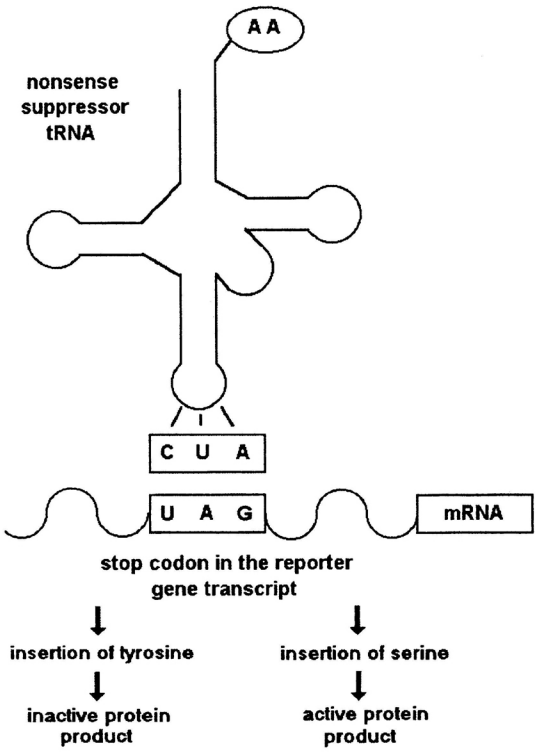

Figure 1. Schematic representation of a suppression assay.

strate in this experiment because this class II tRNA possesses a long variable arm, which is also an important recognition element in the serine system.[8,13,18,38,46] The $tRNA^{Tyr}$ gene was available as a synthetic amber suppressor[47] and it was recloned into the pACYC plasmid (M. Nalaskowska, I. Weygand-Đurašević and D. Söll, in preparation). To demonstrate the suppression activity of the expressed tRNA, the plasmid was transformed into various *E. coli* strains containing nonsense mutations suppressible with tyrosyne, other than *trpA*. However, there was no misacylation with serine detected in JR104, even after cotransformation of the strain with the compatible plasmid carrying the *SES1* gene. We are trying to use this system in our attempts to answer the following questions: 1) Does the alteration of the tRNA-binding domain of SerRS lead to misrecognition of non cognate tRNAs as found in other systems?[48] The experiments related to *SES1* mutagenesis and characterization of SerRS mutants are in progress. 2) Is misrecognition reinforced by overproduction of the yeast SerRS in *E. coli*? This assumption is based on the observation that the overproduction disturbs the competition between synthetases for the acylation of tRNA.[45]

In vitro studies

Since the biochemical and biophysical studies of the recognition process between aminoacyl-tRNA synthetases and tRNAs might require relatively large quantities of both types of macromolecules, we have undertaken the overexpression of synthetic or cloned genes coding for yeast the $tRNA_2^{Ser}$ and seryl-tRNA synthetase.

Overproduction of $tRNA_2^{Ser}$. A DNA fragment corresponding to the sequence of *S. cerevisiae* $tRNA_2^{Ser}$ [26] behind the T7 RNA polymerase promoter[27] was synthesized from six overlapping oligonucleotides (I. Weygand-Đurašević and D. Söll, in preparation). The construct was cloned into pUC119 plasmid for DNA amplification and sequencing. *Bst*NI restriction site was introduced at the 3'-end of the tRNA sequence in the synthetic gene so that the run-off transcription of the *Bst*NI-digested tRNA gene template should produce the correct 3'-CCA terminus in tRNA. After purification of the crude transcript by electrophoresis on a 8% polyacrylamide/urea gel and renaturation by heating to 65 °C and slow cooling to room temperature in a buffer containing magnesium, it was used as a substrate in the aminoacylation assay. The specific activity of the *in vitro* transcribed $tRNA_2^{Ser}$ is about 0.6 nmol/A260 unit, which is only about 50% of the activity of the *in vivo* produced $tRNA^{Ser}$, when isolated from the cell. This discrepancy may be due to the lack of modified bases in the tRNA transcript, which might be important for the recognition with the seryl-tRNA synthetase, as observed for other systems.[49,50] On the other hand, we are trying to improve our purification and renaturation procedure, because lower specific activity could be caused by improper tertiary structure.[12,51]

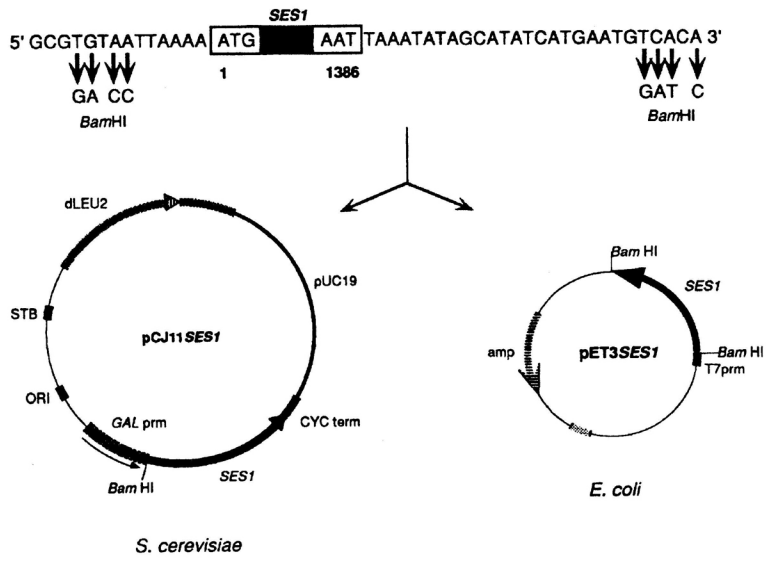

Figure 2. The construction of *SES1* expression plasmids. Two new *Bam*HI sites were introduced into *SES1* flanking regions by *in vitro* mutagenesis. The 1.4-kb *Bam*HI fragment carrying the structural *SES1* gene was recloned into the expression vectors: pCJ11 for the overexpression of SerRS protein in yeast (left) and pET3 for the overexpression in *E. coli* (right).

Overproduction of yeast SerRS in homologous and heterologous system. In order to obtain substantial amounts of yeast SerRS, we first tried the overexpression of the yeast *SES1* gene, and the isolation of its protein product from *E. coli*. *E. coli* is undoubtedly the most commonly used host for recombinant gene expression. An especially powerful system is the one that depends on the T7 RNA polymerase promoter.[52] Thus, the pET3*SES1* construct allows strong transcription of the *SES1* gene and the translation of the transcript from its natural start codon (Figure 2.). Depending on the induction conditions and the growth temperature of bacterial cells, the SerRS overproduction varies between 10 and 20% of the total cell proteins.[19] This facilitates purification of the seryl-tRNA synthetase from *E. coli*, which can be easily achieved by a rapid two step FPLC chromatography on MonoQ and MonoS columns.[19] However, we have observed a dependence of the aminoacylation activity of the isolated enzyme on the overexpression level, being somewhat reduced when the expression system was maximally induced. Several other experiments, like the electrophoresis of the protein under non-denaturing conditions and isoelectric focusing,[24] indicated potential problems in protein folding and/or posttranslational modification of yeast SerRS heterologously overproduced in *E. coli*.

TABLE III

Relative overexpression levels and serylation activities of SerRS in yeast.

Strain					
3A84			S2088		
Plasmid	overpro-duction (-fold)	relative serylation activity	Plasmid	overpro-duction (-fold)	relative serylatyon activity
pVTU-P*SES1*	12	12	pCJ11	1	1
pVTU-103*SES1*	4	4	pCJ11SES1	150	150
pVTU-102*SES1*	4	4			
pUN100*SES1*	1.5	1.5			
pUN70*SES1*	1.6	1.6			
pVTU-103	1	1			
YEp13*SES1*	10	10			

Crude protein extracts were prepared from the indicated yeast strains transformed with plasmids carrying the *SES1* gene. Overproduction of SerRS protein was estimated by scanning the bands on the Western blots. Relative aminoacylation activities were determined with bulk yeast tRNA as, described in Materials and Methods.

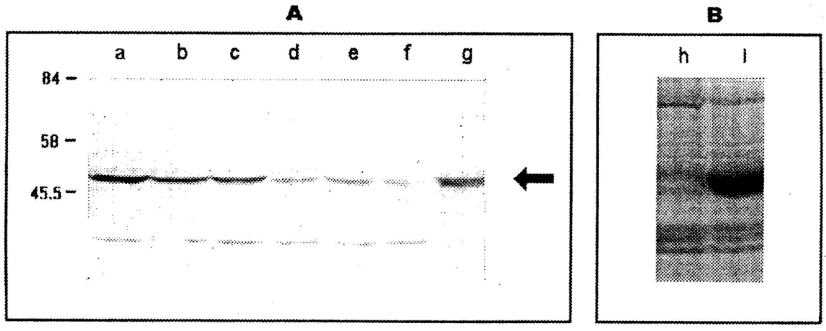

Figure 3. Detection of yeast SerRS expression levels in *S. cerevisiae* strains 3A84 (A) and S2088 (B). An equal amount of the protein extract (50 μg), prepared from the cells transformed with the indicated plasmids, was separated by electrophoresis on SDS/8% polyacrylamide gel. The proteins were either electrically transferred to nitrocellulose and probed with the polyclonal antibodies against yeast SerRS (A), or stained with Coomassie blue (B). The solid arrow indicates the position of yeast SerRS. The positions of the marker proteins are shown on the left margin (fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48.5 kDa). Lane a) pVTU-P*SES1*, b) pVTU-103*SES1*, c) pVTU-102*SES1*, d) pUN100*SES1*, e) pUN70*SES1*, f) pVTU-103, g) YEP13*SES1*, h) pCJ11*SES1*, non induced, i) pCJ11*SES1*, induced.

High level overexpression of yeast SerRS in its natural host, *S. cerevisiae*, is far more complicated and expensive. Besides, we had to determine whether in this system the overproduction of the protein is also followed by irregular folding and modifications, which influence the serylation activity. The expression levels of yeast SerRS, obtained from different constructs carrying the *SES1* gene, are presented in Figure 3. Relative serylation activities of the same protein extracts are given in Table III. It is evident that the overproduction of the protein is proportional to the increased ability of the extract to charge yeast tRNA with serine. Interestingly, the expression of SerRS is somewhat higher when governed from the natural *SES1* promoter than from the commonly used ADH promoter. One of the most powerful systems for the overexpression of protein genes in yeast is based on the *GAL* promoter. The overexpressing plasmid pCJ11*SES1* (Figure 3) was constructed by cloning the *SES1* cassette, obtained by the introduction of two *Bam*HI sites just before and after the *SES1* structural gene,[19] behind the *GAL* promoter. After induction, this construct about 150 times[24] overproduces SerRS protein (Figure 3.) and its activity (Table III). Thus, the amount of the protein is comparable to the one obtained in *E. coli* overexpression system, with a better serylation activity.

Generation of SES1 mutants

A powerful way to study the specificity of serylation is achieved by introduction of mutations either in the tRNA or in the synthetase. The contribution of each mutation to the specificity of $tRNA^{Ser}$: SerRS complex formation can be studied *in vivo*, using the genetic approach like complementation and suppression as described above, or *in vitro*, by the aminoacylation assay. SerRS mutants have been generated by the extensive

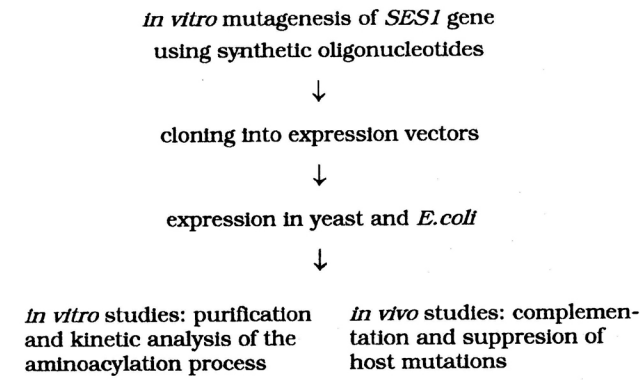

Figure 4. Scheme for the construction of SerRS mutants.

in vitro mutagenesis of the *SES1* gene, using synthetic oligonucleotides, followed by the expression of mutated SerRS enzymes from the relevant plasmids (I. Weygand-Đurašević and D. Söll, in preparation). This is schematically shown in Figure 4. A similar approach will be used for the preparation of mutated tRNAs, using the construct pUC119Ser2.

Outlook

Combining different approaches in studying the specificity of tNRA:synthetase interaction is certainly of great importance. There are two factors operating *in vivo* to ensure the correct aminoacylation of tRNA: competition between synthetases for uncharged tRNA and the specificity of recognition of tRNA by the cognate synthetase. Furthermore, some of the incorrectly charged tRNAs are being proofread and edited *in vivo*.[53,54] All these processes can be significantly disturbed by high overexpression of either tRNA or synthetase. Therefore, we constructed vectors that expressed low and/or comparable amounts of tRNAs and SerRS for *in vivo* studies. It should be noted, however, that the competition between yeast SerRS and bacterial synthetases may be different in a heterologous system than in *S. cerevisiae*, which would require further evaluation of some results in yeast. On the other hand, we have designed powerful overexpressing systems for generating enough material for *in vitro* analyses.

Although *S. cerevisiae* SerRS has not been crystallized yet, our solution studies are faciliated by the existing structures of two SerRS:tRNA^{Ser} complexes from *E. coli*[18] and *T. thermophilus*.[13] Since the yeast enzyme shares significant homology with the seryl-tRNA synthetases from prokaryotic sources, and it seems that all serine tRNAs share some common identity elements, including the long variable arm as the most important one,[8,38] a similar structure of tRNA^{Ser}: synthetase complex could be proposed for *S. cerevisiae*.

Acknowledgements – I am indebted to professor Dieter Söll in whose laboratory the major part of this work was performed. The work was supported by a grant from NIH/FIRCA.

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