# Construction of a *FRS1-FRS2* operon encoding the structural genes for the α and β subunits of yeast phenylalanyl-tRNA synthetase and its use in deletion analysis

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## ABSTRACT

***FRS1* and *FRS2* , the structural genes encoding the large (α) and small (β) subunits of yeast phenylalanyl-tRNA synthetase (PheRS) were placed under the control of the *lacZ* promoter by creating an artificial operon. The *FRS2* gene was fused next to the promoter, followed by a 14 base pair intergenic sequence containing a translation reinitiation site in front of the *FRS1* coding sequences. The engineered PheRS has 16 N-terminal amino acids from β-galactosidase fused to the β subunit. However, the purified protein shows a Km value for $tRNA^{Phe}$ that is indistinguishable from that of the the native enzyme. The product of the *FRS2-FRS1* operon is not able to complement thermosensitive E.coli PheRS, indicating the lack of heterologous aminoacylation *in vivo*. We made a deletion in the *FRS2* gene that removed about 150 amino terminal residues of the β subunit. The truncated protein showed intact ATP-PPi exchange, whereas tRNA aminoacylation was lost. This result is similar to that of limited proteolysis performed on the native enzyme that yielded a tetrameric $\alpha_2\beta'_2$ structure, able to form aminoacyladenylate but unable to bind $tRNA^{Phe}$. A deletion of 50 amino acids from the carboxyl terminus of the β chain resulted in the loss of both enzyme activities; this suggests the participation of the C-terminal end of the β subunit in the active site or in subunit assembly to yield a tetrameric functional enzyme.**

## INTRODUCTION

Nucleotide substitution in $tRNA^{Phe}$ and subsequent aminoacylation by yeast PheRS enabled Uhlenbeck and coworkers (1,2) to identify determinants involved in the specific recognition by the synthetase. These are the five single stranded bases G20, G34, A35, A36 and A73, located on the top and the distal parts of the L- shaped tRNA molecule, indicating that distinct domains of the enzyme are involved in the tRNA recognition. It is also known that the 171 first amino terminal residues of the β-subunit of yeast PheRS having an $\alpha_2\beta_2$ structure, build a domain of Mr 20 000 crucial for tRNA aminoacylation and binding (3). The recent characterization of the genes (*FRS1* and *FRS2*) encoding the α and β subunits ( 4), allows a genetic aproach to study this interaction. However, because of the monocistronic character of yeast mRNAs, it was not easy to design a construction that combines ease of mutation with expression in yeast. To overcome this difficulty we chose to express the α and β subunits of yeast PheRS in *E.coli*. Therefore, we constructed an operon with the *FRS1* and *FRS2* genes using the *E.coli pheST* intergenic region (5) to produce a polycistronic mRNA. The *FRS1* and *FRS2* genes were placed under the control of the *lacZ* promoter and we report here the successful coexpression of the α and β subunits of yeast PheRS.

## MATERIALS AND METHODS

### Strains and plasmids

The *E.coli* strain JM101 (6) was used as recipient for the various plasmids and M13 phages. The *E.coli* strain TG1 was used in transformations by phosphorothioate DNA.The genotype of TG1 is indicated by the supplier (Amersham). The *E.coli* strain IBPC 1651 *pheS5* (7) with a thermosensitive PheRS was a gift from Dr. M. Springer (Institut de Biologie Physico-Chimique, Paris). Plasmids pAS10 and pAS20 ( 4) were the sources of isolation of the *FRS1* and *FRS2* structural genes. Plasmid pUC19 and phage M13mp19 have been described in (6).

### Construction of the phenylalanyl-tRNA synthetase operon

The construction of the *FRS2-FRS1* operon was done using M13 mp19 DNA. The Amersham mutagenesis system based on the method of Eckstein et al. (8) was used to create single base pair mutations. After transformation of TG1 strain, positive clones were identified by sequencing the DNA of four AmpR colonies using the Sanger dideoxy method (9). Directed deletions were obtained by looping out mutagenesis performed essentially as described by Wolfes et al. (10) with a 1:1 ratio of synthetic

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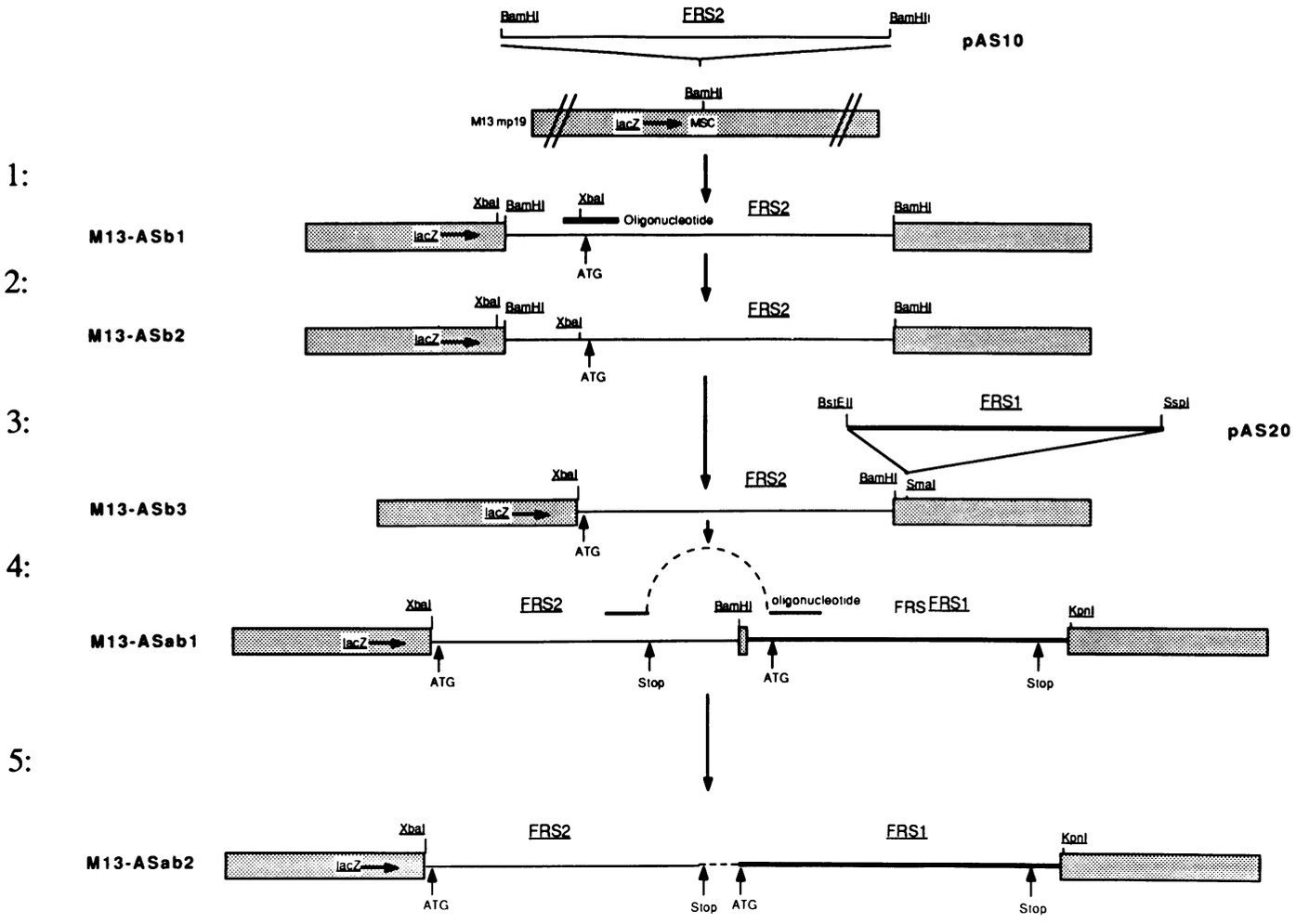


**Figure 1.** Construction of the *FRS2-FRS1* operon. The sequences of the M13 vector, *FRS2* and *FRS1* genes are represented respectively by dotted boxes, thin and thick lines. The dotted line represents the intergenic region of the *pheST* operon. The direction of *lacZ* transcription is indicated by an arrow. Restriction sites of the polylinker region (PCS) used in the construction are indicated. Step 1: a 2.8 Kbp *BamHI* fragment bearing the entire *FRS2* structural gene was isolated from the plasmid pAS10 and cloned into the *BamH1* site of M13 mp19. The construct M13-ASb1 has the *FRS2* gene in the right orientation with respect to the *lacZ* promoter. Step 2 : *in vitro* mutagenesis using the mutant oligonucleotide 5'-GGAAGTCAGACATCTCAGAATTAGGGG-3' and single-stranded M13-ASb1 form to create a *XbaI* site one base upstream from the Met codon of the *FRS2* gene. The presence of the *XbaI* site in M13-ASb2 vector was verified by dideoxy sequencing. Step 3 : digestion of M13-ASb2 by *XbaI*. The linearized vector was recircularized after a tenfold dilution of the digestion mix in the presence of T4 DNA ligase. The resulting M13-ASb3 vector was used to transform JM101 in order to check the removal of the 1.3 kbp *XbaI* fragment from M13-ASb2. Step 4 : a 2 Kbp *BstEII-SspI* fragment bearing the entire *FRS1* structural gene was isolated from plasmid pAS20, filled in by DNA polymerase I (Klenow fragment) and inserted into the *SmaI* site of M13-ASb3 to yield M13-ASab1 vector. Step 5 : insertion of the intergenic region of the *pheST* operon between the *FRS2* and *FRS1* structural genes by gene directed deletion using the 59-mer mutant oligonucleotide described in Materials and Methods. The mutant phage was screened by hybridization with the mutagenic oligonucleotide followed by dideoxy sequencing to confirm the presence of the intergenic region in M13-ASab2 vector. The fragment *XbaI-KpnI* of M13-ASab2 was further subcloned in pUC19 to yield pUC-ASab2.

oligonucleotide to single stranded template instead of 3:1. The mutated M13 phages were identified by colony hybridization of nitrocellulose replicas with the {$^{32}$P} phosphorylated oligonucleotide as described by Wallace et al. (11) and the positive clones were selected. The deletion was further confirmed by DNA sequencing.

## Oligonucleotide design for in vitro mutagenesis

An *XbaI* restriction site in *FRS2* gene was created using oligonucleotide: 5'-GGAAGTCAGACATCTCTAGATTAGGGG-3' (mismatched nucleotides are underlined). Replacement of the 3'-downstream region of the *FRS2* gene and the 5'-upstream region of the *FRS1* gene by the intergenic region of *pheST*, was done using a 59-mer oligonucleotide: 5'-GTTCAAGGAGACGG-TAGGCATAATCTATTCCTGCCTTATTCGTAGAAGTCT-3' (the intergenic region is underlined). Oligonucleotides: 5'-AATCTTGGCGTCAATGCTGAAGTCAGACATCTCTAGA-3' and 5'-AATCTATTCCTGCCTTATTCGTAATCCTTTGGT-AGACCCATGGACTCGA-3' were used to delete respectively the $NH_2$- and COOH -terminal ends of the $\beta$-subunit.

## Determination of enzymatic activities

Yeast PheRS expressed from the *FRS2-FRS1* operon was tested in crude extracts from exponentially growing cells carrying the plasmid-recombinant pUC-ASab2. Cells were resuspended in the aminoacylation buffer and subjected to ultrasonic disintegration.

Aminoacylation reactions were carried out at 25°C under the following conditions : 144mM Tris-HCl pH 7.8; 5 mM dithiothreitol, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM {$^{14}$C} phenylalanine (25 $\mu$Ci/$\mu$mole), 6 mg/ml unfractionated yeast or

*E.coli* tRNA (the fraction of $tRNA^{Phe}$ is 2.5%). At various time intervals 40 $\mu$l aliquots from a 200 $\mu$l reaction mixture were spotted onto Whatman paper discs and quenched by 5% trichloroacetic acid. Radioactivity was counted using a toluene-based scintillant. The ATP-pyrophosphate exchange assays were done at 25°C using : 144 mM Tris-HCl pH 7.8 ; 2 mM ATP, 2 mM {$^{32}$P}PPi (.35 $\mu$Ci/$\mu$mole), 2mM phenylalanine, 10mM potassium fluoride (an inhibitor of endogenous pyrophosphatase) 0.1 mM phenylmethylsulphonylfluoride and various amounts of crude enzyme. Labelled ATP was adsorbed onto charcoal, washed with water and the radioactivity was monitored by scintillation counting as above.

### Western blotting

Protein samples were run on 10% polyacrylamide gels in the presence of 0.1% sodium dodecylsulphate (12). Conditions for the transfer of proteins to nitrocellulose membranes were as described in the Schleicher and Schuell manual n°2. The protein band corresponding to PheRS was detected using DEAE-Sephadex purified antibodies (100 $\mu$g per nitrocellulose membrane of about 150 $cm^2$) and {$^{125}$I}-protein A (0.7 mCi at 50 mCi / mg). Antibodies were obtained from intradermal injections into rabbits of native PheRS emulsified with complete Freund's adjuvent.

## RESULTS AND DISCUSSION

### Construction of the *FRS1-FRS2* operon

In the bacterial *pheST* operon, the *pheS* gene encoding the small subunit precedes *pheT* encoding the large subunit (5). Whether this structural organization represents a polarity in transcription, remains an open question. We kept the same order of transcription for the artificial yeast PheRS operon, because of sequence homologies (30%) between the small subunits of the yeast and bacterial PheRS. In contrast the large subunits show only short streches of homologies (4). Therefore, the *FRS2* gene encoding the small $\beta$-subunit is the first transcribed gene. The steps in the construction are summarized in Fig.1 and below:

1) the entire *FRS2* structural gene was excised from plasmid pAS10 by digestion with *BamH1*. The resulting 2.8 kilo base pair (Kbp) insert bearing the coding sequences of the $\beta$ subunit together with the 5'- and 3'-untranslated sequences was subcloned into the *BamH1* site of M13 mp19 vector in the right orientation with respect to *lacZ* transcription.

2) a unique *XbaI* site was created by site directed mutagenesis onebase upstream from the initiation Met codon of the *FRS2* gene in M13-ASb2 phage.

3) M13-ASb2 phage was digested by *XbaI*. The removal of the *XbaI* fragment in M13-ASb3 brings the *FRS2* gene in phase with *lacZ*. As a result the $\beta$-subunit was expressed as a fusion protein with 16 extra amino acid residues of the $\beta$-galactosidase. These additional amino acids have no influence on the tRNA binding as shown by Km measurements (see below).

4) the entire *FRS1* gene (encoding the $\alpha$ subunit) was isolated from plasmid pAS20 by restriction with the enzymes *BstEII* and *SspI*. The 5'protruding ends were filled in before cloning into the *SmaI* site of M13-ASb3.

5) the 3'-untranslated sequences of the *FRS2* gene and the 5'-upstream sequences of the *FRS1* gene were deleted by looping out mutagenesis.They were replaced by the intergenic region of the *pheST* operon. The oligonucleotide used in this deletion was a 59-mer matching 16 bases of the *FRS2* coding sequences upstream from the stop codon and 20 bases of the *FRS1* coding sequences downstream from the Met codon. The deletion removed about 1.3 kbp of yeast sequences at the desired locus and created a polycistronic mRNA for the $\alpha$ and $\beta$ subunits of yeast PheRS, whose expression is now under the control of the *lacZ* promoter. The intergenic region contains the crucial Shine and Dalgarno sequence to ensure reinitiation of translation of *FRS1* mRNA. The unique *XbaI* and *KpnI* sites of the polylinker were used to reclone the operon into a pUC19 vector to improve its expression.

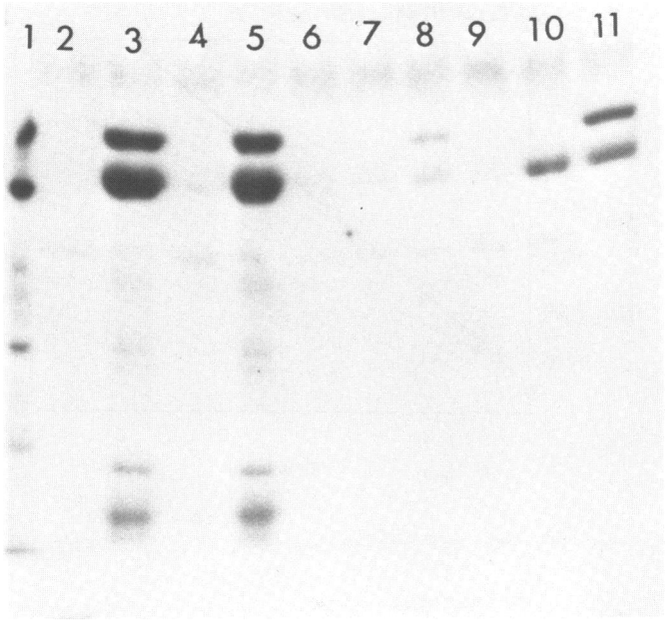


**Figure 2**. Western-blot analysis of yeast PheRS expressed from the *FRS2-FRS1* operon. Crude protein extracts (20 $\mu$g) of control and infected cells were analyzed together with 1$\mu$g of purified yeast PheRS (lane 1 ). Lanes 2 to 5: cells transformed by pUC plasmids. Controls: vector alone in the absence (lane 2 ) and presence of IPTG ( lane 4 ) ; cells transformed by pUC-ASab2 (uninduced, lane 3) and in the presence of IPTG (lane 5). Lanes 6 to 11: JM101 infected by various M13 constructs. Lane 6 and 9 (controls) : cells infected by M13 phage in the absence (lane 6) and presence of IPTG (lane 9). Lanes 7 and 10 : cells infected by M13-ASab1 phage in the absence (lane 7) and presence (lane 10) of IPTG. Lanes 8 and 11: cells infected by M13-ASab2 phage bearing the *FRS2-FRS1* operon, in the absence (lane 8) and presence of IPTG (lane 11). Antibodies directed against the native yeast PheRS were used to detect the small and large subunits expressed from the *FRS2-FRS1* operon.

### Expression of the *FRS1-FRS2* operon

The PheRS from *E.coli* and yeast have similar molecular mass (Mr = 250 000) and quaternary structures ($\alpha_2\beta_2$), but the size of the subunits differ widely: 87 000 and 67 500 for the large subunits from *E.coli* and yeast; 37 000 and 57 000 for the corresponding small subunits (4). As a result the pattern of migration on a SDS-polyacrylamide gel is completely different for the two enzyme species. This difference in migration combined with the absence of cross-reactivity of antibodies between the two enzyme species, allows visualization of yeast PheRS in a crude *E.coli* protein extract by Western blotting. The expression of the *FRS1-FRS2* operon was followed by measuring both the protein level and the enzyme activity. Fig. 2 shows the results of the Western blots. The enzyme expressed in *E.coli* resembles that of the purified yeast PheRS. Only the migration of the plasmid and phage expressed $\beta$ subunit is slightly retarded

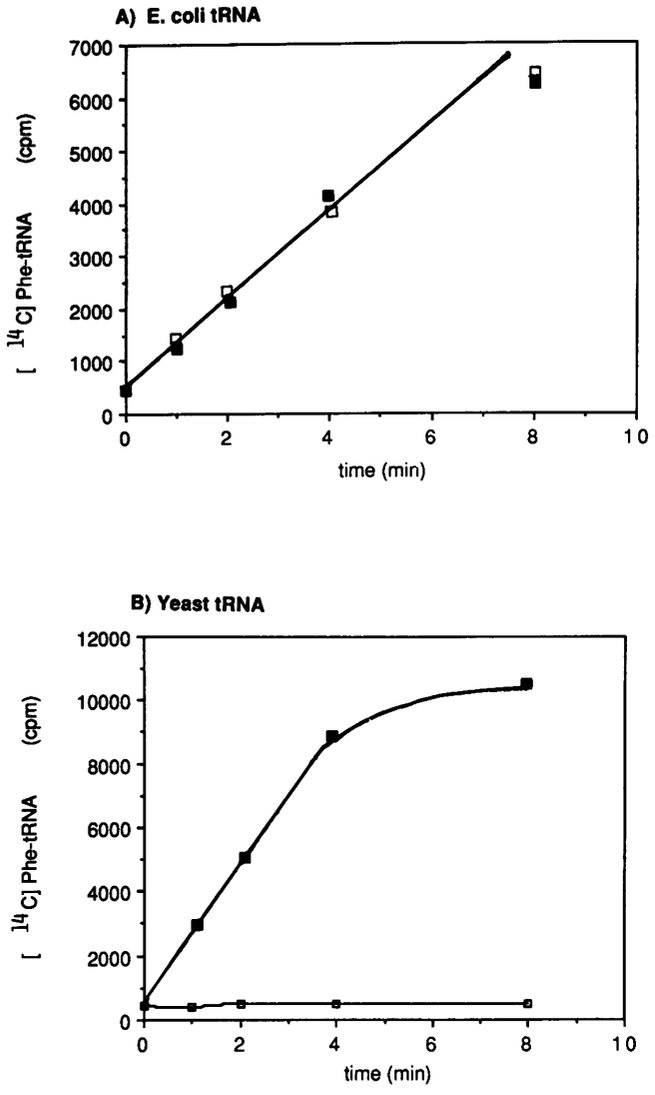


**Figure 3.** Kinetics of aminoacylation using E.coli tRNA (A) and yeast tRNA (B). 1μg of total protein was used in the aminoacylation test. (□) crude protein extract from cells transformed by the vector alone; (■) crude protein extract form cells transformed by pUC-ASab2.

as compared to the corresponding original β structure, but this is compatible with the increase in mass resulting from the addition of 16 residues of β-galactosidase. The level of the expressed PheRS can be modulated by IPTG addition. In M13-infected cells the induction is about 10 fold (lanes 8, 11) whereas no apparent induction is visible in pUC-transformed cells (lanes 3, 5). This is due to titration of the repressor by the lac operator region of the pUC plasmid,which is present in higher copy number than the replicative form of M13 vector. Lane 10 corresponds to the induced level of the β-subunit alone. It is obtained from the construct in which only the *FRS2* gene was in phase with *lacZ* (M13-ASab1). In this case, the level of expression of the β-subunit is comparable to the amount of β-subunit produced from the polycistronic mRNA, indicating that the stability of the β-polypeptide chain is independent of the tetrameric $\alpha_2\beta_2$ structure. Inspection of lanes 3 and 5 shows that the α and β subunits are not present in stoichiometric amounts. This is due to proteolysis of the α subunit yielding a species that comigrates with the β subunit as seen in Fig.4B. We could further establish the comigration of the proteolyzed α subunit with β subunit, using α-specific antibodies (not shown).

## Specificity of aminoacylation

Since yeast PheRS is expressed in an *E.coli* strain having wild type PheRS activity, it was of crucial importance to demonstrate that aminoacylation activity of the operon product is specific for yeast tRNA. Fig. 3A shows the kinetics of aminoacylation using *E.coli* $tRNA^{Phe}$. A crude extract from JM101 transformed by pUC-ASab2 does not show increased level of *E.coli* tRNA aminoacylation over a control strain transformed by the vector alone. This indicates that under the experimental conditions used, there is no heterologous aminoacylation of *E.coli* $tRNA^{Phe}$ by the plasmid expressed yeast PheRS. The kinetics of aminoacylation differ widely when using yeast $tRNA^{Phe}$ (Fig.3B). Only pUC-ASab2 transformed *E.coli* cells show detectable aminoacylation activity, whereas a crude protein extract from the control strain does not. The specific activity measured from the slope of the respective aminoacylation curves, indicates a two-fold higher specific activity for the expressed yeast enzyme compared to the *E.coli* PheRS. From this measurement we can deduce the (functional) protein level of the yeast PheRS since saturating substrate concentrations were used and since kcat values for the *E.coli* and yeast PheRS are similar (in the range of 4 to 6 $sec^{-1}$; 13, 14). This estimation is in agreement with the value determined experimentally by measuring the ATP-PPi exchange. The kinetics obtained with crude protein extracts containing plasmid encoded yeast PheRS show twice the activity of the *E.coli* control strain ( see Fig.4A) indicating that the level of yeast PheRS is similar to that of the chromosomally expressed *E.coli* PheRS. This low production may be attributed to instability of the protein resulting from proteolysis.

To verify that the plasmid expressed yeast PheRS behaves like the native enzyme, we have purified the fusion protein. The enzyme was homogeneous after chromatography of a crude polyethylene glycol fraction (5%−17.5%) on hydroxyapatite-Ultrogel and FPLC-DEAE. The purified enzyme has a specific activity of 700 units / mg. Determination of the Km for tRNA indicates a value in the range of $10^{-7}$M which is similar to that of the native enzyme having a specific activity of 1600 units / mg. This indicates that the decrease in the specific activity does not reflect an alteration of tRNA binding but rather a decrease in the amount of active PheRS. To confirm that a fraction of the enzyme purified from *E.coli* is inactive, we measured the ATP-PPi exchange. As expected, its specific activity (4 800 units / mg) was twice as low as that of the the native enzyme isolated from yeast (10 800 units / mg).

## Inability to complement thermosensitive *E.coli* phenylalanine-tRNA synthetase

The concentration of specific yeast PheRS in the *E.coli* crude extract deduced from the slope of Fig. 3B and the known specific activity of the purified enzyme is about 10nM. To obtain a slope of comparable value with total *E.coli* tRNA, the yeast PheRS has to be increased 100 times (data not shown). This is consistent with an earlier observation by Kern et al. (15) showing that aminoacylation of *E.coli* $tRNA^{Phe}$ by yeast PheRS remains undetectable below one μM enzyme concentration. The increase in non cognate enzyme concentration is necessary to compensate mainly a reduction in kcat of heterologous aminoacylation rather than a low affinity for tRNA since yeast PheRS shows only a 7-fold elevation in Km for *E.coli* $tRNA^{Phe}$ (16). Therefore, the

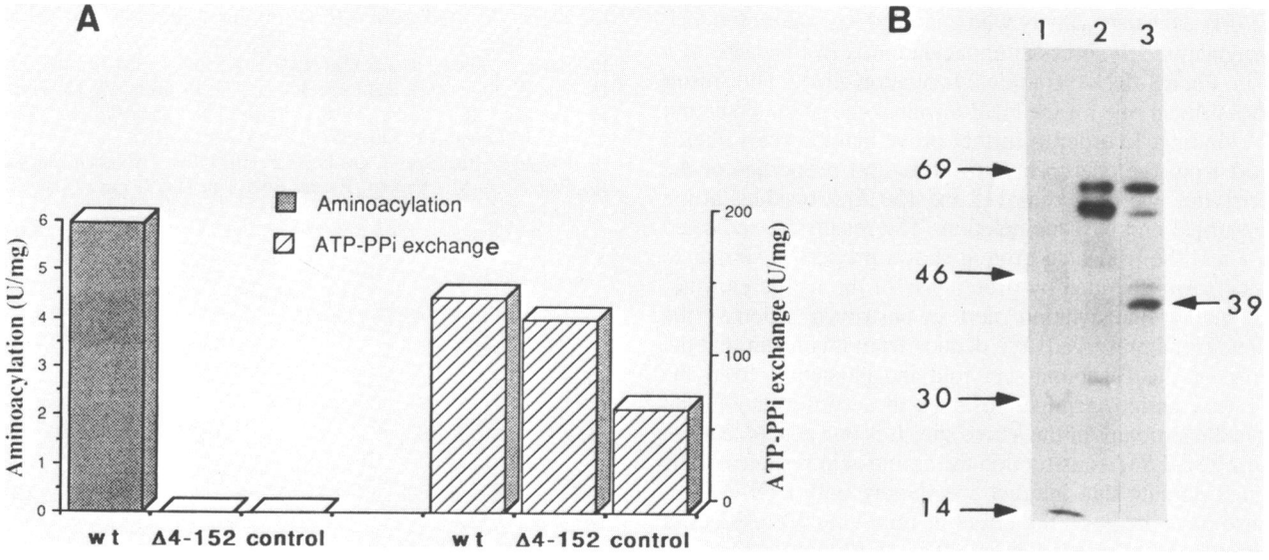


**Figure 4**. N-terminal deletion from the β-subunit of yeast PheRS. Removal of residues 4−152 from the N-terminus of the β subunit was done at the gene level using the mutant oligonucleotide described in the Experimental section. The XbaI-KpnI fragment of the resulting M13-ASab3 phage was excized and subcloned into pUC19 to yield pUC-ASab3. This plasmid was used together with pUC-ASab2 to express truncated and wild type PheRS in JM101 cells. A) Effect of the deletion on the enzyme activities. The wild type (wt) and truncated (Δ4−152) PheRS were tested in tRNA aminoacylation and ATP-PPi exchange. Control: crude protein extract from JM101 cells transformed by pUC19. The activities are expressed in U/mg crude extract (1 unit corresponds to one nmole of product formed/min under the standard conditions). B) Western blot analysis of the wild type and truncated PheRS. Visualization of the protein bands was done using antibodies directed against the native enzyme. Lane 1: protein standards; lane 2: wild type PheRS; lane 3: truncated PheRS. The numbers refer to Mr values of the proteins (× 1/1000). The Mr of the truncated β subunit (39000) is consistent with the removal of 150 amino acid residues.

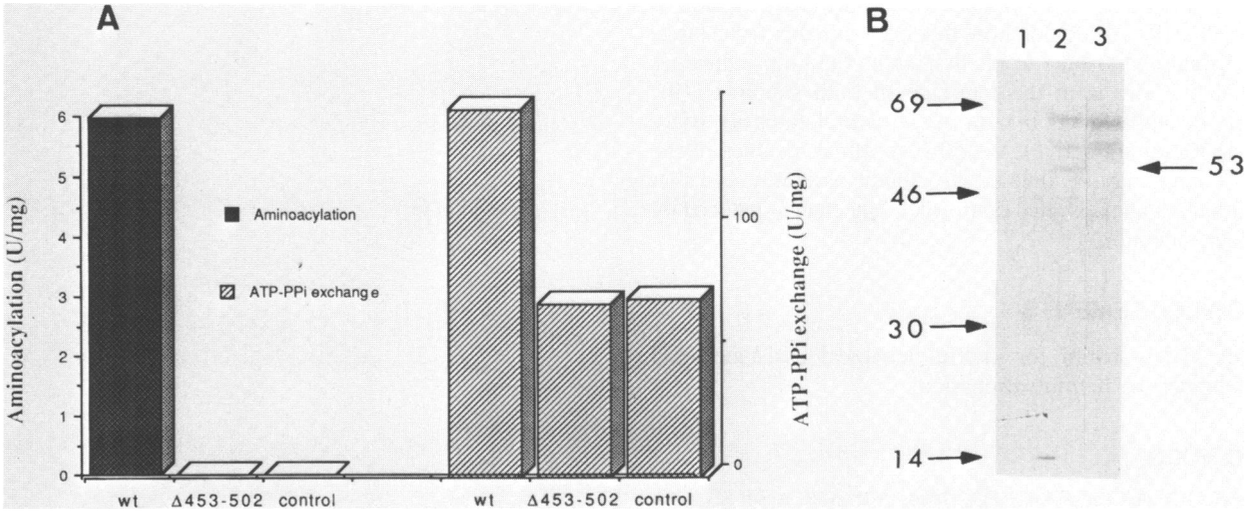


**Figure 5**. C-terminal deletion from the β-subunit of yeast PheRS. Removal of residues 453−502 from the carboxyl-terminus of the β subunit was done at the gene level using the mutant oligonucleotide described in the Experimental section. Expression of the truncated enzyme and activity measurements were carried out as described in Fig.4. A and B: same as in Fig.4 except that numbers 453−502 replace 4−152. The Mr of the truncated β-subunit (53000; lane 2) is consistent with the removal of 50 amino acid residues. Lane 1: protein standards; lane 2: truncated PheRS; lane 3: wild type PheRS.

level of yeast PheRS expressed in the *E.coli* cell may not be sufficient to complement a thermosensitive *E.coli* PheRS. Indeed, pUC-ASab2 transformed mutant pheS5 strain was unable to grow at 42°C and this can be attributed mainly to the low kcat value in *E.coli* $tRNA^{Phe}$ aminoacylation. By no means can the misaminoacylation of $tRNA^{Val}$ or $tRNA^{Ala}$, that has been reported for yeast PheRS *in vitro* (15,16, 17), be responsible for lethality at 42°C, since the cells grow well at permissive temperature.

## Deletion of the amino- and carboxyl-terminal ends of the β-subunit

The comparison of three small subunit sequences of PheRS from different sources (the cytoplasmic and mitochondrial isoenzymes from yeast and the *E.coli* bacterial enzyme) has indicated an N-terminal extension of about 150 residues for the yeast cytoplasmic PheRS (4). This extension is part of a N-terminal domain that can be cleaved by mild proteolysis removing the 171 first amino acid residues (3). It results in a truncated $\alpha_2\beta'_2$ form that retains

full ATP-PPi exchange activity with unaltered Km values for ATP and phenylalanine, whereas aminoacylation is lost because of a failure in PheRS-tRNA complex formation (18). This result suggests a critical role for the NH2-terminal end of the β-subunit in tRNA binding. In order to further prove that the yeast PheRS expressed from the operon has the structural properties of the native enzyme, we have removed the 150 first residues at the amino terminal end by gene deletion. The results are indicated in Fig. 4 and the truncated protein shows properties similar to the tryptic form obtained by proteolysis of the native enzyme. That is, the aminoacylation step is destroyed whereas the activation step is preserved. We deduce from this result that the remaining of the β-subunit can fold independently from the 150/171 first amino terminal residues in a conformation that preserves the structure of the active site. It is our goal to analyze with our *FRS2-FRS1* construction the amino acid residues of the N-terminal domain that interact specifically with $tRNA^{Phe}$.

We also have analysed the effect of removing 50 residues at the carboxyl-end of the β-subunit. It is worth mentioning that the homology between the three small subunits is the highest in the middle and carboxyl- regions suggesting a common structural and /or functional role. Fig.5 shows the result of the analysis. No activity could be measured either in the aminoacylation reaction or in the ATP-PPi exchange; thus the loss in tRNA charging results from a primary defect in the amino acid activation step. This defect may have arisen from the removal of important catalytic residues present in the C-terminal region of the β-subunit. Such interpretation is consistent with a previous study on the distribution of binding sites for the different ligands of the yeast PheRS. We could show that determinants from the α and the β chain contribute to phenylalanine binding, indicating that the active site is at the interface of both subunits (19). However, because function requires structural integrity (only tetrameric forms are active), the 50 C-terminal residues of the yeast β-subunit may be involed in subunit assembly and that activity requires an additional conformational change induced by the partner subunit .

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