THE ROLE OF THE FOURTH NUCLEOTIDE FROM THE 3'END IN THE YEAST PHENYLALANYL TRANSFER RNA SYNTHETASE RECOGNITION SITE: REQUIREMENT FOR ADENOSINE.*

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Summary

Purified yeast phenylalanyl tRNA synthetase (PRS) can aminoacylate highly purified E. coli $tRNA^{Met}_{M}$ and E. coli $tRNA^{Ile}$, but cannot aminoacylate highly purified E. coli $tRNA^{Gly}_{3}$, E. coli $tRNA^{His}$ and yeast $tRNA^{Lys}$. It was previously proposed that a group of nine nucleotides comprising the region adjacent to the dihydrouridine loop were directly involved in the PRS recognition site. In addition to confirming this hypothesis, we now show that a second region of the tRNA molecule, consisting of the fourth nucleotide from the 3'end, is also directly involved in the PRS recognition site. This synthetase has an absolute requirement for adenosine at this location.

One approach to the elucidation of the aminoacyl tRNA synthetase recognition site is to compare the sequences of several transfer RNAs, all of which are aminoacylated by a single synthetase. Those regions which are common to these tRNAs are then most likely candidates to be involved in the recognition site for that synthetase.

Yeast phenylalanyl tRNA synthetase (PRS) can aminoacylate the phenylalanine tRNAs of E. coli, yeast and wheat and E. coli $tRNA^{Val}_{1}$ (1-4). The latter reaction, the aminoacylation of E. coli tRNA $tRNA^{Val}_{1}$ by PRS is called heterologous mischarging for it refers to the instance in which a synthetase from one source aminoacylates an "incorrect"tRNA from another source. Heterologous mischarging, first discussed by Barnett and Jacobson (5), can be a most valuable tool in elucidating the nucleotides which are involved in the aminoacyl tRNA synthetase recognition site for they allow the comparison of very different tRNAs, all of which are aminoacylated by a single synthetase. Using this approach, the sequences of the phenylalanine tRNAs of E. coli, yeast and wheat and E. coli $tRNA^{Val}_{1}$ were compared and it was proposed that a group of nine

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nucleotides located in the double stranded region adjacent to the dihydrouridine loop (diHU stem region) (Fig. 1) were directly involved in the PRS recognition site (1). To examine this hypothesis further and to determine what other regions are also involved in the recognition of a tRNA by this particular synthetase we have sought to extend this method of sequence comparison. We now report a more extensive sequence compariosn based upon eight tRNAs which are aminoacylated by PRS. This more extensive comparison a) confirms the role of the nucleotides of the diHU stem region in the PRS recognition site and b) shows that a second region of the tRNA molecule, specifically the fourth nucleotide from the 3'end is also directly involved in the recognition of a tRNA by PRS. Indeed PRS has an absolute requirement for adenosine at this location.

To eliminate the possibility of artifacts in this approach to determining the synthetase recognition site, each of the components have been extensively purified. Thus, each of the tRNAs have been obtained in highly purified form as is described below, and the synthetase, PRS, was either a 200-fold purified preparation prepared as previously described (4) or a highly purified preparation obtained by a three step chromatographic procedure involving DEAE cellulose, phosphocellulose and hydroxylapatite column chromatography. This latter procedure which will be described in detail elsewhere (6) gave PRS which showed only one major band on polyacrylamide disc gel electrophoresis in the location expected for this synthetase (7).

E. coli $tRNA^{Val}_{2A}$ and $tRNA^{Val}_{2B}$ were both extensively purified and are both aminoacylated by PRS as is shown in Table 1. (8). We have now found that E. coli $tRNA^{Met}_{M}$, the non-formylatable species of methionine tRNA and E. coli $tRNA^{Ilu}$ are aminoacylated by PRS as shown in Table 1. E. coli $tRNA^{Met}_{M}$ was purified as follows: crude E. coli B tRNA (Schwarz-Mann) was fractionated on benzolated DEAE cellulose (BD-cellulose) at neutral pH as previously described (4). The $tRNA^{Met}_{M}$, now free from $tRNA^{Met}_{F}$, was further fractionated on DEAE

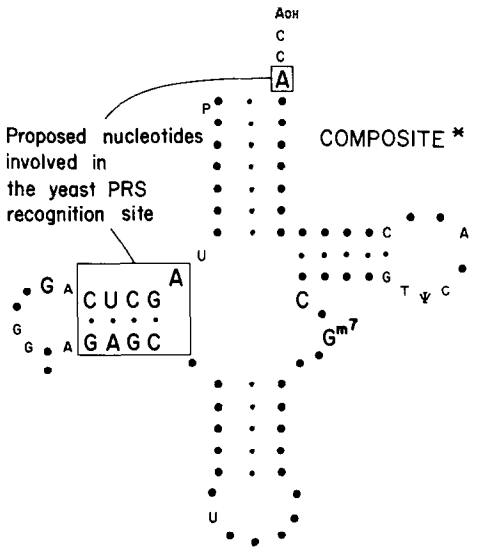

Figure 1. Composite tRNA. Those nucleotides that are not the same in the first eight tRNAs of Table 1 are shown in the composite with a dot. Those nucleotides that are found in the same position in all tRNAs are shown in the composite in light type. Those nucleotides that are uniquely common to the first eight tRNAs of Table 1 are shown in BOLDFACE.

The extra dot near the dihydrouridine loop denotes the fact that three tRNAs (E. coli $tRNA^{Val}_{2A}$, E. coli $tRNA^{Val}_{2B}$, and E. coli $tRNA^{Ilu}$) have one extra nucleotide in the dihydrouridine loop.

*The central nucleotide of the anticodon of the eight tRNAs upon which this composite is based is adenosine. This has been eliminated from the composite since, based on the genetic code, it could not be present in that position in any species of alanine tRNA. Indeed, E. coli $tRNA^{Ala}_{1}$ has a guanosine at that location (19).

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sephadex according to the procedure of Nishimura (9). The final purification step was fractionation on plaskon chromatography at pH 4.5 (10). This procedure gave E. coli $tRNA^{Met}_{M}$ (1.2 nmoles/OD unit) which could be heterologously aminoacylated with PRS to 80% of the level of the homologous aminoacylation reaction (Table 1).

E. coli $tRNA^{Ilu}$ was purified by a four step procedure as follows: crude E. coli B tRNA was fractionated on BD-cellulose as previously described. The enriched $tRNA^{Ilu}$ fraction was then chromotographed on plaskon first at pH 4.5 and then at pH 7.0. The final purification step was the phenoxyacetylation procedure of Tener and coworkers (11) as shown in Fig. 2. This procedure

Table 1. tRNAs Aminoacylated By Yeast Phenylalanyl tRNA Synthetase*

tRNA	homologous aminoacylation nmoles/OD unit	heterologous aminoacylation nmoles/ OD unit	heterologous aminoacylation % homologous
yeast phenylalanine	1.2	-	-
wheat phenylalanine	1.2	1.2	100%
E. coli phenylalanine	1.2	1.2	100%
E. coli valine 1	1.4	1.25	90%
E. coli valine 2A	1.2	1.0	85%
E. coli valine 2B	1.2	1.0	85%
E. coli methionine (M)**	1.2	0.96	80%
E. coli isoleucine	1.25	1.0	80%
E. coli alanine 1	1.5	1.35	90%
E. coli alanine 2	1.0	0.85	85%
E. coli lysine	1.2	1.1	90%

*Homologous and heterologous aminoacylation reactions were performed as previously described (8).

**E. coli $tRNA^{Met}_{M}$ was distinguished from E. coli $tRNA^{Met}_{F}$ by the procedure of Schofield (22).

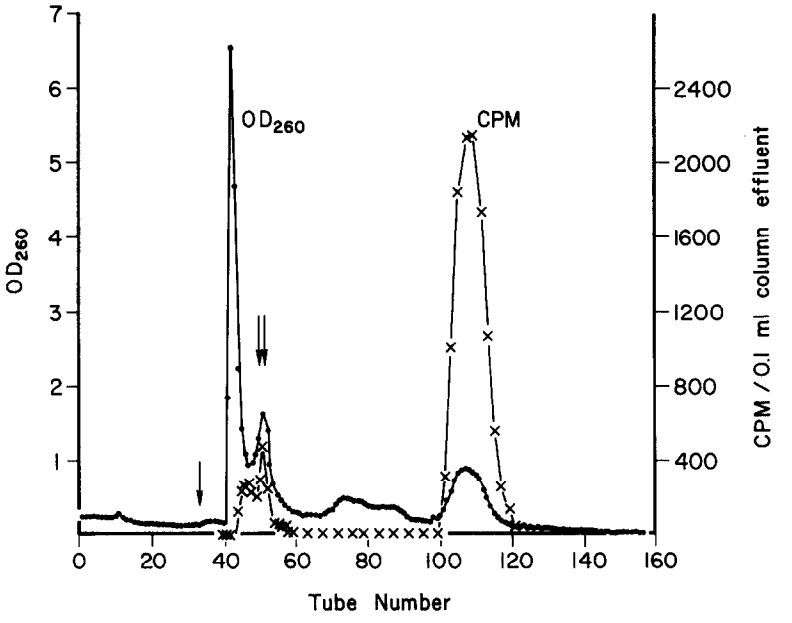

Figure 2. Chromotography of Phenoxyacetylated E. coli $tRNA^{Ilu}$. Partially purified E. coli $tRNA^{Ilu}$ was aminoacylated with [^{14}C] isoleucine (10 Ci/mole) and then phenoxyacetylated according to the procedure of Gillam, et al., (11) and then applied to a 0.4 x 50 cm BD cellulose column. The column was washed with 50 ml of 0.01M sodium acetate pH 4.5, 0.01M magnesium chloride, 0.001M sodium thiosulfate (Buffer A) containing 0.3M sodium chloride. It was then washed with 50ml of Buffer A containing 1.0M sodium chloride (started at single arrow). Highly purified E. coli $tRNA^{Ilu}$ was eluted using a linear gradient (started at double arrow), 400ml total volume, of Buffer A containing 1M sodium chloride and Buffer A containing 1M sodium chloride and 50% v/v ethanol. Flow rate: 2ml/3 min/tube. 0.1ml aliquots were plated on Whatman GF/A filters, dried and counted in omnifluor-toluene.

gave highly purified $tRNA^{Ilu}$ (1.25 nmoles/OD unit) which was aminoacylated with PRS to 80% of the level achieved in the homologous reaction (Table 1).

E. coli $tRNA^{Val}_{2A}$, $tRNA^{Val}_{2B}$, $tRNA^{Met}_{M}$ and $tRNA^{Ilu}$ each have the exact nine nucleotides located in the diHU stem region as was found previously (see composite tRNA, Fig. 1) (12-14). Thus, these four tRNAs strongly confirm the hypothesis that this region of the molecule is directly involved in recognition of a tRNA by PRS. We now report that a second region of the tRNA molecule, consisting of the fourth residue from the 3'end, is also directly involved in the recognition site for this particular synthetase. The evidence is based first on the observation that all eight tRNAs aminoacylated by PRS, whose sequences are known (the first eight tRNAs of Table 1) all contain adenosine at the fourth position from the 3' end (see Fig. 1). Second, are the results obtained with three tRNAs which have nucleotide sequences very similar to the composite tRNA (Fig. 1), but which have nucleosides other than adenosine at the fourth position from the 3' end. Thus, E. coli $tRNA^{Gly}_{3}$ differs from the composite tRNA (Fig. 1) in only one nucleotide (15). Each of the nucleotides shown in the composite tRNA (Fig. 1) are also present in the same exact location in E. coli $tRNA^{Gly}_{3}$, except for one nucleotide; E. coli $tRNA^{Gly}_{3}$ has a uridine at the fourth position from the 3' end instead of adenosine as is shown in the composite tRNA. E. coli $tRNA^{Gly}_{3}$ was isolated in purified form (1.2 nmoles/OD unit) by chromatography on BD-cellulose at pH 7.5 followed by plaskon chromatography at pH 7.0 and is not an acceptable substrate for PRS as is shown in Table 2.

Further evidence for the role of this adenosine in the PRS recognition site is provided by E. coli $tRNA^{His}$. This tRNA was isolated in highly purified form (1.5 nmoles/OD unit) by chromatography on BD-cellulose at pH 7.5 and on plaskon at pH 7.0 and pH 4.5. E. coli $tRNA^{His}$ differs from the composite tRNA (Fig. 1) only in that it has a cytidine instead of an adenosine at the fourth position from the 3' end and, in addition, this tRNA has an extra residue, guanosine, at the 5' end (16,17). In all other respects this tRNA

Table 2. tRNAs Not Aminoacylated By Yeast Phenylalanyl tRNA Synthetase*

tRNA	homologous aminoacylation (nmoles/OD unit)	heterologous aminoacylation (nmoles/OD unit)
E. coli glycine 3	1.2	0.025
E. coli histidine	1.5	0.050
yeast lysine	1.35	0.025
E. coli methionine (F)**	1.1	0.025
wheat glycine	1.65	0.050

*The reaction conditions for homologous and heterologous aminoacylation have been described previously (8).

**E. coli $tRNA_F^{Met}$ was distinguished from E. coli $tRNA_M^{Met}$ by the procedure of Schofield (22)

is also identical to the composite tRNA (Fig. 1). This purified histidine tRNA is also not aminoacylated by PRS (Table 2).

Finally, the series is completed by yeast $tRNA^{Lys}$. This tRNA is also identical to the composite tRNA (Fig. 1) in that each nucleotide shown in the composite tRNA is also found in the same exact location in yeast $tRNA^{Lys}$ except for one nucleotide (18). Yeast $tRNA^{Lys}$ has a guanosine at the fourth position from the 3' end instead of adenosine as is present in the composite tRNA. Pure yeast $tRNA^{Lys}$, a gift of Dr. J. Madison, is not aminoacylated by PRS as shown in Table 2.

These results, in addition to showing that adenosine at position four from the 3' end is involved in the PRS recognition site also illustrates the high degree of specificity which is apparent in these heterologous aminoacylation reactions. The heterologous reactions differ from the standard homologous aminoacylation reactions in three respects: the heterologous reactions are incubated at pH 5.8, they require more enzyme (10 to 50 times more than in the homologous reaction), and they require more time (occasionally up to 2 hours). In other respects the heterologous and homologous aminoacylation reaction conditions are identical. The detailed kinetics of the heterologous aminoacylation reactions with the eight tRNAs discussed above will be published elsewhere (6).

The use of sequence comparisons to determine which nucleotides and regions of the tRNA molecule are involved in the synthetase recognition site has certain disadvantages. For instance any compensatory changes, i.e., a change in nucleotides in two regions, one change compensating for the other, which occur in these tRNA sequences could easily be missed by this approach. Secondary and tertiary structure must play a part in the recognition process but to what extent this method is unable to determine. Finally, the effects of modified nucleotides in the system as currently employed has been eliminated since we are drawing our composite based on the parent nucleotide rather than on its modification.

Several E. coli tRNAs ($tRNA^{Ala}_1$, $tRNA^{Ala}_2$, $tRNA^{Lys}$) whose sequences have not been determined are aminoacylated by PRS (1,8). Based on the observations discussed here and previously (1) we would conclude that these tRNAs should have the 10 nucleotides involved in the PRS recognition site. E. coli $tRNA^{Ala}_1$, under investigation in our laboratory, indeed has these exact 10 nucleotides in their expected location (19).

Several tRNAs have adenosine at the fourth position from the 3' end but not the specific nine nucleotides of the diHU stem region as shown in Fig. 1. These tRNAs include E. coli $tRNA^{Met}_F$ (20) and wheat $tRNA^{Gly}_1$ (21) both of which were isolated in purified form and both are not aminoacylated by PRS (Table 2). Thus, specific nucleotides in both regions are required for a tRNA to be aminoacylated by PRS.

The current model of the yeast phenylalanyl tRNA synthetase recognition site is that it consists, in part, of nine nucleotides in the diHU stem region and adenosine as the fourth residue from the 3' end. This model must be considered fluid. We do not know, for example, whether all nine nucleotides in the diHU stem region are required for the recognition process, or whether some of these nucleotides are dispensable. We do not know if in the tertiary structure, the nucleotides of the diHU stem region and the fourth nucleotide from the 3'end are part of a single site which is recognized by PRS. We do

not believe that nucleotides in these two regions of the tRNA molecule are the only parameters involved in this recognition process. The important roles of other nucleotides (see Fig. 1), the size of various loops, base modifications and the tertiary structure all require further study.

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