Lysine tRNAs from *Bacillus subtilis* 168: function of the isoacceptors in a rabbit reticulocyte cell-free protein-synthesizing system

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ABSTRACT

The two principal tRNA^{Lys} isoaccepting species of *Bacillus subtilis* were compared in their functional activity in translating rabbit globin. Although neither species demonstrates any preference in reading either of the lysine codons, there is an overall preference for tRNA^{Lys}₃ in lysine incorporation. The ratios of lysine incorporated by the two species into the different lysine-containing sites in the globin subunits vary over a more than two-fold range. As described in the accompanying paper, tRNA^{Lys}₁ is a hypomodified form of tRNA^{Lys}₃. Consistent with studies on other tRNA species, the fully modified isoacceptor functions preferentially. In contrast to these results, however, the fully modified isoacceptor (tRNA^{Lys}₃) is found predominantly in rapidly dividing cells while the hypomodified isoacceptor (tRNA^{Lys}₁) predominates in the stationary cells and spores of *B. subtilis*.

INTRODUCTION

There are two principal tRNA^{Lys} isoaccepting species as resolved by reversed phase column chromatography in *Bacillus subtilis* 168 and several other strains¹⁻³. tRNA^{Lys}₁ predominates in spores and tRNA^{Lys}₃ in cells in exponential growth. The shift from one isoacceptor to the other occurs during stationary growth and is associated with little overall change in tRNA^{Lys} abundance in the cells⁴. There is an additional tRNA^{Lys} isoacceptor designated tRNA^{Lys}₂ which is not considered in the present study because it accounts for less than 10% of total tRNA^{Lys} in cells at any growth stage.

The function in protein synthesis of the tRNA^{Lys} isoacceptors from *B. subtilis* 168 was investigated in the present study using rabbit reticulocyte lysates in which the added tRNA species incorporate labeled lysine into globin. The known amino acid sequences of the rabbit globin subunits⁵ and the base sequences of the subunit mRNA's^{6,7} permit correlation of amino acid residues with their codons. Our approach can be used to compare three aspects of the function in translation of competing tRNA's as described previously^{8,9,10} including codon preferences, overall usage, and the comparative activity of each

species in incorporating lysine into each lysine-containing site in the globin subunits. Structural studies on the tRNA^{Lys} isoacceptors will be presented in the accompanying paper to provide some basis for the functional differences described here.

MATERIALS AND METHODS

Growth conditions and preparation of tRNA^{Lys} fractions

Bacillus subtilis 168 trpC2 was grown to late exponential phase on tryptone-yeast extract medium containing 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl, 10^{-3} mole CaCl₂, 10^{-5} mole MnCl₂, 10^{-6} mole FeCl₃, and 40 mg L-tryptophan per liter. After the cells were harvested and washed, they were broken with lysozyme, and the tRNA was extracted with phenol and fractionated on a column of Sephadex G-100. Mixed tRNA's from the Sephadex column were then chromatographed on a reversed phase-5 (RPC-5) column¹¹ using a gradient of 0.4 to 0.8 M NaCl in 10 mM sodium acetate, pH 4.5, 10 mM MgCl₂, 1 mM EDTA, 20 mM 2-mercaptoethanol, and 0.1 ml/l isoamylacetate. Fractions containing lysine acceptance activity were identified by post-charging the eluted fractions using an aminoacylation reaction for lysine which has been described previously¹. Fractions containing tRNA₁^{Lys} and tRNA₃^{Lys} were precipitated with ethanol and collected on Millipore nitrocellulose filters. Aminoacylated preparations of each tRNA^{Lys} isoacceptor were made both with [¹⁴C]lysine (New England Nuclear, 345 mCi/mmol) and with [³H]lysine (New England Nuclear, 80.5 Ci/mmol).

After aminoacylation the contents of each reaction mixture were loaded onto 1 ml DEAE-cellulose columns packed in syringes. After washing with a solution of low salt, the tRNA of each reaction was eluted, precipitated with ethanol, and collected on a Millipore nitrocellulose filter. These aminoacylated fractions were then added to the protein synthesizing systems described below.

Incorporation of lysine from *B. subtilis* lysyl-tRNA^{Lys} into rabbit globin tryptic peptides

The present study follows in detail the procedure described previously for the incorporation of amino acids from added aminoacyl-tRNA's into globin in a rabbit reticulocyte lysate^{8,9,10}. Incorporation both from the two major lysyl-tRNA^{Lys} species added singly and from the tRNA^{Lys} isoacceptors with contrasting amino acid labels added in competition were studied. One hundred to 200 pmol of each exogenous labeled lysyl-tRNA^{Lys} were added. Added exogenous tRNA^{Lys} accounted for 5 to 10 times more tRNA^{Lys} than the endogenous tRNA^{Lys}

present in reticulocyte lysates⁹. In experiments in which the two tRNA^{Lys} isoacceptors were added in competition with each other, equal amounts of each isoacceptor were added. Duplicate and reciprocal (isotopic labels reversed on the isoacceptors) experiments were performed, and duplicate fingerprints from each experiment were obtained.

After 30 minutes of incubation of the reticulocyte lysates with added tRNA at 25°, hemoglobin was prepared, and heme was removed from globin as described previously^{8,9}. At that point determinations were made of the relative "usage" of the two competing tRNA isoacceptors by determining the fmoles of lysine incorporated from each and using the formula of Rosenbloom and his coworkers^{12,13} as previously described by our laboratory¹⁰.

Globin subunits were then prepared and digested with trypsin, and the tryptic peptides were resolved by fingerprinting and identified as previously described⁸. Since there is no more than a single lysine residue per tryptic peptide, it is possible to identify single lysine residues unambiguously. In competition experiments double labeling techniques were used to distinguish incorporation from the two tRNA^{Lys} isoacceptors. In order to avoid the consequences of variable quenching of ³H in different globin subunit and tryptic peptide preparations, all samples were counted after heme removal, and when reciprocal experiments were done, the results were averaged.

RESULTS

Relative usage of the tRNA^{Lys} isoacceptors in globin synthesis

Three to 10% of the labeled lysine added as lysyl-tRNA was incorporated into globin. This is a smaller percentage of incorporation than occurs from similar amounts of animal aminoacyl-tRNA in which 20 to 30% of histidine^{8,10} and lysine⁹ are incorporated under similar conditions. Lysine incorporation from *B. subtilis* tRNA continued through the incorporation period but at a decreasing rate. At the end of the period some labeled lysyl-tRNA still remained.

Based on three separate experiments, 58% of the total lysine incorporated was from tRNA₃^{Lys} and 42% from tRNA₁^{Lys} although the two isoaccepting species were added to lysates in equal amounts. The usage ratio of tRNA₃^{Lys}/tRNA₁^{Lys} is, therefore, 1.38.

Site-specific incorporation of lysine from the tRNA^{Lys} isoacceptors

Experiments in which the tRNA^{Lys} isoacceptors were added singly revealed that each incorporates lysine into every lysine-containing site in each subunit

of rabbit globin. The experiments indicate that the minor isoacceptor, tRNA^{Lys}₂, does not function uniquely in the incorporation of any lysine residue.

The results of incorporation by the two tRNA^{Lys} isoacceptors in competition with each other are shown in Table I. In competition, each of the two isoacceptors incorporates lysine into every lysine-containing site in rabbit

TABLE I. Incorporation of Lysine from *B. subtilis* Isoaccepting tRNA^{Lys} Species into Globin Tryptic Peptides

Ninhydrin staining spot corresponding to globin peptide	Lysine residue in amino acid sequence	Lysine codon from mRNA ^{6,7}	Lysine incorporated by tRNA isoacceptor (fmol)		Incorporation ratio $\frac{tRNA^{Lys}_3}{tRNA^{Lys}_1}$
			$tRNA^{Lys}_1$	$tRNA^{Lys}_3$	
Alpha subunit					
αT1	7	AAG	7.91	15.69	1.984
αT1-2 ^a			5.48	16.93	3.089*
αT2	11	AAG	2.42	3.66	1.512
αT3	16	AAG	4.54	19.30	4.251**
αT4					
αT5	40	AAG	17.96	22.98	1.279
αT6	56	AAA	38.75	80.91	2.088
αT7	60	AAG	16.41	22.25	1.356
αT8	61	AAG	66.63	64.05	0.961*
αT8-9 ^a			25.09	36.71	1.463
αT9	68	AAG	12.16	24.60	2.023
αT10	90	AAG	13.72	40.36	2.941*
αT11					
αT11-12 ^a			15.28	33.83	2.214
αT12	99	AAG	11.66	19.98	1.713
αT13	127	AAG	Core Peptide		
αT14	139	AAA	16.68	34.03	2.040
αT15					
Beta subunit					
βT1	8	AAG	11.59	26.98	2.327
βT2	17	AAG	10.64	18.01	1.693
βT3					
βT4					
βT5	59	AAG	19.95	35.14	1.761
βT6	61	AAG	11.81	14.80	1.253
βT7	65	AAG	19.86	24.06	1.211
βT8	66	AAG	140.06	135.90	0.970*
βT9	82	AAA	29.28	65.77	2.248
βT10	87	AAG	39.86	79.47	1.994
βT11 ^b	95	AAG	44.81	57.63	1.286
βT12			Core Peptide		
βT13-14 ^a	120 and 132	AAA and AAG	55.10	71.39	1.296
βT15	144	AAA	60.49	129.48	2.140
βT16					
Mean of incorporation ratios: 1.884 + 0.738					

^aPeptide incompletely split by trypsin.

^bPeptide βT11, which contains cysteine, migrates as two different spots as previously shown^{8,9}. The counts in both spots have been combined for this table.

*Incorporation ratios differing from the mean of ratios by more than 1 or 2 (**) standard deviations.

[¹⁴C] and [³H]lysine esterified to *B. subtilis* tRNA^{Lys} and tRNA^{Lys} were incorporated into the tryptic peptides of the globin subunits as described in the text. The results shown are the average of lysine found in duplicate fingerprints as incorporated by two lysates with opposite labels in the lysine on the two Lys-tRNA^{Lys} isoacceptors. Counts in peptides which do not contain lysine were taken as background levels and have been subtracted from the values shown.

globin just as they do when added separately. A possible exception could be the lysine residue in the core peptides which are insoluble and are therefore not prepared with the other peptides or observed on fingerprints. It should be noted that the amounts of labeled lysine found in the different peptides varies considerably. In the case of the α peptides, this variation is determined by peptide recovery and occurs randomly, but in the peptides of the β subunit the amount of labeled lysine found generally increases from the amino- to the carboxyl-end of the subunit. This suggests that most of the incorporation was into partially completed β subunits and that relatively little reinitiation of translation of the β subunit occurred. Differences in the processes of initiation of the two subunits are recognized¹⁴.

The ratios of lysine incorporated by the two isoacceptors are independent of peptide recovery and reflect the general preference for $\text{tRNA}_3^{\text{Lys}}$ over $\text{tRNA}_1^{\text{Lys}}$. The mean value of the ratios of incorporation of $\text{tRNA}_3^{\text{Lys}}/\text{tRNA}_1^{\text{Lys}}$ is 1.88, consistent with the usage ratio described above which was determined with intact globin subunits. While the preference for $\text{tRNA}_3^{\text{Lys}}$ is apparent in most sites, there is a site in each subunit in which the usage of each isoacceptor is approximately equal. In each subunit the residue is in peptide 8 which consists of a single lysine which is in tandem with the carboxy-terminal lysine of peptide 7. Interestingly, our previous work⁹ indicates that these same residues are incorporated preferentially by the hypomodified $\text{tRNA}_3^{\text{Lys}}$ species of rabbit liver which is generally less preferred. The table indicates those ratios which differ from the mean of all ratios by more than one and by more than two standard deviations.

There is little, if any, evidence of codon-related preference by either of the $\text{tRNA}_1^{\text{Lys}}$ isoacceptors. The AAG encoded residues are incorporated by the two isoacceptors in a variety of ratios. The AAA encoded sites show a small but consistent preference for $\text{tRNA}_3^{\text{Lys}}$ which is in excess of the mean preference for this isoacceptor.

It is uncertain why the incompletely split peptide $\alpha\text{Tl-2}$ has an incorporation ratio which is not consistent with those of the peptides as seen separately on the fingerprints. We suggest that the separate peptide αTl is contaminated by surrounding lysine containing peptides such as αT9 and αT12 (see fingerprint in ref. 8), thus reducing the apparent preference for $\text{tRNA}_3^{\text{Lys}}$ in incorporation into this position. αTl is more likely to be contaminated than αT2 or $\alpha\text{Tl-2}$, and we believe that the incorporation ratio of the unsplit peptide is a better indication of the incorporation ratio of αTl than the apparent ratio of the separate αTl spot.

Miscoding by the *B. subtilis* tRNA^{Lys} isoacceptors in rabbit globin synthesis

Each fingerprint was examined for the possible occurrence of labeled lysine outside of the globin peptides and in globin peptides which do not contain lysine. Only 10 to 20% of the label was found in such locations on the fingerprints. This is consistent with the amount of non-globin synthesis in reticulocytes¹⁵.

DISCUSSION

The validity of studies of *B. subtilis* tRNA^{Lys} function in a translational system in which all other components are of animal cell origin must be considered. The advantage of the reticulocyte lysate as a system is that virtually the only protein synthesized is globin¹⁵, of which both amino acid sequences and mRNA base sequences are known. Such significant concepts as the adaptor hypothesis of tRNA function¹⁶ and the unique functions of certain tRNA isoaccepting species in translation^{17,18} have resulted from similar experiments with microbial tRNA's in reticulocyte lysates. Although our studies indicate that the bacterial tRNA isoacceptors do not function as efficiently in the system as animal tRNA's, the point of our studies is the comparison of the function of two homogeneous^{19,20} tRNA's which differ in known post-transcriptional modifications (see accompanying paper). There is enough incorporation by the isoacceptors that the comparisons can be made, however as the opportunities become available, the studies reported here should be confirmed in homologous systems. It should be noted that the modifications seen in this study occur in both bacterial and animal cells.

During the growth and differentiation of *B. subtilis* ten different sets of isoaccepting tRNA groups show changes in relative amounts or show new isoaccepting species²¹. The only sets of tRNA isoacceptors for which sequences have been determined are the lysine tRNA's described here and a pair of tyrosine isoacceptors²². The two tyrosine isoacceptors also have the same primary base sequence and differ in a post-transcriptional modification. It was shown that the fully modified tRNA^{Tyr} is more efficient in binding to ribosomes than its hypomodified counterpart, consistent with the present study and with published results on a variety of other tRNA groups²³.

Usually fully modified isoacceptors are found in slowly growing cells and hypomodified isoacceptors in rapidly dividing cells including tumor cells²³⁻²⁵. This is the case with the tRNA^{Tyr} isoacceptors of *B. subtilis*, however, in contrast, the fully modified tRNA^{Lys} isoacceptor is found in exponentially growing cells and the hypomodified in spores. There are functional differ-

ences between the isoacceptors, and it is possible that the synthesis and modification of tRNA species are independent processes with each serving specific needs in the translation of the proteins synthesized in different cellular conditions.

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