IDENTIFICATION OF TWO LYSINE tRNA CISTRONS IN BACILLUS SUBTILIS BY HYBRIDIZATION OF LYSYL-tRNA WITH DNA

Ronald Chuang, Toshio Yamakawa, and Roy H. Doi
Department of Biochemistry and Biophysics
University of California
Davis, California 95616

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Summary. Two lysyl-tRNA species were obtained from Bacillus subtilis by benzoylated-DEAE-cellulose column chromatography. They were found to hybridize in an additive fashion to B. subtilis DNA. These results indicate that the base sequences of these two lysine tRNA species differ sufficiently to prevent the saturation of both genetic sites by either and indicate that there are at least two cistrons for lysine tRNA.

Many examples of multiple tRNA species for an amino acid have been reported (1,2). Two general assumptions are that the tRNA species either are products of different genes or are different modified forms of a tRNA transcribed from one gene. In a sequence study it was found that two serine tRNAs from brewer's yeast differed by 3 base changes (3) indicating the presence of two serine tRNA genes. In this particular case it appears that gene duplication had occurred followed by three mutations resulting in two serine tRNAs which were still very similar in structure and function.

In previous studies of the lysine tRNA species of Bacillus subtilis during sporulation, it was reported that two peaks of lysyl-tRNA were resolved by chromatographic columns (4-6). In a continuing study of lysine tRNA patterns and functions of B. subtilis cells (7), we have found from tRNA-DNA hybridization studies that two lysine tRNA species separated by column chromatography hybridized to DNA in an additive fashion. This method of analysis allows one to distinguish two isoaccepting tRNA species which differ sufficiently in base sequence to prevent the saturation of both genetic sites by either.

MATERIALS AND METHODS

Organism and medium: B. subtilis W23 was used as the source of tRNA,

aminoacyl synthetase, and DNA. Cells were grown to a density of 5 x 10^8 cells per ml in Penassay medium (Difco) at 37° C with vigorous shaking.

Preparation of lysyl-tRNA: The tRNA was prepared from cells as described by von Ehrenstein and Lipmann (8). The aminoacyl synthetase was prepared generally as described by Zubay (9) followed by chromatography through Sephadex G75 and DEAE-sephadex A-50 to a stage where no RNase activity could be detected (Chuang and Doi, manuscript in preparation). The reaction mixture for the preparation of lysyl-tRNA for the hybridization experiments contained in μmoles: 50, Tris-HCl buffer, pH 8.0; 2.5, $MgCl_2$; 5, KCl; 0.5, ATP; 0.5, glutathione; 100-500 μg, tRNA; 58 μg, partially purified synthetase; 50 μg, bovine serum albumin; and 25 μCi ^{3}H-lysine (50 Ci/mmole); in a total volume of 0.20 ml. The reaction mixture was incubated at 37° C for 12 min. The reaction was stopped by adding 0.2 ml of phenol saturated with 0.1 M sodium acetate buffer, pH 5. The lysyl-tRNA was extracted by the phenol method (8) and the final precipitate was dissolved in 0.05 M sodium acetate buffer, pH 5.0.

Column chromatography: The benzoylated diethylaminoethyl-cellulose (B-DEAE) column was prepared according to Gillam et al. (10). The T_1 RNase treatment of lysyl-tRNA and chromatography of oligonucleotides on DEAE-cellulose columns were performed by the method described by Ishida and Miura (11).

DNA-RNA hybridization: DNA-RNA hybridization was carried out essentially as described by Gillespie and Spiegelman (12) and Weiss et al. (13). Alkaline denatured DNA (200 μg) was fixed onto nitrocellulose filters (27 mm) and incubated in a total volume of 1.5 ml with 50% formamide, 1 mg/ml yeast RNA, 2XSSC buffer (0.3 M NaCl + 0.03 M Na citrate), pH 5.0, and ^{3}H-lysyl-tRNA as indicated for 3 hr at 30° C. The filters were washed with 2XSSC, treated with RNase T_1 (2.5 μg/ml) for 30 min at room temperature, washed again with 2XSSC, dried, and then counted in a scintillation counter. The filters with E. coli DNA or without any DNA were used as controls.

RESULTS

When tRNA obtained from log phase cells of B. subtilis was charged with ^{3}H-lysine and chromatographed through a B-DEAE-cellulose column, two peaks of ^{3}H-lysyl-tRNA were obtained as illustrated in Figure 1. The fractions in these peaks were pooled, concentrated by flash evaporation, deacylated by treatment at pH 8.8, and recharged with ^{3}H-lysine illustrating that they were both functionally active tRNA fractions. Furthermore when the first or second peak was passed through a B-DEAE-cellulose column a second time, it eluted from the column in the original position. The lysine tRNA from peaks 1 (fractions 130-150) and 2 (fractions 186-220) are designated as lysine $tRNA_1$ and lysine $tRNA_2$ through the rest of this paper.

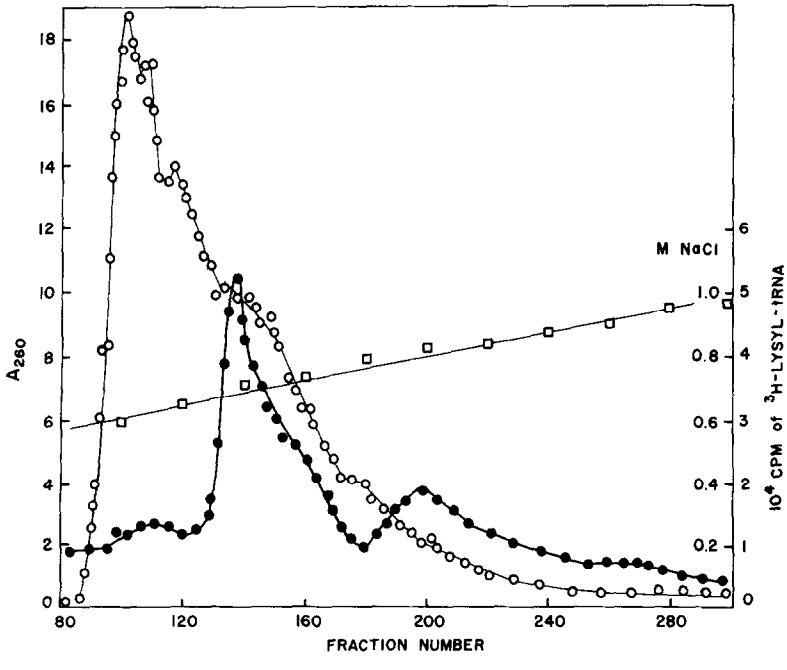

Figure 1. Elution profile of ^{3}H-lysyl-tRNA from a B-DEAE-cellulose column. The open and closed circles represent A_{260} and counts per minute, respectively. The squares indicate NaCl molarity for the elution gradient.

In a preliminary test to determine whether the two lysine tRNA species were different in sequence, lysine $tRNA_1$ and lysine $tRNA_2$ were charged with ^{3}H-lysine and ^{14}C-lysine, respectively, mixed together, and then digested

with T_1 RNase; the resulting lysyl-oligonucleotides were chromatographed through a DEAE-cellulose column to determine whether the two lysine tRNAs differed in sequence from the first guanine residue from the 3'terminus. The results showed that lysyl-oligonucleotides (with a chain length of 3 bases) from lysine $tRNA_1$ and lysine $tRNA_2$ eluted in exactly the same position. These results in contrast to the previous results with B. subtilis valine tRNA (14) indicated that the lysine tRNA species had identical T_1 RNase susceptible sequences near the amino acid accepting terminus.

To test whether lysine $tRNA_1$ and lysine $tRNA_2$ contained different base sequences in the rest of the molecule, hybridization between lysyl-$tRNA_1$ and lysyl-$tRNA_2$ and DNA was tested by the method of Weiss et al. (13). Each of the pooled fractions from the B-DEAE-cellulose column (Fig. 1) was charged separately with ^{3}H-lysine and the amount of ^{3}H-lysyl-$tRNA_1$ and of ^{3}H-lysyl-$tRNA_2$ which would hybridize with DNA was determined. These results are shown in Figure 2. The ratio of tRNA to DNA above 0.3 (i.e. 60 µg of tRNA to 200 µg of DNA) resulted in saturation of DNA sites for both tRNA preparations under the conditions used. Since the same specific activity of ^{3}H-lysine was used during charging of both tRNA preparations, the almost equal number of hybridized counts with both tRNA preparations suggested

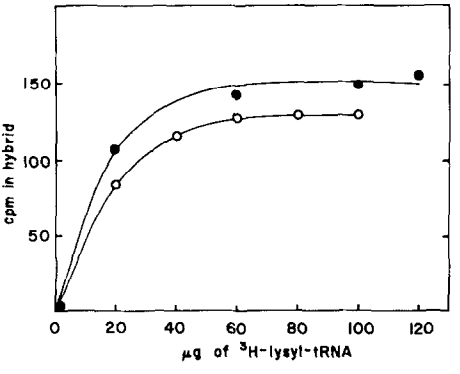

Figure 2. Hybridization of ^{3}H-lysyl-$tRNA_1$ and ^{3}H-lysyl-$tRNA_2$ with B. subtilis W23 DNA. The open and closed circles represent ^{3}H-lysyl-$tRNA_1$ (760 cpm per µg) and ^{3}H-lysyl-$tRNA_2$ (1,170 cpm per µg), respectively.

that equal amounts of lysyl-$tRNA_1$ and lysyl-$tRNA_2$ were hybridized. Only 14% deacylation occurred with control samples of both tRNA preparations during the experiment under incubation conditions identical to that of the hybridization reactions.

To determine whether the two lysine tRNA species were hybridizing with two distinct DNA sites, the additive nature of hybridization was tested by adding saturating levels of lysyl-$tRNA_1$ and lysyl-$tRNA_2$ simultaneously to the hybridization reaction mixture. The results from two experiments are presented in Table 1. For each experiment, the lysyl-tRNA preparations were tested first to determine the concentration of tRNA required for saturation

Table 1. Hybridization of lysyl-$tRNA_1$ and lysyl-$tRNA_2$ to DNA

Exp. No.	DNA on filter	^{3}H-lysyl-tRNA hybridized		
		$tRNA_1^{lys}$	$tRNA_2^{lys}$	
		cpm	cpm	net cpm
1 a	no DNA	231		
	B. subtilis W23	361		130
b	no DNA		353*	
	B. subtilis W23		501	148
c	no DNA	567*		
	B. subtilis W23	870		303
2 a	no DNA	110		
	E. coli	100		
	B. subtilis W23	233		123
b	no DNA		245	
	E. coli		209	
	B. subtilis W23		366	121
c	no DNA	320		
	E. coli	339		
	B. subtilis W23	533		213

^{3}H-lysyl-$tRNA_1$ (80 µg) and ^{3}H-lysyl-$tRNA_2$ at saturating concentrations (80 µg or 100 µg*) were hybridized with denatured DNA (200 µg), respectively. In Exps. 1c and 2c, both aminoacyl-tRNAs were added to the incubation mixture.

of the DNA before testing for additive hybridization. The same preparations were used for the results reported in Figure 2 and Table 1. In other experiments no aberrant hybridization was noted when up to 200 μg of lysyl-tRNA was added to the reaction mixture, i.e. a large excess of lysyl-tRNA did not result in greater hybridization than the saturation levels noted in Figure 2. These results illustrate that lysine $tRNA_1$ and lysine $tRNA_2$ each hybridized to a given saturation level and that when they were added together to the reaction mixture, they hybridized in an additive fashion.

DISCUSSION

Two significant conclusions can be reached from these data:

1) These results indicate that two lysine tRNA genes are present whose base sequences differ sufficiently to allow additive hybridization with two isoaccepting tRNA species. If the genes were originally derived by gene duplication, these results suggest that sufficient base changes have occurred during evolution to prevent hybridization of one tRNA species to the genetic locus of the other. At first glance these results are surprising; however, a change of only a few bases could lead to unstable heterologous hybrid formation in the usual manner or the base changes could give rise to two tRNA species which are modified quite differently by the various base-modifying enzymes with subsequent enhanced loss of stable hybrid formation with the heterologous DNA sequence.

2) In this particular case the hybridization technique was able to distinguish the origin of two isoaccepting tRNA species. Although this may not be the case with other isoaccepting tRNAs, it would be judicious to apply this additive hybridization test to other bacterial systems, in which isoaccepting tRNAs have been reported to determine their genetic origin. Obviously those isoaccepting tRNAs which differ by only one or a few bases may still bind to heterologous sites and this particular test will not distinguish their origin. However, the clear and readily obtained results in this particular case indicate that it is worth the effort to acquire this basic information.

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