Lysine tRNAs from *Bacillus subtilis* 168: structural analysis

Barbara S.Vold*, Duane E.Keith, Jr.*, Martin Buck+, James A.McCloskey† and Henriana Pang†

*Biomedical Research, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025, +Department of Biochemistry, University of California, Berkeley, CA 94720, and †Department of Medicinal Chemistry, College of Pharmacy, University of Utah, Salt Lake City, UT 84112, USA

Received 23 February 1982; Revised and Accepted 26 April 1982

ABSTRACT

The primary sequence was established for two lysine tRNA isoacceptors which differ in abundance during development in Bacillus subtilis. Both tRNAs shared the same primary sequence but differed in the degree of post-transcriptional modification in the anticodon loop. The earlier eluting species, $tRNA_1^{Lys}$, had an unmodified C in position 32 and a mixture of N-[9-β-ribofuranosyl) purin-6-ylcarbamoyl]-L-threonine, t^6A, and N-[(9-β-D-ribofuranosyl-2-methylthio-purin-6-yl)carbamoyl]threonine, ms^2t^6A, in position 37. The later eluting species, $tRNA_3^{Lys}$, which is the more efficient in protein synthesis, had a modified C in position 32 and only ms^2t^6A in position 37. The possibility exists that modification to make a more efficient tRNA species may be part of a functional interaction between the translational and transcriptional changes that are part of the differentiation process in B. subtilis.

INTRODUCTION

During differentiation in Bacillus subtilis, changes in transcriptional and translational processes occur (1,2), among these are changes in the relative amounts and kinds of isoaccepting tRNA species (3-6). This could be caused by the expression of new tRNA genes at various growth stages of differences in the expression of the genes which code for tRNA modifying enzymes. To obtain more data about this phenomenon, we chose to compare the primary sequence of two lysine isoacceptors from B. subtilis 168 that showed differences in relative abundance as a function of growth stage (6).

MATERIALS AND METHODS

Isolation of lysine tRNAs and sequence analysis. tRNA was extracted from cells of B. subtilis 168 trpC2. The lysine isoacceptors were purified on columns of RPC-5 or Aminex A-28 with polyacrylamide gel electrophoresis as the final purification step (7). $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$ were sequenced by the method of Gupta and Randerath (8). When two-dimensional TLC was required, the oligonucleotide bands were extracted from the gel, digested with nuclease

0305-1048/82/1010-3125$2.00/0

P_1 and analyzed as described by Kuchino et al. (9).

Analysis of modified nucleosides by mass spectrometry. Purified $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$ were hydrolyzed using RNase T_2 followed by alkaline phosphatase and venom phosphodiesterase. Digests were dried, then trimethylsilylated by heating with N,O-bis-(trimethylsilyl)acetamide-trimethylchlorosilane-pyridine (100:1:10) for 1 h at 100°C. Samples of the resulting mixture equivalent to 5 µg tRNA were introduced into a Varian MAT 731 mass spectrometer by direct probe, and their high resolution mass spectra photographically recorded at resolution 15,000. Modified nucleoside components were identified from predetermined exact mass values (10).

Analysis of modified nucleosides by HPLC. Nucleosides were fractionated on a Supelco C_{18} 5 µ column, 250 x 4.6 mm ID. HPLC solvents were A 0.25 M ammonium acetate pH 6.0 and B 40/60 (v/v) acetonitrile/water. Nucleosides were resolved by gradient elution with a flow rate of 2 mℓ/min. The absorbance at 254 nm and 280 nm was integrated with a Spectra physics 4020/4000 system. Nucleosides were identified by retention time, the 254/280 spectral ratios and co-chromatography with standards. Full details of those methods will be published elsewhere (M. Buck and B. N. Ames, manuscript in preparation).

RESULTS

Primary sequence of $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$. The method of Stanley and Vassilenko (11), as modified by Gupta and Randerath (8) and Kuchino et al. (9) was used to determine the primary sequence of $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$. The 5'-terminus of both isoacceptors had previously been determined to be pG (12). In the majority of analyses, the modified nucleosides labeled well enough to locate by autoradiography. Final identification and quantitation were done using HPLC and mass spectrophotometric analysis. For the initial analysis of primary sequence, lysine tRNAs purified through a denaturing gel were hydrolysed, the 5'-end of each oligonucleotide labeled with [γ-^{32}P]ATP using polynucleotide kinase, and the oligonucleotides fractionated by polyacrylamide gel electrophoresis. These oligonucleotides were transferred to PEI-cellulose, digested with RNase T_2 and separated in one dimension using either the ammonium sulfate or ammonium formate solvent systems of Gupta and Randerath (8). Both species contained 2 pseudouridines, one in position 55 and one in position 39; 2 dihydrouridines, one in position 17 and one in position 20; m^7G at position 46; and a mixture of U and a modified U at position 34. The anticodon loop region was not well resolved by one-dimensional TLC, therefore, a two-dimensional TLC was performed (9). The results are shown in Fig. 1.

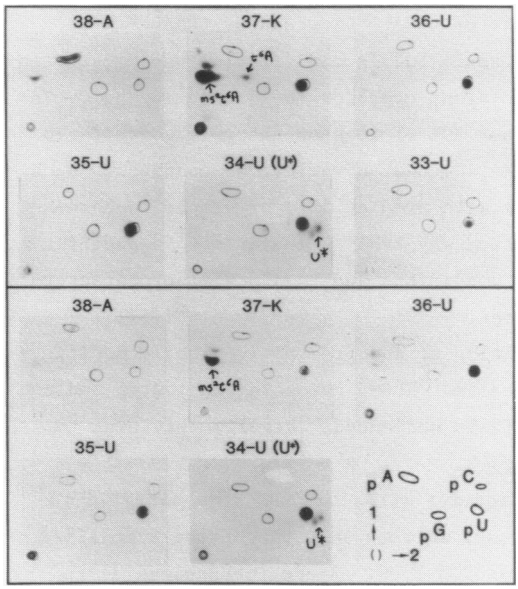

Figure 1. Analysis of the 5'-terminal nucleotides released by nuclease P_1 digestion of oligonucleotides extracted from a polyacrylamide gel. The upper section shows results of nucleosides derived from $tRNA_1^{Lys}$ and the lower section shows results with nucleosides derived from $tRNA_3^{Lys}$.

The designations K and U* were given by Yamada and Ishikura (13) in their original report of the sequence of B. subtilis $tRNA_1^{Lys}$. Upon subsequent investigation, U*, which was found at about 0.3 mol/mol tRNA, was identified as 5-(carboxymethylaminomethyl)-2-thiouridine, $cmnm^5s^2U$, based on its ultraviolet absorption spectra and mass spectrometric analysis of the trimethylsilyl derivative (14). Yamada and Ishikura identified K as N-[(9-β-D-ribofuranosyl-2-methylthiopurin-6-yl)carbamoyl]threnonine, ms^2t^6A, based on analysis of alkaline hydrolyzate of K and comparison of chromatographic properties with those of authentic ms^2t^6A from rabbit liver (15).

Both species have a small amount of label appearing as a double spot near U at position 34. This is assumed to represent U* or $cmnm^5s^2U$, although we did not have a standard to which to compare it. Also, in both cases, there is an area of radioactivity representing ms^2t^6A. One difference is that position 37 in $tRNA_1^{Lys}$ also contains t^6A. This assignment is based on the chromatographic mobility published for t^6A by Kuchino et al. (16) and by our HPLC analysis revealing the presence of t^6A in $tRNA_1^{Lys}$. Since there were no detectable changes so far in the primary sequence or minor modifications of the purified $tRNA_1^{Lys}$ or $tRNA_3^{Lys}$ except that $tRNA_1^{Lys}$ had a mixture of t^6A and ms^2t^6A, the chromatographic properties of the two species were re-examined by co-chromatography with mixed tRNAs aminoacylated with lysine. Although $tRNA_1^{Lys}$ co-eluted exactly with the $tRNA_1^{Lys}$ from mixed tRNAs, purified $tRNA_3^{Lys}$

co-eluted in a position characteristic of $tRNA_1^{Lys}$ instead of $tRNA_3^{Lys}$. We interpreted this result to indicate that a labile modification was present in $tRNA_3^{Lys}$ which was lost under the conditions used for the denaturing gel. In fact, $tRNA_3^{Lys}$ is so unstable that about 30% of it spontaneously converts to either the position of $tRNA_1^{Lys}$ or $tRNA_2^{Lys}$ under a variety of conditions such as withdrawal of magnesium, treatment with urea at 45°, or simple re-chromatography on RPC-5. We do not understand why $tRNA_3^{Lys}$ converts to a position characteristic of $tRNA_1^{Lys}$ under some conditions and to a position characteristic of $tRNA_2^{Lys}$ under other conditions.

In an attempt to locate the position of the presumptive labile modification, $tRNA_3^{Lys}$ purified as far as the last RPC-5 chromatography step and not subject to a denaturing gel was partially sequenced. Results pertinent to the anticodon region are shown in Fig. 2.

In the absence of the gel purification step, the tRNAs do not give a clear nucleotide pattern. Nevertheless, we were able to observe a modified nucleoside at position 32 in the anticodon loop. Since the nucleoside in this position chromatographed as C in $tRNA_3^{Lys}$ purified on the denaturing gel, C is the parent nucleoside. In ammonium formate or ammonium sulfate, the modified nucleoside ran ahead of C.

Identification of modified nucleosides from mass spectra of unfractionated tRNA hydrolysates. Modified nucleosides were identified by exact mass

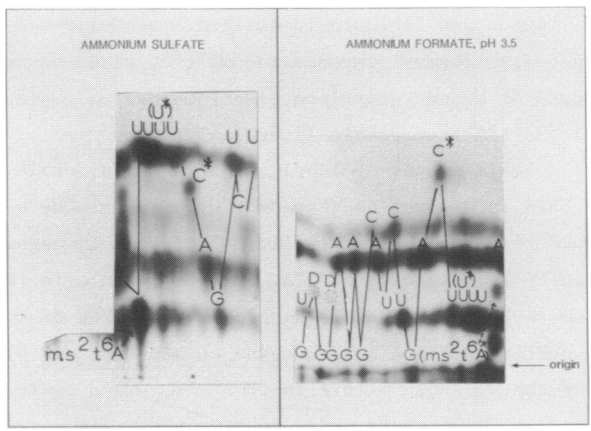

Figure 2. One dimensional PEI-cellulose TLC analysis of 5'-^{32}P-labeled complete RNase T2 digestion products of oligonucleotides derived from a $tRNA_3^{Lys}$ sample which had not been purified on a denaturing gel. Ammonium formate was the solvent for TLC shown in the left panel and ammonium sulfate in the right panel.

values of characteristic ions (17,18) for each component (10) from an unfractionated hydrolysate of a purified tRNA. Examples of the resulting data are represented by the profiles from a small section of the photographic plate shown in Figure 3. The identifying peaks for m^7G are the molecular ion (M) and $M-CH_3$ ion from the pentasilyl derivative of the imidazole ring-opened form which is characteristically observed in silylated hydrolysates (10). The identification of ms^2t^6A is based on the $M-CH_3$ and $M-C_2H_4O$ (from the threonine side chain) ions which are characteristic for the t^6A family of nucleosides (19,20). In similar fashion, ms^2t^6A was found in $tRNA_3^{Lys}$ (10); m^7G was not found, but the absence of appropriate peaks is not indicative of its absence (10). It was found in both tRNAs by HPLC and in determination of the primary sequence.

D, T and Ψ were observed in both tRNA species, based on ions of the molecular ion series (17) (data not presented). No evidence was found for $cmnm^5s^2U$ in either species, although both showed m/z 488.2069, which is characteristic of 5-carboxymethylaminomethyluridine ($M-CH_2CO_2Si(CH_3)_3$), which may have arisen from the chemically plausible oxidation of $cmnm^2s^5U$.

Analysis of modified nucleosides by HPLC. Nucleosides were analyzed by HPLC as described in Materials and Methods. The data showed that both $tRNA_1^{Lys}$ and $tRNA_3^{Lys}$ contained equal amounts of ψ, m^5U, and m^7G in molar ratios of 2:1:1. The nucleoside t^6A was found in $tRNA_1^{Lys}$ at about 0.17 mol t^6A/mol m^5U and in $tRNA_3^{Lys}$ at about 0.03 mol t^6A/mol m^5U. Some ms^2t^6A was also found in

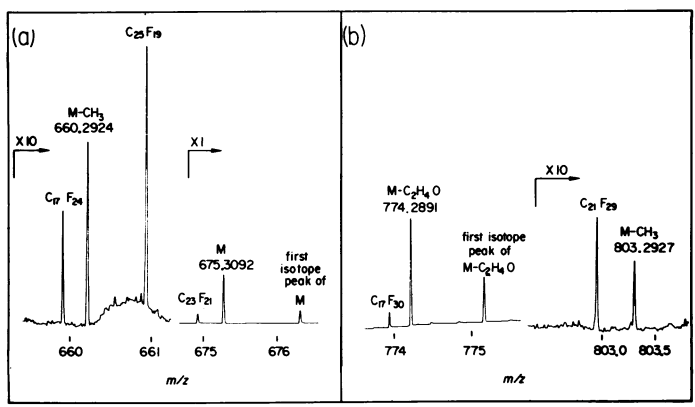

Figure 3. Selected regions of the high resolution mass spectra of trimethylsilylated digests of $tRNA_1^{Lys}$ showing presence of (a) m^7G, and (b) ms^2t^6A. Mass values shown are experimental; theoretical values are (a) 660.2922, 675.3157; (b) 774.2936, 803.2963.

both species, but it seemed to undergo a chemical change during the preparation of the material for analysis and the species co-chromatographing with an authentic standard from rabbit liver tRNA was only detectable in very low amounts. There was no mt^{6}A detectable in either species.

An unidentified nucleoside eluting between U and m^{5}U was also found in both species. Its elution position is compatible with it being a modified uridine such as cmnm^{5}s^{2}U. It was shown not to be 5-methylaminomethyl-2-thiouridine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methyl-2-thiouridine, or 5-methoxycarbonylmethyl uridine.

DISCUSSION

The structure for tRNA$_3^{Lys}$ from B. subtilis is given in Fig. 4. The structure for tRNA$_1^{Lys}$ is the same as that for tRNA$_3^{Lys}$ in primary sequence but differs in post-transcriptional modification in that tRNA$_1^{Lys}$ has an unmodified C in position 32 and both t^{6}A and ms^{2}t^{6}A in position 37. The sequence of tRNA$_1^{Lys}$ has been published by Yamada and Ishikura (13). Our sequence of tRNA$_1^{Lys}$ is the same as theirs except that we detected a mixture of t^{6}A and ms^{2}t^{6}A whereas Yamada and Ishikura (13) reported the presence of only ms^{2}t^{6}A. Both laboratories found a partly replaced uridine residue at position 34 which Yamada, Murao, and Ishikura (14) identified

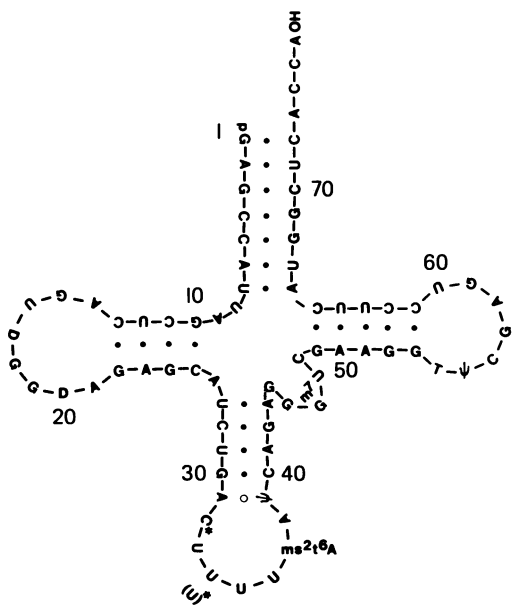

Figure 4. Primary sequence of tRNA$_3^{Lys}$ from B. subtilis.

as 5-(carboxymethylaminomethyl)-2-thiouridine. Although we have not sequenced $tRNA_2^{Lys}$, it seems most likely that this minor species is generated through the degeneration of $tRNA_3^{Lys}$. Therefore, all three lysine isoacceptors in B. subtilis probably arise from structural genes of the same sequence. Similarly, a change in the two tyrosine isoacceptors at various growth stages in B. subtilis is due to a post-transcriptional modification (21) and there is only one type of gene sequence for tyrosine tRNAs in B. subtilis. Hopefully, when more is known about the structure and function of the other eight sets of isoacceptor tRNAs which show chromatographic variations during development (6), we will be able to relate these changes to other alterations in the translational and transcriptional apparatus which occur as part of sporulation (22,23).

ACKNOWLEDGEMENTS

The technical assistance of Annette Wen is gratefully acknowledged. We are indebted to Kurt and Erika Randerath for their many helpful discussions concerning the rapid read-out gel technique. We thank Bruce N. Ames in whose laboratory the HPLC analyses were performed, and Dr. T.-W. Chou for doing fermentor runs for us.

This work was supported by the following sources: Public Health Services Research Grant GM 27732 from the National Institutes of Health (B.V.), NIH Grant GM 19993 (B.A.), and GM 21584 (J.M.).

REFERENCES

1. Sonenshein, A.L. and Campbell, K. M. (1978) In Spores VII, pp. 179-192, American Society for Microbiology, Washington, D.C.
2. Trowsdale, J., Shiflett, M. and Hoch, J. (1978) Nature (London) 272, 179-181.
3. Lazzarini, R.A. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 185-190.
4. Doi, R. H. and Kaneko, I. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 581-582.
5. Arecneaux, J. L. and Sueoka, N. (1969) J. Biol. Chem. 244, 5959-5966.
6. Vold, B. S. (1973) J. Bacteriol. 113, 825-833.
7. Chia, L.-L. S. Y., Randerath, K., and Randerath, E. (1973) Anal. Biochem. 55, 102-113.
8. Gupta, R. C., and Randerath, K. (1979) Nucleic Acids Res. 6, 3443-3458.
9. Kuchino, Y., Kato, M., Sugisaki, H. and Nishimura, S. (1979) Nucleic Acids Res. 6, 3459-3469.
10. Pang, H., McCloskey, J. A. presented at the 28th Annual Conference on Mass Spectrometry and Allied Topics, May 1980, New York, pp. 384-385.
11. Stanley, J., and Vassilenko, S. (1978) Nature 274, 87-89.
12. Vold, B. (1978) J. Bacteriol. 135, 124-132.
13. Yamada, Y., and Ishikura, H. (1977) Nucleic Acids Res. 4, 4291-4303.
14. Yamada, Y., Murao, K., and Ishikura, H. (1981) Nucleic Acids Res. 9, 1933-1939.

15. Yamada, Y. and Ishikura, H. (1981) J. Biochem. 89, 1589-1591.
16. Kuchino, Y., Mita, T., and Nishimura, S. (1981) Nucleic Acids Res. 9, 4557-4562.
17. McCloskey, J. A., Lawson, A. M., Tsuboyama, K., Krueger, P. M., Stillwell, R. N. (1968) J. Am. Chem. Soc. 90, 4182-4184.
18. McCloskey, J. A. In "Basic Principles in Nucleic Acid Chemistry," Ts'o, P.O.P. Ed., Academic Press: New York, 1974; vol. I; pp 209-309.
19. Yamaizumi, Z., Nishimura, S., Limburg, K., Raba, M., Gross, H. J., Crain, P. F., McCloskey, J. A. (1979) J. Am. Chem. Soc. 101, 2224-2225.
20. Kasai, H., Muraeo, K., Nishimura, S., Liehr, J. G., Crain, P. F., McCloskey, J. A. (1976) Eur. J. Biochem. 69, 435-444 and supplemental material A. O. 553: Archives originales du centre de documentation du C.N.R.S., F-75971 Paris-Cedex 20, France.
21. Menichi, B., Arnold, H. H., Heyman, T., Dirheimer, G. and Keith, G. (1980) Biochem. Biophys. Res. Commun. 95, 461-467.
22. Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961) Virology 14, 54-58.
23. Hirochika, H. and Kobayashi, Y. (1978) J. Bacteriol. 136, 983-993.