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PRIMARY STRUCTURE OF *BACILLUS SUBTILIS* tRNAs^{Tyr}

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SUMMARY

tRNA$_I^{Tyr}$ and tRNA$_{II}^{Tyr}$ have been purified from B.subtilis and their nucleotide sequence determined. tRNA$_I^{Tyr}$ differs from tRNA$_{II}^{Tyr}$ only by the extent of modification of the adenosine in 3' position adjacent to the anticodon, i^{6}A and ms^{2}i^{6}A respectively.

INTRODUCTION

Two species of tRNA^{Tyr} appear during *B.subtilis* growth. They have different chromatographic properties, and their relative proportions change during development. Indeed tRNA$_I^{Tyr}$ is more important in vegetative cells whereas tRNA$_{II}^{Tyr}$ is predominant in stationary cells and spores (1-3). Previous work showed that their nucleotide compositions differ only by a modification of an adenosine residue : i^{6}A in tRNA$_I^{Tyr}$ and ms^{2}i^{6}A in tRNA$_{II}^{Tyr}$ (4). In *E.coli* also two tRNA^{Tyr}-isoacceptors have been found : Under normal growth conditions (maximal medium and sufficient aeration) these bacteria contain ms^{2}i^{6}A tRNA^{Tyr} but in unfavourable conditions i^{6}A tRNA^{Tyr} is accumulated (5). An identical result has been found previously in *B.subtilis* tRNA^{Phe} prepared under different growth conditions (7). Furthermore in *E.coli* infected by Ø 80 dsu$^+$ bacteriophage three different species of tRNA$_{SuIII}^{Tyr}$ have been found, differing only by the adenosine derivative adjacent to the 3' of the anticodon : A,i^{6}A or ms^{2}i^{6}A (5). These differences seem to account for variations in *in vitro* ribosomes binding of the tRNAs^{Tyr} in presence of specific polynucleotides (5). The same observation was made with *Bacillus subtilis* (6) tRNA$_{II}^{Tyr}$ (ms^{2}i^{6}A) which binds 2-3 times more than tRNA$_I^{Tyr}$ (i^{6}A).

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It was therefore interesting to localize the position of the modified adenosine and to determine whether the two species have identical or different primary structures. Primary structure analyses of different $tRNAs^{Tyr}$ allowed us to compare *Bacillus subtilis* $tRNA^{Tyr}$ with its known counterparts from neighbour organisms such as *B.stearothermophilus* (8) which is also $gram^+$, and *E.coli* ($gram^-$) (9) or with yeast $tRNA^{Tyr}$ the only known $tRNA^{Tyr}$ (10) from eukaryotic cells and to get informations concerning the recognition sites of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetases. Preliminary results have shown, that *Bacillus subtilis* and *B.stearothermophilus* $tRNAs^{Tyr}$ are charged to the same extent by the enzymes from *E.coli* and *B.stearothermophilus*.

MATERIALS AND METHODS

Purification of $tRNA^{Tyr}$. The $tRNAs^{Tyr}$ (I and II) have been purified as previously published (6) and further subjected to 20% polyacrylamide slab gel electrophoresis at pH 8.3.

Sequencing methods. 5-20 A 260 units of the $tRNA^{Tyr}$ were digested either with pancreatic RNAase or with T_1 RNAase (11), and the fragments separated by DEAE-cellulose column chromatography and DEAE-cellulose paper electrophoresis or by two-dimensional electrophoresis using cellogel (Chemetron, Milano) in the first dimension and DEAE-cellulose paper in the second dimension (Fig. 1) (12). After elution (12) each U.V. absorbing spot was further studied by determining its nucleoside composition (13).

The sequences of the oligonucleotides longer than trinucleotides were determined by 5' $|^{32}P|$ post-labelling followed by the "wandering spot" technique. 5' $|^{32}P|$ labelling was performed as described by Silberklang *et al.* (14) with T_4 polynucleotide kinase prepared according to Richardson (15) and Panet *et al.* (16). $\gamma|^{32}P|$ATP (400-2000 Ci/mmole) was prepared by a method derived from Glynn and Chappel (17) and Maxam and Gilbert (18) and purified on a DEAE-cellulose column (0.1 ml) eluted by a triethylammonium (pH 8) gradient from 0 to 0.5 M (100 ml total volume). The separation of the 5' labelled fragments was done by two-dimensional electrophoresis as mentioned above, or by electrophoresis on cellogel followed by homochromatography on DEAE-cellulose thin layer. The methods used for sequencing each oligonucleotide were identical to those already reported (19).

Partial digests of $tRNA^{Tyr}$ were obtained by incubating 2-10 µg tRNA in 10 µl dimethylsulfoxyde (DMSO) at 100°C for 10 min. The digestion products were post-labelled and separated by electrophoresis on cellogel and homochromatography as mentioned above, or by two-dimensional electrophoresis on polyacrylamide gel (18, 20) followed by total hydrolysis with P_1 nuclease (P.L. Biochemicals) and analysis of the 5' $|^{32}P|$ nucleotides on thin layer chromatography (11, 22). The latter procedure was used especially for confirmation of the minor nucleotides and for obtention of overlapping sequences in the D-, anticodon-, and T-ψ regions.

The *Bacillus subtilis* $tRNA^{Tyr}$ could not be dephosphorylated and 5' $|^{32}P|$ post-labelled as described for many other tRNAs. However smaller fragments, quarters and halves, from the 5' end, could be labelled. They were prepared by digesting the tRNA with S_1 nuclease (Miles) (23) under the following conditions : 50 µl Na acetate 0.05 M pH 4.5, NaCl 0.3 M, $ZnCl_2$ 1 mM and glycerol 5% containing 125 units S_1 nuclease and 25 µg tRNA were incubated overnight at 18°C. After phenol extraction and several washings with ethanol, the fragments were dephosphorylated, 5'$|^{32}P|$ postlabelled and separated on 20% polyacrylamide sequencing gels (18). The radioactive spots were eluted (18) and further analysed and sequenced by (i) the "wandering spot" technique (14),

(ii) by two-dimensional electrophoresis on polyacrylamide gel (18, 20), after partial hydrolysis in 10 µl DMSO at 100°C for 60 min in presence of 5-10 µg carrier tRNA, (iii) partial digestions with T_1 RNAase, U_2 RNAase and pancreatic RNAase and analysis on 20% polyacrylamide gel (21).

RESULTS

To determine unambiguously the rare nucleotides (s^4U, m^1A, i^6A, ms^2i^6A and Q) containing oligonucleotides, exhaustive digestions of non-radioactive $tRNA_I^{Tyr}$ and $tRNA_{II}^{Tyr}$ were done. The digestion products were separated by column chromatography on DEAE-cellulose (not shown) or electrophoresis on cellulose acetate, followed by electrophoresis on DEAE-cellulose paper. The nucleotide compositions of these non-radioactive spots were studied. For the determination of the sequence of the oligonucleotides we used the $|^{32}P|$ postlabelling techniques as mentioned in Material and Methods. The fingerprints and the found sequences for the oligonucleotides originating from the two $tRNAs^{Tyr}$ are shown in Fig. 1 : both non-radioactive and 5'$|^{32}P|$ labelled digests give identical fingerprints. These results show that the only difference between $tRNA_I^{Tyr}$ and $tRNA_{II}^{Tyr}$ is the hypermodified adenosine in the corresponding fragments. This

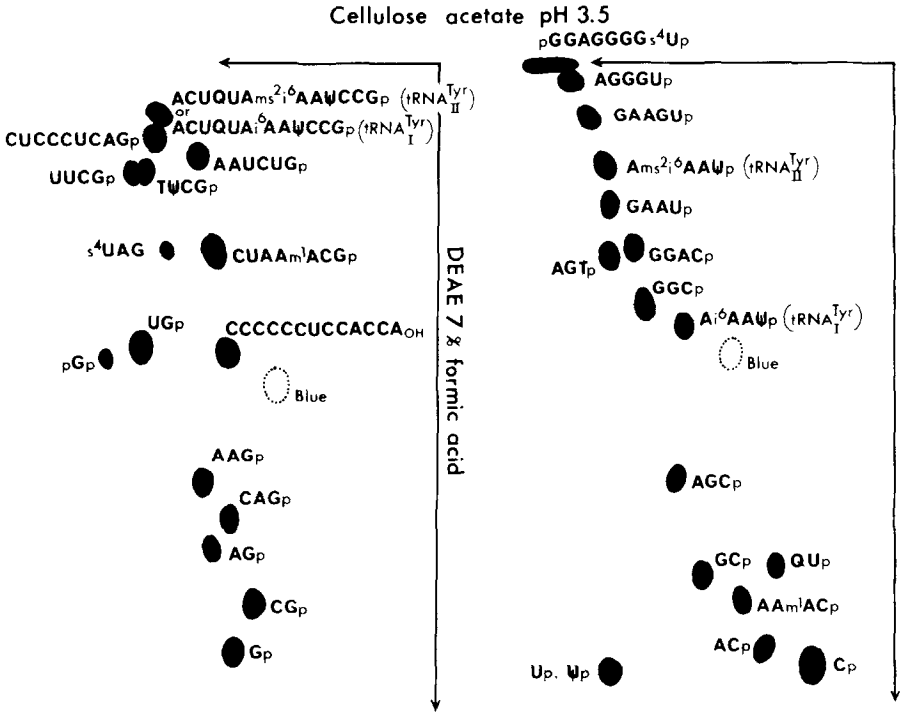

Fig. 1. Two-dimensional fractionation patterns of complete RNAase T_1 digest (A) and RNAase A digest (B) of *B. subtilis* $tRNAs_{I \text{ and } II}^{Tyr}$.

is best shown on the pancreatic RNAase fingerprint because of the big difference of mobility of A-ms^{2}i^{6}A-A-ψp and A-i^{6}A-A-ψp.

All the oligonucleotide sequences could be established directly by classical methods or by the wandering spot technique. But special attention had to be taken for the study of the oligonucleotides pG-G-A-G-G-G-G-s^{4}Up and C-U-A-A-m^{1}A-C-Gp. Concerning the first one, the minor base s^{4}U was found despite its high lability in a RNAase T_1 trinucleotide s^{4}U-A-Gp, but its characterization in the pancreatic RNAase digest was much more difficult. Indeed the G rich oligonucleotide pG-G-A-G-G-G-G-s^{4}U remained fixed on DEAE-cellulose column when eluted with urea and NaCl. However, using 1 M triethylammonium bicarbonate, small amounts (20-30%) of it could be recovered. An identical situation was on DEAE-cellulose paper electrophoresis. Finally s^{4}U was found in this pancreatic octanucleotide, because it gave the s^{4}U characteristic spectrum with a maximum of absorption at 336 nm. Concerning the structure, 5'|^{32}P| labelled fragment gave unambiguously the position of A in the G track on homochromatography after partial alcali digestion. The sequence of the m^{1}A containing oligonucleotide, because of the presence of this positively charged minor nucleotide, was done by comparing the 5' labelled oligonucleotide to its alcali treated counterpart (triethylammonium carbonate pH 9 for a few hours at 65°). In these conditions m^{1}A is transformed into m^{6}A. By homochromatography of a partial digest (see Materials and Methods) m^{1}A or m^{6}A were found in position 5' from the 5' labelled extremity of the oligonucleotide C-U-A-A-m^{1}A-C-Gp. Because of the extreme complexity due to m^{1}A found in that region (19 to 25) of the structure of *B.subtilis* tRNA^{Tyr} on sequencing gels (star in Fig. 2) or on homochromatographies of large fragments (not shown) the corresponding sequence readings were impossible. This oligonucleotide C-U-A-A-m^{1}A-C-Gp permits to link nucleotide 19 to nucleotide 25 of tRNA^{Tyr}.

The primary structure of tRNA$^{Tyr}_{I \text{ and } II}$ was deduced from (i) the overlapping sequences of the oligonucleotides obtained by exhaustive nuclease digestions (ii) the sequences of long fragments obtained by partial S_1 nuclease digestion (iii) partial digestion in DMSO followed by 5'|^{32}P| labelling as previously described. Several structure determinations by different methods are shown in Fig. 2. The cloverleaf model of tRNA^{Tyr} is shown in Fig. 3.

DISCUSSION

The only difference between the primary structure of tRNA$^{Tyr}_I$ and tRNA$^{Tyr}_{II}$ is the extent of modification of the adenosine in 3' position adjacent to the anticodon. *B.subtilis* tRNA^{Tyr} is 85 nucleotides long like *E.coli* tRNA$^{Tyr}_{SuIII^+}$ (9) and *B.stearothermophilus* tRNA^{Tyr} (8). *B.subtilis* and *B.stearothermophilus*

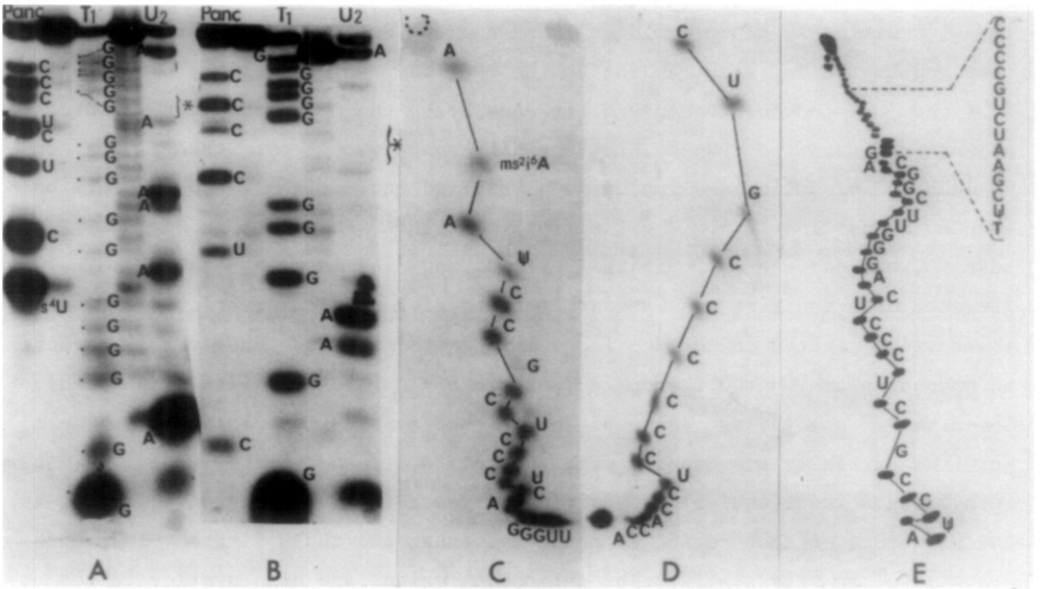

Fig. 2. Autoradiograms of partial digest of post-labelled fragments obtained by S_1 nuclease cleavage of *B.subtilis* $tRNA^{Tyr}$.

A and B. Sequence analysis on 20% polyacrylamide gel. Partial enzymatic digestions were done as described in ref. 8 (A : 1 to 31 - B : 10 to 33).

C and D. Two-dimensional homochromatography of a partial digest of 5' ^{32}P - labelled fragments (C : 37 to 56 - D : 70 to 85).

E. Two-dimensional gel electrophoresis of a partial digest of 5' ^{32}P -labelled 3' half molecule of $tRNA^{Tyr}$ (E : 39 to 75)

* Region of m^1A, for sequence see remarks in text.

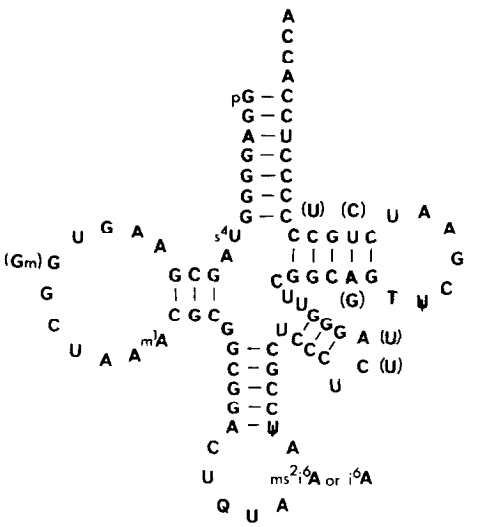

Fig. 3. Cloverleaf model of *B.subtilis* $tRNAs^{Tyr}_{I \text{ and } II}$. Between brackets are shown the differences with *B.stearothermophilus* $tRNA^{Tyr}$ (8).

tRNAs^{Tyr} differ by 6 nucleotides. These nucleotides are located in the extra loop and in the T-ψ stem. As far as the modified nucleotides are concerned, 7 of them are common, the only difference being the Gm in position 17 which is absent in *B.subtilis* tRNA^{Tyr}. But the difference with *E.coli* tRNA^{Tyr} concern as much as 29 nucleotides including 3 modified nucleotides : s^4U in position 8 and Gm in position 17 are modified in *E.coli* tRNA^{Tyr} and not in *B.subtilis* tRNA^{Tyr}, whereas m^1A in position 23 is not modified in *E.coli* tRNA^{Tyr}. The presence of a modified m^1A is characteristic for many eukaryotic tRNAs, but there it is located in the T-ψ loop. In *B.subtilis* tRNA^{Tyr} however it occupies an unusual place in the D loop as found in *B.stearothermophilus* tRNA^{Tyr} (8). The *B.subtilis* methylase responsible for that type of methylation has been purified partially and studied with respect to its specificity (24). The enzyme methylates A in an equivalent position in yeast tRNA^{Tyr}, suggesting as far as the structure of that region is concerned, that the tRNAs^{Tyr} from yeast and *B.subtilis* are very similar. The anticodon regions are also similar in the known prokaryotic and eukaryotic tRNAs^{Tyr}.

Most of the remaining structures of prokaryotic and yeast tRNA^{Tyr} are very different, especially the small extra loop of yeast tRNA^{Tyr} opposed to the large extra loop of prokaryotic tRNAs^{Tyr}. These structural differences can account for the differences in the aminoacylation. The bacterial tRNA synthetases (*E.coli* and *B.stearothermophilus*) aminoacylate to the same extent *B.subtilis* tRNA^{Tyr}, but the yeast tyrosyl tRNA synthetase aminoacylates the prokaryotic tRNAs to a much lower extent (not yet published). These results agree with the hypothesis which proposes that the structural forms of tRNAs rather than specific nucleotides could account for interactions with the cognate aminoacyl-tRNA synthetases (8, 25).

As already quoted the thiomethylation of i^6A enhances the codon ribosome interaction with tRNA^{Tyr} in the presence of specific polynucleotides (6). The relative amount of $tRNA^{Tyr}_{II}$ (ms^2i^6A) increases during sporulation of *B.subtilis*. Therefore the thiomethylation of i^6A to ms^2i^6A in tRNA^{Tyr} has been considered to play an important (as yet still unknown) role in the regulation of protein synthesis during this developmental process.

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