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CONTROLLED DEGRADATION OF YEAST tRNA^{Phe} BY SPLEEN PHOSPHODIESTERASE IN THE PRESENCE OF ETHIDIUM BROMIDE

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SUMMARY: Degradation of yeast tRNA^{Phe} with spleen phosphodiesterase in the presence of ethidium bromide has been studied. It was found that in the presence of the intercalating dye, the digestion is halted after a limited number of nucleotides is removed. Possible explanations of the observed phenomenon in connection with tRNA-ethidium bromide complex formation are discussed.

Routine application of the total synthesis for preparation of natural and structurally modified tRNAs, in spite of the first successful efforts (1-3) is still a matter of future. It is one of the reasons why in the studies of tRNA's structure-function relationship, chemically and enzymatically altered natural tRNAs and their fragments are in a wide use (4-6).

tRNA fragments can be obtained by endonucleolytic cleavage or exonucleolytic digestion. Both, chemical (7-9) as well as enzymatic methods (10-13) are employed. The anticodon replacement based on specific anticodon loop cleavage (6,10,11), 3'-terminal amino acid stem base changes (5) or synthesis of chemically misacylated yeast tRNA^{Phe} (14), both based on limited degradation of tRNAs from their 3'-end, provide useful tools for tRNA studies.

Relatively less attention has been paid to the preparation of tRNAs with a shortened 5'-terminus of the aminoacyl stem. The method described in the literature is based on the digestion of tRNA with spleen phosphodiesterase (15). In the case of yeast tRNA^{Phe}, the tRNA truncated by 27 nucleotides is isolated as a major product (15). Unfortunately this approach usually yields several fragments according to the "retardation sites" pattern (15,16).

One of the main goals of our study was to work out the method of removing possibly short oligoribonucleotides from the tRNA 5'-terminus. The sensitivity of spleen phosphodiesterase towards the three-dimensional structure of the sub-

Abbreviations: yeast tRNA$^{Phe}_{SPDE}$: yeast tRNA^{Phe} treated with spleen phosphodiesterase.

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strate (17) and the known fact of protection of particular sites in nucleic acids by intercalating agents (18,19), prompted us to approach this problem by making use of the promotion of new retardation site(s) as a result of tRNA-intercalating dye interaction. For the controlled digestion of yeast $tRNA^{Phe}$ by spleen phosphodiesterase, ethidium bromide was chosen. This intercalating agent is believed to have major binding sites in the acceptor stem of yeast $tRNA^{Phe}$ (20,21).

MATERIALS AND METHODS

Yeast $tRNA^{Phe}$ was purchased from Boehringer Mannheim (1100 pmol/A_{260} unit) and Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland [1170 pmol/A_{260} unit after pretreatment with CTP (ATP): tRNA nucleotidyltransferase]. Alkaline phosphatase from E.coli (EC 3.1.3.1), polynucleotide kinase from T4 infected E.coli B (EC 2.7.1.7.8) and nuclease P1 from Penicillium citrinum (EC 3.1.30.x) were from Pharmacia. Phosphodiesterase II from Bovine Spleen (EC 3.1.16.1) and ethidium bromide were purchased from Sigma. [γ-^{32}P] ATP (5000 Ci/mmol) was purchased from Amersham. Acrylamide and N,N'-bisacrylamide were from Biomol Feinchemikalien GmbH, urea was from BRL, tris(hydroxymethyl)aminomethane was from J.T.Baker Chemicals B.V., boric acid, EDTA, magnesium chloride, sodium acetate were Merck products.

Gel electrophoresis

Denaturating polyacrylamide gels (200x200x0.5 mm) contained 10% (w/v) acrylamide/N,N'-methylenebisacrylamide (19:1), 7M urea, 90 mM Tris-borate, pH 8.3, and 2 mM EDTA (22). Electrophoresis was usually carried out at 300-500 V. The runs were terminated when the front of bromophenol blue marker was at the bottom of the gel. Analytical gels were stained with methylene blue or autoradiographed by using Agfa-Gevaert Osray M3 film. In the case of preparative gels, RNA bands were located by UV shadowing or autoradiography, cut out with a razor blade and recovered from the gel by extraction (23).

Dephosphorylation

The reaction mixture containing 20.0 A_{260} units of yeast $tRNA^{Phe}$ and 14 units of E.coli alkaline phosphatase in 400 µl of 25 mM Tris-HCl, pH 8.0, was incubated for 30 min at +50°C, then EDTA and dithiothreitol were added to final concentration of 4 mM and 3 mM, respectively. The incubation was continued for 15 min at room temperature and the reaction mixture was extracted with 200 µl of phenol saturated with buffer containing 25 mM Tris-HCl, pH 8.0, 4 mM EDTA and 3 mM dithiothreitol. The phenol fraction was reextracted with 200 µl of buffer as above saturated with phenol. Water fractions were combined, extracted six times with 200 µl portions of ethyl ether then 1 M NaOAC, pH 6.2, was added to final concentration 0.2 M and the product was precipitated with four volumes of ethanol. The resulting solution was chilled with liquid nitrogen and left for 30min at -20°C. The pellet of dephosphorylated yeast $tRNA^{Phe}$ was centrifuged off, washed with ethanol and ethyl ether and dried under vacuum.

Preparative degradation of 5'-dephosphorylated yeast $tRNA^{Phe}$ by spleen phosphodiesterase in the presence of ethidium bromide

Dephosphorylated yeast $tRNA^{Phe}$, 5.0 A_{260} units in 20 µl 10 mM NaOAc, pH 6.2, was heated for 10 min at +60°C then it was allowed to cool down slowly to room temperature in the course of about 2 h. To this solution 14.5 µl 100 mM NaOAc, pH 6.2, and 66 µl 5 mM ethidium bromide were added. The resulting mixture was incubated for 30 min at +4°C. The reaction was then initiated by the addition of 66 µl (6.6 units) of spleen phosphodiesterase. Final volume of reaction mixture was 165 µl, concentration of NaOAc, ethidium bromide and spleen phosphodiesterase were 10 mM, 2 mM and 40 units/ml, respectively. After 15-20 min

of the incubation at +20°C 1 M NaOAc pH 6.2 was added to a final concentration of 0.2 M and the product was precipitated with four volumes of ethanol. The resulting solution was cooled down with liquid nitrogen and left for 30 min at -20°C. The pellet of 5'-truncated yeast $tRNA^{Phe}$ was centrifuged down, washed with ethanol and ethyl ether, and dried under vacuum.

Labeling of 5'-ends with T4 polynucleotide Kinase/[γ-^{32}P]ATP was performed according to the literature method (24).

Two dimensional analysis of the 5'-terminal nucleotide was performed as described (25) except that for the chromatography in the second dimension, n-butyl alcohol/acetic acid/H_2O (5:2:3, v/v/v) as solvent system was used instead of isopropyl alcohol/concentrated HCl/H_2O (70:15:15, v/v/v).

RESULTS AND DISCUSSION

Spleen phosphodiesterase hydrolyses nucleic acids from the 5'-end by releasing 3'-mononucleotides (17). The treatment of tRNAs with the enzyme leads to several fragments shortened at their 5'-end (15,16). We have found that if the digestion of yeast $tRNA^{Phe}$ is performed in the presence of 1-2 mM ethidium bromide, the degradation process is halted after only a limited number of nucleotides is removed, yielding merely slightly shortened tRNA (FIGURE 1 and FIGURE 2).

The 5'-terminal nucleoside of 5'-truncated yeast $tRNA^{Phe}$ (yeast $tRNA^{Phe}_{SPDE}$) was determined as A by means of two dimensional thin-layer chromatography analysis of a nuclease P1 digest of 5'-[^{32}P]-labeled yeast $tRNA^{Phe}_{SPDE}$. Prolonged autoradiography revealed also traces of G and U (FIGURE 3). There are three adenosine residues in the 5'-part of the aminoacyl stem of yeast $tRNA^{Phe}$ namely A5, A9 and A14. In an effort to determine the ethidium bromide promoted retardation site in tRNA, the exact position of the yeast $tRNA^{Phe}$ 5'-terminal A was established. By comparing the chain lengths of the 5'-truncated and of the intact yeast $tRNA^{Phe}$ with the ladder obtained on a 8% polyacryl-

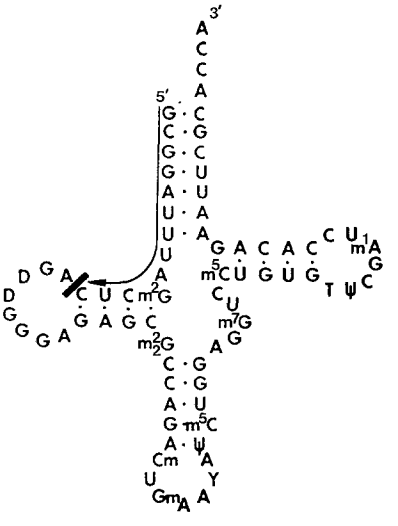

FIGURE 1. Secondary structure of yeast $tRNA^{Phe}$ showing presumable ethidium bromide induced spleen phosphodiesterase retardation site.

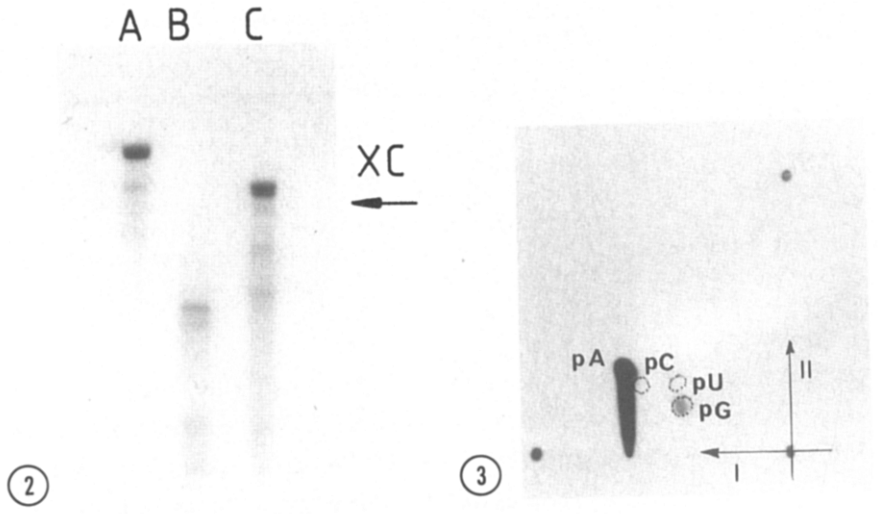

FIGURE 2. 10% Polyacrylamide-7M urea gel.
Lane A: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.15 A_{260}).
Lane B: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.3 A_{260}), spleen phosphodiesterase (0.4 unit), 10 mM sodium acetate, pH 6.2 (10 µl), 30 min, 20°C.
Lane C: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.3 A_{260}), spleen phosphodiesterase (0.4 unit), 10 mM sodium acetate, pH 6.2, 1 mM ethidium bromide (10 µl), 30 min, 20°C.

FIGURE 3. Analysis of the 5'-terminal nucleotide of 5'-[^{32}P]-labeled 5'-truncated yeast $tRNA^{Phe}$ by two dimensional cellulose thin-layer chromatography (Nuclease P1 digest). First dimension: isobutyric acid/0.5 M ammonia (5:3, v/v), second dimension: n-butyl alcohol/acetic acid/H_2O (5:2:3, v/v/v).

amide sequencing gel (data not shown) it was found that the 5'-truncated $tRNA^{Phe}$ is shorter than the intact molecule by 13 nucleotides. The 5'-terminal adenosine residue in yeast $tRNA^{Phe}_{SPDE}$ should be thus A14.

The effect of the concentration of ethidium bromide on the cleavage of dephosphorylated yeast $tRNA^{Phe}$ by spleen phosphodiesterase is shown in FIGURE 4. In the range of 0-1 mM intercalating agent, the specific resistance of the substrate against digestion by the enzyme increased as the ethidium concentration was increased. As a result more yeast $tRNA^{Phe}_{SPDE}$ 76-13 and fewer short fragments were observed. Concentrations of ethidium higher than 2 mM do not improve the specificity of degradation and therefore for practical reasons 1-2 mM ethidium bromide was applied. The effect of Mg^{2+} on the digestion of tRNA in the presence of ethidium is shown in FIGURE 5. Even though Mg^{2+} (as well as spermine and spermidine) competes for ethidium binding sites in tRNA (26), we have not observed a decrease of the digestion specificity in the range of 0.25-5 mM Mg^{2+} concentration (five-fold excess of Mg^{2+} over ethidium). At 50 mM concentration of Mg^{2+} (fifty-fold excess) the degradation of yeast $tRNA^{Phe}$ has almost ceased, most probably due to enzyme inhibition (17). Monovalent Na^{+} ion alone has little effect on digestion specificity, however at a high concentration of sodium acetate buffer (60 mM) and in the absence of ethidium,

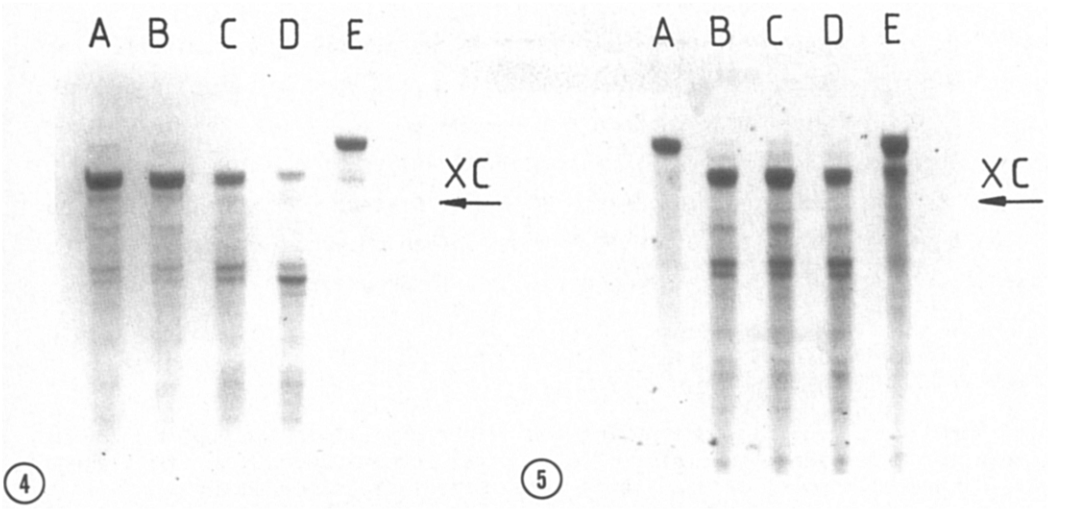

FIGURE 4. 10% Polyacrylamide-7M urea gel.
Digestion of 5'-dephosphorylated yeast $tRNA^{Phe}$ by spleen phosphodiesterase in the presence of ethidium bromide - effect of ethidium concentration.
Lane A: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.3 A_{260}), spleen phosphodiesterase (0.4 unit), 10 mM sodium acetate, pH 6.2, 1 mM ethidium bromide (10 µl), 30 min, 20°C.
Lane B: as above, 0.5 mM ethidium.
Lane C: as above, 0.25 mM ethidium.
Lane D: as above but without ethidium.
Lane E: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.15 A_{260}).

FIGURE 5. 10% Polyacrylamide - 7M urea gel.
Digestion of 5'-dephosphorylated yeast $tRNA^{Phe}$ by spleen phosphodiesterase in the presence of ethidium bromide - effect of Mg^{2+} concentration.
Lane A: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.15 A_{260}).
Lane B: 5'-dephosphorylated yeast $tRNA^{Phe}$ (0.3 A_{260}), spleen phosphodiesterase (0.4 unit), 10 mM sodium acetate , pH 6.2, 1 mM ethidium bromide, 15 min, 20°C.
Lane C: as above, 0.5 mM $MgCl_2$.
Lane D: as above, 5 mM $MgCl_2$.
Lane E: as above, 50 mM $MgCl_2$.

traces of yeast $tRNA^{Phe}_{SPDE}$ 76-13 fragment were detected (data not shown) in addition to yeast $tRNA^{Phe}$ 76-27 (?) fragment as a major product. One of the possible explanations for the observed limited digestion of yeast $tRNA^{Phe}$ by spleen phosphodiesterase in the presence of ethidium bromide may be changing (27) and stabilization (28) of tRNA conformation by the intercalating agent in such a mode that after removing a defined number of nucleotides the remaining part of the molecule is not accessible to enzyme action.

Crystallographic data shows that one of the ethidium binding sites is located in the P-10 loop of yeast $tRNA^{Phe}$, hydrogen bonded with its exocyclic amino groups to backbone phosphate P-8 and P-15 (29). This finding is consistent with another possible explanation of limited degradation of yeast $tRNA^{Phe}$ by spleen phosphodiesterase in the presence of ethidium: that a molecule of intercalating dye acts as "a sprag". The exact interpretation of the observed phenomena would require more detailed studies. The results presented in this report show the possibility of specific and effective shortening of yeast

$tRNA^{Phe}$ at the 5'-end yielding yeast $tRNA^{Phe}$ 76-13 fragment suitable for structure - function relationship studies and also for further 5'-end modification. Our results also support the assumption that one of the major ethidium bromide binding sites of yeast $tRNA^{Phe}$ exists at the P-10 loop. Studies on the reconstruction of the 5'-end of 5'-truncated yeast $tRNA^{Phe}$ as well as on the digestion of other tRNAs with spleen phosphodiesterase in the presence of different intercalating agents are in progress in our Laboratory.

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