The EMBO Journal vol.5 no.5 pp.1105–1109, 1986

Enzymatic 2′-O-methylation of the *wobble* nucleoside of eukaryotic tRNA^{Phe}: specificity depends on structural elements outside the anticodon loop

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Communicated by R.H.Buckingham

We have investigated the specificity of the enzyme tRNA (*wobble* guanosine 2′-O-)methyltransferase which catalyses the maturation of guanosine-34 of eukaryotic tRNA^{Phe} to the 2′-O-methyl derivative Gm-34. This study was done by microinjection into *Xenopus laevis* oocytes of restructured yeast tRNA^{Phe} in which the anticodon GmAA and the 3′ adjacent nucleotide 'Y' were substituted by various tetranucleotides. The results indicate that (i) the enzyme is cytoplasmic; (ii) the chemical nature of the bases of the anticodon and its 3′ adjacent nucleotide is not critical for the methylation of G-34; the size of the anticodon loop is however important; (iii) structural features beyond the anticodon loop are involved in the specific recognition of the tRNA by the enzyme since *Escherichia coli* tRNA^{Phe} and four chimeric yeast tRNAs carrying the GAA anticodon are not substrates; (iv) unexpectedly, the 2′-O-methylation is not restricted to G-34 since C-34, U-34 and A-34 in restructured yeast tRNA^{Phe} also became methylated. It seems probable that the tRNA (*wobble* guanosine 2′-O-)methyltransferase is not specific for the type of nucleotide-34 in eukaryotic tRNA^{Phe}; however the existence in the oocyte of several methylation enzymes specific for each nucleotide-34 has not yet been ruled out.

Key words: anticodon/maturation/2′-O-methyl nucleoside/oocyte microinjection/tRNA

Introduction

The 2′-O-methyl derivatives of guanosine, cytosine or uridine are found in several positions of the tRNA molecule. The modification occurs enzymatically during the process of tRNA maturation after the transcription of the tRNA gene. The presence of 2′-O-methylated nucleotides is more frequent in tRNA from eukaryotes than from prokaryotes (see Sprinzl *et al.*, 1985). Several distinct 2′-O-methylating enzymes are probably involved but little information is available about them (reviewed in Söll and Kline, 1982; Björk, 1984). A major difficulty in studying this class of enzymes is obtaining a substrate lacking the corresponding 2′-O-methylation. The only tRNA 2′-O-methyltransferase that has been well characterized was extracted from *Thermus thermophilus* and assayed *in vitro* with tRNA species of various origins using S-adenosyl-L-[methyl-^{14}C] methionine as a donor of the labelled methyl group (Kumagai *et al.*, 1980, 1982). This enzyme (EC 2.1.1.34) was shown to methylate the guanosine-18 in the D-loop of tRNA. The structural parameters of the tRNA species recognized by this particular ribose methylating enzyme are still largely unknown. The only information available comes from the work of Matsumoto *et al.* (1984): using fragments of the D-loop and D-stem containing the G-18 G-19 sequence of *Escherichia coli* tRNA^{Met} and various species of tRNA tested at different temperatures, the authors conclude that this thermophilic tRNA methyltransferase has an absolute requirement for a tRNA with a correct tertiary structure.

To approach the same problem in a different way, we have taken advantage of techniques for excising the anticodons of several tRNAs and replacing them by synthetic oligonucleotides using a procedure adapted from that of Bruce and Uhlenbeck (1982). During the reconstruction, a ^{32}P-label was selectively introduced 5′ adjacent to the nucleoside-34 (*wobble* nucleoside) (Carbon *et al.*, 1982). This allowed us to study the specificity of the *Xenopus laevis* oocyte tRNA (*wobble* guanosine 2′-O-)-methyltransferase in methylating the 2′ hydroxyl group of the ribose of the guanosine-34 in tRNA^{Phe} after its microinjection into the *X. laevis* oocytes. The nucleoside Gm-34 has been found in all species of eukaryotic tRNA^{Phe} sequenced so far namely in *X. laevis* tRNA^{Phe} (Mazabraud, 1982) and in yeast tRNA^{Phe} (RajBhandary and Chang, 1968) except in tumor-specific tRNA^{Phe} (Kuchino *et al.*, 1982). *E. coli* tRNA^{Phe} does not contain Gm-34 (Barrell and Sanger, 1969), which however exists in several other species of prokaryotic tRNA^{Phe}, such as *Bacillus stearothermophilus* and *B. subtilis* (see Sprinzl *et al.*, 1985).

We have studied the effect of the nucleotide sequence in the vicinity of the modification site in yeast tRNA^{Phe}. It is generally believed that the nucleotides around the site of a given post-transcriptional modification in tRNA are of prime importance in the recognition by the maturation enzyme (see Tsang *et al.*, 1983). We provide evidence that this is not the case for tRNA (*wobble* guanosine 2′-O-)methyltransferase, and that this enzyme clearly recognizes structural parameters outside the anticodon loop.

Results

Yeast tRNA^{Phe} is a substrate for tRNA (wobble guanosine 2′-O-)-methyltransferase from the X. laevis oocyte

To measure the activity of the tRNA (*wobble* guanosine 2′-O-)-methyltransferase in *X. laevis* oocytes we constructed *in vitro* the appropriate substrate by replacement of the yeast tRNA^{Phe} anticodon and its 3′ adjacent nucleotide (GmAAY) by the synthetic tetranucleotide GAAG. During reconstruction, a ^{32}P-label of high specific radioactivity was introduced adjacent to G-34 (Figure 1a). The reconstructured tRNA was microinjected into the cytoplasm of *X. laevis* oocytes and recovered by phenol extraction after different periods of time. The labelled tRNA^{Phe} was then isolated by denaturing polyacrylamide gel electrophoresis, completely hydrolysed by nuclease P1 and the resulting nucleotides were analysed by two-dimensional t.l.c. followed by autoradiography. The conversion of G-34 into Gm-34 was almost complete, reaching 90% after 24 h of incubation (see Figure 2).

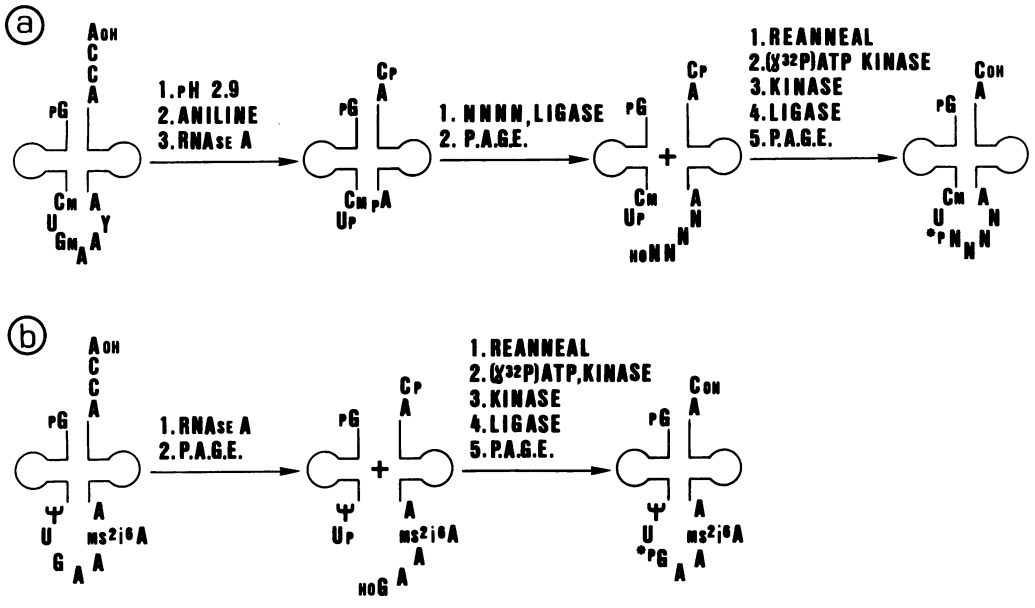

Fig. 1. Schematic representation of the procedures used for cleavage and reconstruction of yeast $tRNA^{Phe}$ **(a)** and *E. coli* $tRNA^{Phe}$ **(b)**. Details of these procedures are described in Materials and methods.

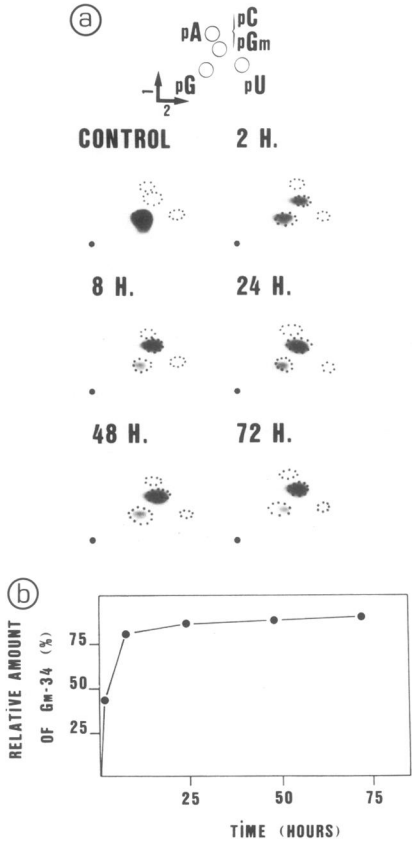

Fig. 2. Kinetics of guanosine-34 methylation in yeast $tRNA^{Phe}$ restructured with the oligonucleotide GAAG and micro-injected into the cytoplasm of *X. laevis* oocytes. **(a)** Autoradiograms of the two-dimensional thin layer chromatograms of complete P1 digestion of 5′ [^{32}P]G-34-labelled yeast $tRNA^{Phe}$ before (control) and after micro-injection into *X. laevis* oocytes. Times of incubation at 19°C in the oocytes are given in the figure. Circles in dotted lines show the migration of the four canonical nucleotides used as u.v. markers. At the top is the reference map used to identify the compounds (Nishimura, 1979). **(b)** Ratio of Gm-34 to G-34 (expressed as a percentage) plotted as a function of incubation time of the restructured yeast $tRNA^{Phe}$ in *X. laevis* oocytes.

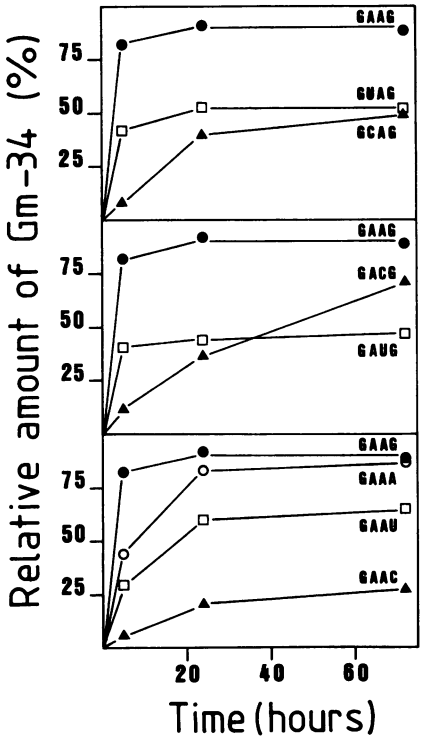

Fig. 3. Comparison of guanosine-34 methylation in reconstructed yeast $tRNA^{Phe}$ micro-injected into *X. laevis* oocytes. The oligonucleotides used to restructure the tRNA are indicated. GAAG is the normal sequence of the anticodon and 3′ adjacent nucleotide in the precursor $tRNA^{Phe}$.

The type of nucleotide in positions 35, 36 and 37 is not critical for the enzymatic methylation of the wobble guanosine in yeast $tRNA^{Phe}$

Figure 3 shows the results obtained for G-34 methylation in restructured $tRNA^{Phe}$ in which nucleotides 35, 36 and 37 were replaced by other nucleotides. Evidently, none of these three nucleotides were essential for the action of the maturation enzyme since all the restructured tRNAs became methylated to some

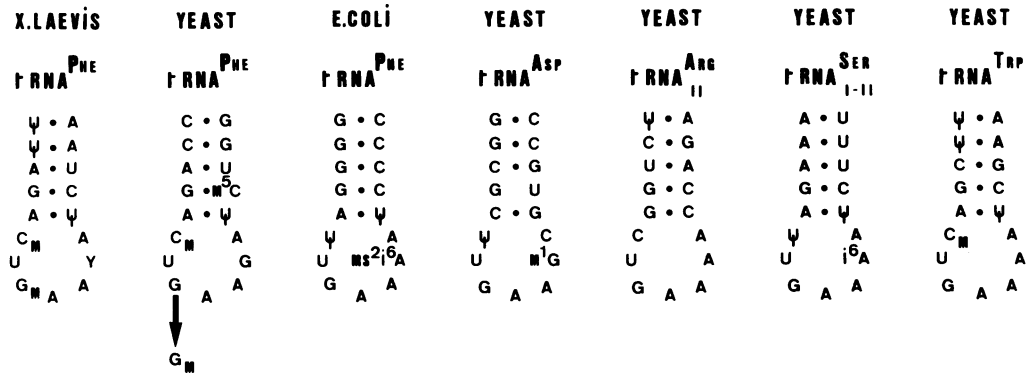

Fig. 4. Comparison of the nucleotide sequences of the anticodon stems and loops of the restructured tRNAs (with a GAA anticodon) assayed for guanosine-34 methylation *in vivo*. On the left is the anticodon stem and loop of *X. laevis* $tRNA^{Phe}$, the normal substrate for the tRNA (*wobble* guanosine 2'-O-)-methyltransferase. The arrow indicates which G in yeast $tRNA^{Phe}$ is efficiently modified to the 2'-O-methyl derivative (for details, see Figure 2). For all other restructured tRNAs micro-injected into *X. laevis* oocytes no trace of Gm-34 appeared even after 72 h of incubation (results not shown).

extent. However, the tRNA containing the GAAG sequence was the best substrate for the oocyte enzyme.

Interestingly, when the trinucleotide GAA was used to construct a tRNA with an anticodon loop of six nucleotides instead of seven as in the original tRNA, the G-34 was no longer methylated, indicating that the size and therefore the conformation of the loop is critical for the action of the enzyme.

In the case of the $tRNA^{Phe}$ reconstructed with the oligonucleotide GUAG, we observed the presence of 20% of queuosine (Q) after 72 h of incubation in the oocytes (result not shown). This result is consistent with those obtained earlier for the structural requirements of the tRNA-guanine ribosyltransferase, namely, that the nucleotide sequence U-33 G-34 U35 is an essential target for this particular maturation enzyme (Carbon *et al.*, 1983).

Specificity of tRNA (wobble guanosine 2'-O-)methyltransferase depends on structural elements outside the anticodon loop

The G-34 in the anticodon of *E. coli* $tRNA^{Phe}$ is not naturally modified. One explanation is the absence of the corresponding maturation enzyme in *E. coli*. In order to check whether this tRNA could be a substrate for the oocyte maturation enzyme, we prepared an *E. coli* $tRNA^{Phe}$ with a ^{32}P-label adjacent to guanosine 34 by the procedure shown in Figure 1b. After microinjection into *X. laevis* oocytes, this tRNA was not a substrate for the eukaryotic methyltransferase since no trace of Gm-34 appeared after 72 h of incubation in the cytoplasm of *X. laevis* oocytes (see Figure 4). Furthermore, we have inserted the phenylalanine anticodon GAA in several species of yeast tRNA, namely, $tRNA^{Asp}$ (normal anticodon GUC), $tRNA^{Arg}_{II}$ (ICG), $tRNA^{Ser}_{I-II}$ (IGA) and $tRNA^{Trp}$ (CmCA). As in the case of *E. coli* $tRNA^{Phe}$, none of these tRNAs was a substrate for the maturation enzyme (see Figure 4). The logical interpretation of these results is that structural parameters beyond the anticodon loop are implicated in the recognition of the eukaryotic $tRNA^{Phe}$ by the oocyte tRNA (*wobble* guanosine 2'-O-)methyltransferase.

2'-O-Methylation of nucleotide-34 in reconstructed yeast $tRNA^{Phe}$ is not restricted to guanosine

To determine whether the methylation of the ribose of nucleotide-34 is dependent on the base moiety, we have replaced the normal anticodon and the 3'-adjacent nucleotide (GmAAY) of yeast $tRNA^{Phe}$ by the oligonucleotides XAAG (where X is C, U or A). As shown in Figure 5, each of the X-34 nucleotides became 2'-O-methylated. The methylations of C-34 and U-34 were as fast and as complete as the methylation of G-34 (compare with Figure 2). The presence of Um-34 in reconstructed yeast $tRNA^{Phe}$ recovered from *X. laevis* oocytes was further confirmed by co-chromatography of the labelled nucleotide from the hydrolysate with authentic pUm as a marker. The possibility of the presence of a 2-thio derivative was discarded since the chromatographic behaviour of the labelled nucleotide was not affected by cyanogen bromide treatment as described by Kimura-Harada *et al.* (1971) (result not shown). The maturation of A-34 was slower and clearly more complex than with the three other nucleotides-34: as well as the appearance of an appreciable amount of 2'-O-methyl adenosine (Am), 2'-O-methyl inosine (Im) and a trace of inosine (I) were also detected after 72 h of incubation in the oocytes.

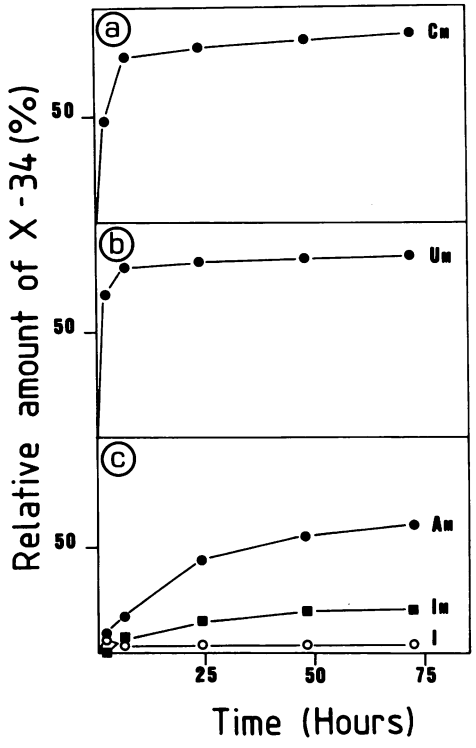

Fig. 5. Kinetics of nucleotide-34 modification in yeast $tRNA^{Phe}$ reconstructed with the oligonucleotide XAAG (where X is C, U or A). **(a)** CAAG; **(b)** UAAG; **(c)** AAAG. The type of modification is indicated at the end of the curve.

Discussion

We have studied the specificity of the oocyte enzyme that methylates the 2'-hydroxyl group of the ribose of guanosine-34 in $tRNA^{Phe}$. This nucleotide modification occurs in all species of eukaryotic $tRNA^{Phe}$ sequenced so far (except those from tumor cells), in certain species of prokaryotic $tRNA^{Phe}$ (*B. subtilis* and *B. stearothermophilus*) but does not occur in *E. coli* $tRNA^{Phe}$. So far, no details of the enzymes catalysing this kind of modification in the *wobble* position have ever been described: only for the 2'-O-methylation of guanosine-18 in the D-loop of certain tRNAs has the enzymatic activity been characterized (Kumagai *et al.*, 1980, 1982; Matsumoto *et al.*, 1984).

Our strategy was to micro-inject into the cytoplasm of *X. laevis* oocytes restructured yeast $tRNA^{Phe}$ in which the normal anticodon GmAA and the 3' adjacent wyosine nucleotide had been replaced by various synthetic oligonucleotides, or other tRNAs restructured with the phenylalanine anticodon (GAA). These experiments lead us to conclude the following. (i) The tRNA (*wobble* guanosine 2'-O-)methyltransferase clearly occurs in the cytoplasm of *X. laevis* oocytes. This observation is consistent with the general idea that modifications occurring in the *wobble* position are among the last modifications in the long stepwise maturation process (Nishikura and De Robertis, 1981; Tranquilla *et al.*, 1982) after the tRNA precursor has been actively transported from the nucleus into the cytoplasm (Tobian *et al.*, 1985). (ii) Reconstructed yeast $tRNA^{Phe}$ with G-34 in place of the naturally occurring Gm-34 is an excellent substrate for the oocyte tRNA (*wobble* guanosine 2'-O-)methyltransferase, the enzymatic modification being almost complete after 24 h of incubation. The size of the anticodon loop (seven nucleotides) is very important for the recognition of the tRNA by the enzyme. Interestingly, the kind of nucleotide in positions 35, 36 and 37 is not critical for G-34 to become 2'-O-methylated. In contrast, microinjected *E. coli* $tRNA^{Phe}$ (anticodon GAA) is not a substrate for the oocyte maturation enzyme. This observation clearly demonstrates that features other than the three bases of the anticodon are important for the recognition by the 2'-O-methylation enzyme. (iii) Yeast $tRNA^{Trp}$ (anticodon CmCA) naturally contains a 2'-O-methylation on C-34; furthermore, the nucleotide sequence of its anticodon loop and stem is very similar to that of *X. laevis* $tRNA^{Phe}$ (see Figure 4). Nevertheless, reconstructed yeast $tRNA^{Trp}$ with a GAA anticodon was not a substrate for any of the oocyte modification enzymes acting on the *wobble* guanosine.

In addition to these results none of the other yeast tRNAs reconstructed with a GAA anticodon instead of GUC (aspartic acid), ICG (arginine) or IGA (serine) was a substrate for the maturation enzyme.

Taken together, these results suggest that the tRNA (*wobble* guanosine 2'-O-)methyltransferase is very specific for $tRNA^{Phe}$ of eukaryotic type. They contrast with the observation of Kumagai *et al.* (1980) that tRNA (guanosine-18 2'-O-)methyltransferase acts on several tRNAs from different organisms. Therefore, a given modification (2'-O-methylation of guanosine in tRNA) can be performed by different enzymes with clearly different targets and specificities. We propose to distinguish these two tRNA (guanosine 2'-O-)methyltransferases by indicating the exact location of the modification site i.e. the *wobble* guanosine or the guanosine-18. (iv) Of particular interest is the observation that the methylation of nucleotide-34 in yeast $tRNA^{Phe}$ in the oocyte is not restricted to guanosine: it also occurs on C-34, U-34 and A-34 in reconstructed tRNAs. Since our data result from *in vivo* measurement using heterologous tRNAs, we do not know whether one or several enzymes are implicated in the 2'-O-methylation of the four nucleotides-34. However Am-34 has never been identified in any tRNA so far sequenced. This suggests that the maturation enzyme responsible for methylating A-34 normally modifies a different nucleotide (or nucleotides). Work is in progress to elucidate this important aspect of tRNA maturation.

The above results differ from those we reported previously for the specificities of two other maturation enzymes, tRNA-guanine ribosyltransferase (the so-called Q-insertase, EC 2.4.2.29) and tRNA-hypoxanthine ribosyltransferase of the *X. laevis* oocyte. In these cases, we observed a strong dependence on the nature of the neighbouring nucleotides on the substrate activity of the tRNA for the corresponding enzymes (Carbon *et al.*, 1983; Haumont *et al.*, 1984). Thus, it has become evident that the structural features of a tRNA may be recognized in many different ways by different modification enzymes, a situation somewhat analogous to the intricate recognition of a tRNA by its cognate aminoacyl-tRNA synthetase (see Schimmel and Söll, 1979).

Materials and methods

Enzymes and chemicals

T4 RNA ligase (EC 6.5.1.3) was purchased from P-L Biochemicals, T4 polynucleotide kinase (EC 2.7.1.78), *E. coli* A19 alkaline phosphatase (EC 3.1.3.1) and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham, UK; RNase A (EC 3.1.27.5), RNase T1 (EC 3.1.27.3), RNase P1 (EC 3.1.4.X), RNase T2 (EC 3.1.27.1), dinucleosides monophosphates, 2'-O-methyluridine 5'-phosphate, guanosine 5',3'-diphosphate and adenosine 5',3'-diphosphate from Sigma Chemicals (St. Louis, MO). Yeast and *E. coli* $tRNA^{Phe}$, polynucleotide phosphorylase (EC 2.7.7.8), calf intestine alkaline phosphatase (EC 3.1.3.1) and mononucleosides 5'-diphosphates were purchased from Boehringer, Mannheim. Yeast $tRNA^{Asp}$, $tRNA^{Trp}$ and $tRNA^{Scr}_{I-II}$ were generous gifts of G.Keith and yeast $tRNA^{Arg}_{II}$ was received from J.Gangloff (Strasbourg, France).

Oligonucleotide synthesis

Trinucleotides AAA, UAA, CAA, GAA, GUA and GCA were obtained at equilibrium of the polynucleotide phosphorylase reaction as described by Thach (1966); a typical reaction mixture was described in a previous paper (Haumont *et al.*, 1984). GAU and GAC were prepared in the same way but RNase A (100 μg/ml) was added. Tetranucleotides AAAG, UAAG and CAAG were synthesized by RNase T1-assisted polynucleotide phosphorylase (Mohr and Thach, 1969), using the corresponding trinucleotides and GDP. GAAU and GAAC were obtained by RNase A-assisted polynucleotide phosphorylase using GAA and UDP or CDP. After treatment with calf intestine alkaline phosphatase (12 units/ml, 1 h at 37°C) all these oligonucleotides were purified by DEAE – Sephadex A-25 column chromatography (Sprinzl *et al.*, 1976). Tetranucleotides GAAG, GUAG, GAUG and GACG were obtained by ligation of the mononucleotide 5',3'-diphosphate pGp to the corresponding trinucleotides under the conditions described by Bruce and Uhlenbeck (1982); GAAA was prepared in the same way using GAA and pAp; after treatment with *E. coli* alkaline phosphatase (6 units/ml, 1 h at 37°C), purifications were performed on cellulose thin layer plates using 1 M ammonium acetate pH 7:ethanol (1:1; v/v) as liquid phase.

In vitro enzymatic restructuration of tRNAs

The procedure for restructuring yeast $tRNA^{Phe}$ (Figure 1a) was adapted from that described by Bruce and Uhlenbeck (1982); preparation of anticodon and nucleotide-37 deprived molecule was performed as described. After ligation of a tetranucleotide to the 3' fragment, the two half molecules were purified by denaturing 15% polyacrylamide gel electrophoresis to discard any trace of non-radioactive ATP present in the ligase reaction mixture. Annealing of the two fragments followed by labelling of nucleoside-34 with [γ-^{32}P]ATP, 3' dephosphorylation by T4 polynucleotide kinase and sealing of the anticodon loop were performed as described by Carbon *et al.* (1982). Limited hydrolysis of *E. coli* $tRNA^{Phe}$ (Figure 1b) was conducted under the following conditions: 100 mM Tris-Cl pH 7.5; 200 mM KCl; 10 mM $Mg(OAc)_2$; 700 μg/ml *E. coli* $tRNA^{Phe}$ and 10 μg/ml RNase A. After 1 h of incubation at 0°C and phenol extraction, the two fragments (from pG-1 to U-33p and from HOG-34 to C-74p) were purified by denaturing 15% polyacrylamide gel electrophoresis.

The subsequent steps were the same as for the construction of substituted yeast $tRNA^{Phe}$. The construction of the anticodon-substituted yeast $tRNA_{II}^{Arg}$ and $tRNA^{Asp}$ were described previously (Fournier *et al.*, 1983; Haumont *et al.*, 1984).

The construction of substituted yeast $tRNA^{Trp}$ and yeast $tRNA_{I-II}^{Ser}$ will be described elsewhere; they are based on the experimental procedures reported above.

Acknowledgements

We thank Dr R.H.Buckingham, Dr E.Kubli and Professor H.Chantrenne for improvements to the manuscript. L.D. was supported by a pre-doctoral fellowship from the IRSIA (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture). This work was supported by grants from the Belgian Government 'Actions Concertées', from the 'Fondation D. et A. Van Buuren' and from the University of Brussels.

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Received on 25 February 1986