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Enzymatic conversion of guanosine 3′ adjacent to the anticodon of yeast tRNA^{Phe} to N^1-methylguanosine and the wye nucleoside: dependence on the anticodon sequence

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N^1-Methylguanosine (m^1G) or wye nucleoside (Y) are found 3′ adjacent to the anticodon (position 37) of eukaryotic tRNA^{Phe}. The biosynthesis of these two modified nucleosides has been investigated. The importance of the type of nucleosides in the anticodon of yeast tRNA^{Phe} on the potentiality of this tRNA to be a substrate for the corresponding maturation enzyme has also been studied. This involved microinjection into *Xenopus laevis* oocytes and incubation in a yeast extract of restructured yeast tRNA^{Phe} in which the anticodon GmAA and the 3′ adjacent Y nucleoside were substituted by various tetranucleotides ending with a guanosine. The results obtained by oocyte microinjection indicate: (i) that all the restructured yeast tRNAs^{Phe} are efficient substrates for the tRNA (guanosine-37 N^1)methyltransferase. This means that the anticodon sequence is not critical for the tRNA recognition by this enzyme; (ii) in contrast, for Y nucleoside biosynthesis, the anticodon sequence GAA is an absolute requirement; (iii) the conversion of G-37 into Y-37 nucleoside is a multienzymatic process in which m^1G-37 is the first obligatory intermediate; (iv) all the corresponding enzymes are cytoplasmic. In a crude yeast extract, restructured yeast tRNA^{Phe} with G-37 is efficiently modified only into m^1G-37; the corresponding enzyme is a S-adenosyl-L-methionine-dependent tRNA methyltransferase. The pure *Escherichia coli* tRNA (guanosine-37 N^1) methyltransferase is unable to modify the guanosine-37 of yeast tRNA^{Phe}.

Key words: maturation/N^1-methylguanosine/oocyte microinjection/tRNA/wye nucleoside

Introduction

In almost all tRNAs sequenced so far, there is a modified (often hypermodified) purine nucleoside adjacent to the 3′ end of the anticodon (position 37). Among them, the so-called wye nucleosides (Y) are certainly the most complex. They are found exclusively 3′ adjacent to the anticodon of most eukaryotic tRNA^{Phe}; known exceptions are tRNA^{Phe} from *Bombix mori* and *Drosophila melanogaster* which contain N^1-methylguanosine (m^1G) instead of a Y nucleoside (see Sprinzl *et al.*, 1985).

Different variants of Y nucleosides have been characterized; Figure 1 shows the structure of the Y nucleoside of yeast tRNA^{Phe} (Blobstein *et al.*, 1975). tRNA^{Phe} of *Torulopsis utilis* contains a Y nucleoside lacking the side chain attached to carbon 10 (Kasai *et al.*, 1976) while tRNA^{Phe} extracted from *Lupinus luteus* or beef, rat, calf and chicken liver contain a hydroxylated form of Y nucleoside (Nakanishi *et al.*, 1971; Wiewiorowski *et al.*, 1974; Kasai *et al.*, 1979), the hydroxyl group being attached to the carbon in position 14 of the side chain. This latter form of Y nucleoside is probably the one occurring in *X. laevis* oocyte tRNA^{Phe} (Mazabraud, 1982).

The biosynthetic pathways of the various Y nucleosides are only partially understood. Early work of Li *et al.* (1973) showed that the Y base of yeast tRNA^{Phe} is a derivative of guanine. Further studies by Münch and Thiebe (1975) indicated an incorporation of the carbonyl group of methionine in this Y nucleoside, most probably at position 16 of the side chain (see Figure 1). Additional information was obtained recently by Smith *et al.* (1985) who enriched yeast tRNA^{Phe} with ^{13}C by growing a methionine auxotroph strain of *Saccharomyces cerevisiae* in the presence of [^{13}C]methyl-L-methionine. Using n.m.r. they clearly showed that the methyl groups of the side chain and in position N^3 were derived from methionine, suggesting that they could be transferred from S-adenosyl-L-methionine (SAM) by a tRNA methyltransferase. The same may occur for either carbon 10 or carbon 11 of the third ring. However, the methyl group attached on carbon 11 is clearly not derived from methionine. These authors hypothesize that one mechanism of biosynthesis in yeast would be an initial methylation of the N^1 position of guanosine resulting in N^1-methylguanosine, a frequently occurring methylated nucleoside in tRNA, followed by insertion of a two-carbon molecule between the N^1 carbon and the N^2 amino group to close the third ring of Y.

On the other hand, Pergolizzi *et al.* (1979) showed an incorporation of radioactive lysine into the Y base of tRNA^{Phe} from Vero cells (a monkey kidney cell line). From their results, the suggestion was made that the third ring and the lateral chain of Y nucleoside is essentially derived from lysine. This latter idea

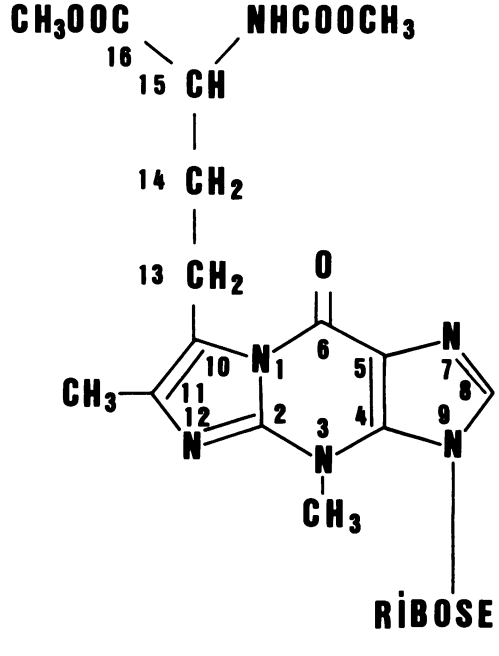

Fig. 1. Chemical structure of the Y nucleoside from yeast.

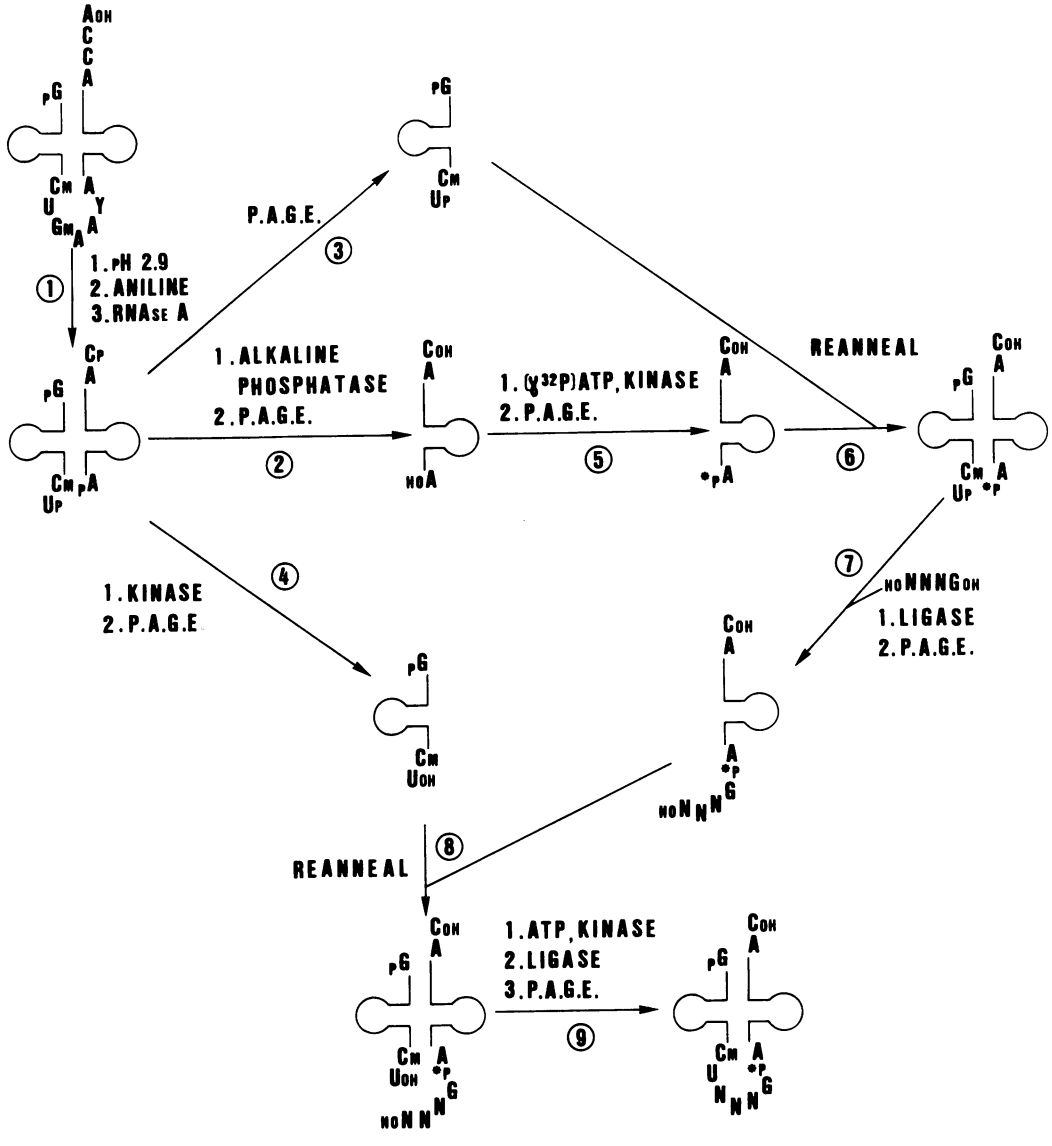

Fig. 2. Schematic representation of the procedure used for anticodon and nucleotide-37 replacement in yeast $tRNA^{Phe}$. Details are described in Materials and methods.

may not be easily compatible with the results of Münch and Thiebe (1975) concerning methionine incorporation into the Y nucleoside from yeast. However, different biosynthetic pathways for Y nucleoside may exist in different cell types.

Interestingly enough, in physiological states in which the Y nucleoside is not synthesized, such as in tumour cells, the $tRNA^{Phe}$ generally contains m^1G in position 37 (Kuchino *et al.*, 1982); these authors proposed that when m^1G-37 is present, Y nucleoside synthesis cannot proceed.

In the present paper we have reinvestigated the problem of m^1G and Y nucleoside biosynthesis in eukaryotic $tRNA^{Phe}$ using a completely different approach. Our methodology is based on earlier work in which the anticodon of several tRNAs were excised and replaced by synthetic oligonucleotides. During the reconstruction, a ^{32}P-label was specifically introduced adjacent to the nucleoside of interest. Microinjection of these ^{32}P-labelled restructured tRNAs into *X. laevis* oocytes allowed us to follow the kinetics of several enzymatic nucleoside modifications in the anticodon loop (reviewed in Grosjean *et al.*, 1987).

In a previous paper, we have used this methodology to study the specificity of the tRNA (*wobble* guanosine 2′-*O*-)methyltransferase acting on eukaryotic $tRNA^{Phe}$ (Droogmans *et al.*, 1986). We have now taken advantage of a similar procedure to study the maturation of guanosine-37 into the Y nucleoside and the specificities of the corresponding enzymes. The results indicate that, contrary to the earlier prediction of Kuchino *et al.* (1982), the first step in Y nucleoside biosynthesis is the enzymatic formation of m^1G-37 which will proceed independently of the nucleotide sequence in the anticodon. However, the enzymes involved in the subsequent reactions leading to Y-37 strictly depend on the GAA anticodon.

Results

Restructured yeast $tRNA^{Phe}$ with $G_{34}AAG_{37}$ is a substrate for the X. laevis oocyte enzymes involved in Y nucleoside biosynthesis

To obtain a suitably unmodified tRNA that can be used as a substrate to aid detection of the modifying enzymes involved in Y nucleoside biosynthesis, we have replaced the anticodon and 3′ adjacent nucleotide (G_mAAY) of yeast $tRNA^{Phe}$ by the syn-

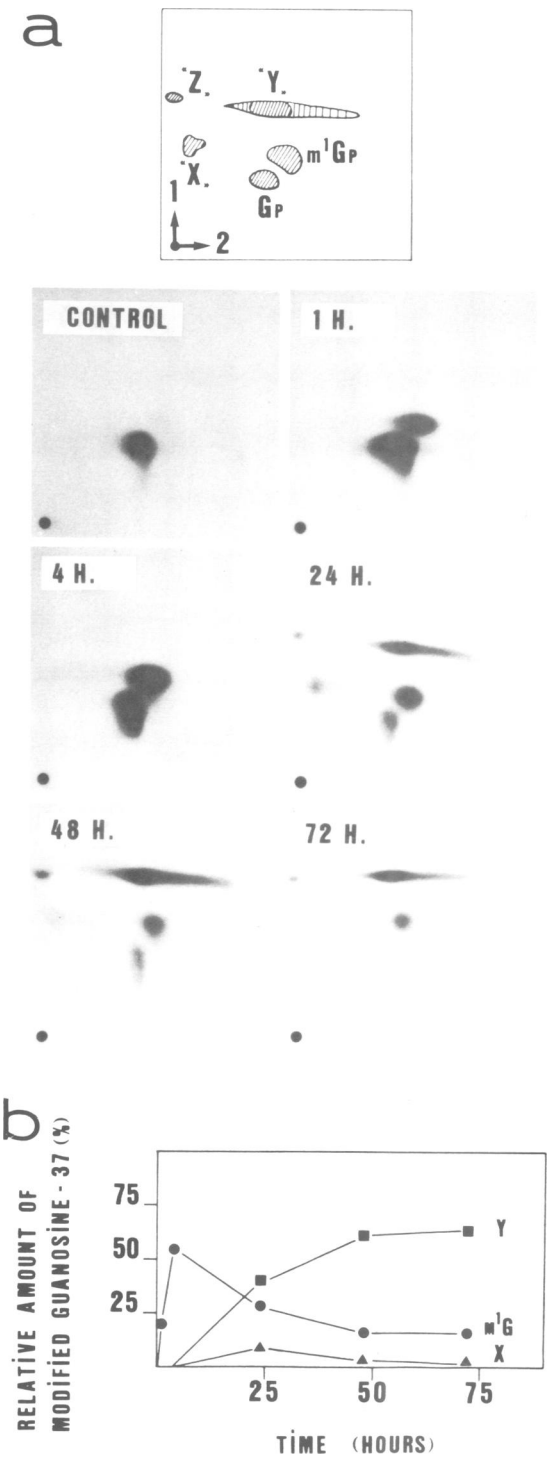

Fig. 3. Kinetics of guanosine-37 modification in yeast $tRNA^{Phe}$ restructured with the oligonucleotide GAAG and microinjected into the cytoplasm of *X. laevis* oocytes. The tRNA was recovered from the oocytes by phenol extraction and isolated by polyacrylamide gel electrophoresis. The tRNA was then hydrolysed for 2 h at 37°C by 0.2 units of RNase T2 in the presence of 5 μg total yeast tRNA as carrier. (**a**) Autoradiograms of the two-dimensional thin-layer chromatograms (using chromatographic system I; see Materials and methods) of T2 digestion of 3′ [^{32}P]G-37-labelled yeast $tRNA^{Phe}$ before (control) and after microinjection into *X. laevis* oocytes. Times of incubation at 19°C in the oocytes are given in the figure. At the top is a diagram showing the relative migrations of all the ^{32}P-labelled nucleotides found in position 37 of yeast $tRNA^{Phe}$. (**b**) The radioactivity found in each spot was expressed as the percentage of total radioactivity found on the t.l.c. plates and plotted as a function of incubation time of yeast $tRNA^{Phe}$ in the oocytes.

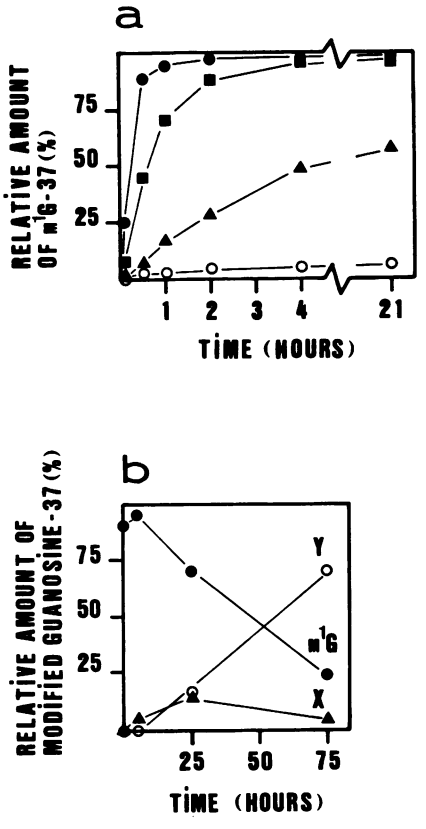

Fig. 4. Kinetics of G-37 conversion into m^1G-37 in a yeast extract (**a**) and of m^1G-37 into Y-37 in *X. laevis* oocytes (**b**). (**a**) The tRNA substrate is yeast $tRNA^{Phe}$ reconstructed with the oligonucleotide GAAG. All experiments were performed at 25°C in a crude yeast extract (prepared as described in Materials and methods) and in the presence of 5 μM SAM. Increasing amounts of the inhibitor SAH are added in the incubation mixture (●, control without SAH; ■, 5 μm SAH; ▲, 50 μm SAH; ○, 500 μM SAH). (**b**) The tRNA substrate is yeast $tRNA^{Phe}$ containing m^1G-37, isolated from the above reaction mixture (after a 5-h incubation) by ethanol precipitation and polyacrylamide gel electrophoresis. The ^{32}P-labelled material was microinjected into the cytoplasm of *X. laevis* oocytes. The oocytes were then processed as described in the legend of Figure 3.

thetic tetranucleotide GAAG. During reconstruction, a ^{32}P-label of high specific radioactivity is introduced 3′ ajacent to G-37. The procedure, as detailed in Figure 2, is very similar to those already described in several other papers (Bruce and Uhlenbeck, 1982; Carbon *et al.*, 1982; Fournier *et al.*, 1983; Haumont *et al.*, 1984; Droogmans *et al.*, 1986).

The restructured $tRNA^{Phe}$ is microinjected into the cytoplasm of the *X. laevis* oocytes. Each oocyte receives 2×10^{-15} moles of foreign tRNA, corresponding to ~1% of the normal amount of *X. laevis* oocyte $tRNA^{Phe}$ (Gatica *et al.*, 1975). At different times of incubation at 19°C, the total RNA of the oocytes is recovered. The presence of modified guanosine-37 is detected as described in the legend of Figure 3.

The results indicate that the maturation of G-37 in yeast $tRNA^{Phe}$ proceeds in several steps (Figure 3). The first one corresponds to a fast accumulation of m^1G-37 up to a maximum of ~60% (almost 100% in other experiments) of the amount of the original guanosine-37. The second step corresponds to a transitory formation of a product marked 'X' on the autoradiograms. The chemical nature of this latter compound is completely unknown. The third step corresponds to the slower formation of the highly hydrophobic acid-labile 'Y' compound which spreads out all along the acidic second dimension of chromatographic system I (see Materials and methods).

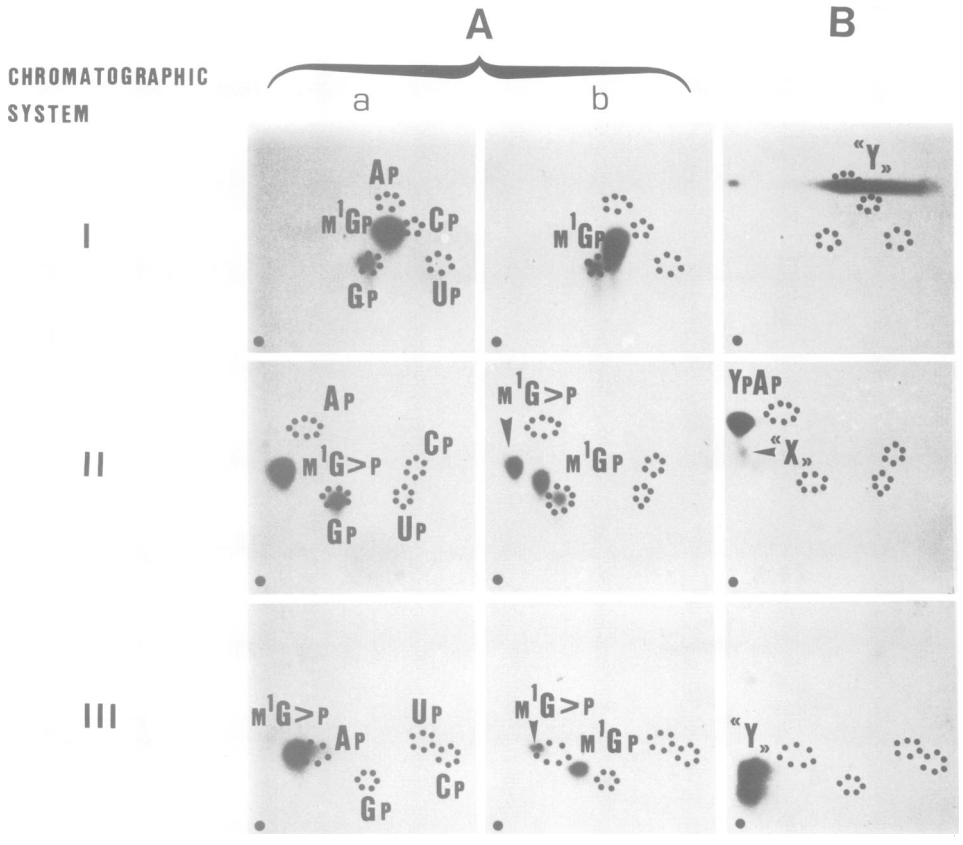

Fig. 5. Two-dimensional t.l.c. characterization in different solvent systems of the modified nucleotides found in position 37 of microinjected yeast $tRNA^{Phe}$. The incubation in the oocytes was for 4 h in **A** (essentially only m^1G is formed) or 3 days in **B** (essentially only Y-37 is present, see Figure 3). The phenol-extracted tRNA (from five oocytes) was isolated by polyacrylamide gel electrophoresis; it was then digested by 0.2 units of RNase T2 for 2 h (with 5 μg total yeast tRNA as carrier (**a**) or 20 h without carrier (**b**) at 37°C. The hydrolysates were subjected to two-dimensional t.l.c. using the solvent systems I, II or III described in Materials and methods. Circles in dotted lines show the migration of the four canonical nucleotides used as u.v. markers.

Enzymatic formation of m^1G-37 $tRNA^{Phe}$ is catalysed by a S-adenosyl-L-methionine-dependent tRNA methyltransferase in yeast

Considering the above observation that the enzymatic formation of m^1G-37 is the first step in Y nucleoside biosynthesis, it was of particular importance to investigate the possibility that it was also the case in a homologous system involving both $tRNA^{Phe}$ and the enzymes from yeast. Indeed, up to now, no m^1G-37-containing yeast $tRNA^{Phe}$ has been reported. Figure 4a clearly shows that reconstructed yeast $tRNA^{Phe}$ ($G_{34}AAG_{37}$) is a very good substrate for an enzyme present in a crude yeast extract which transforms G-37 into m^1G-37. The enzymatic reaction is fast and quantitative. Addition of SAM up to 5×10^{-6} M does not stimulate the enzyme of the crude, fresh, undialysed yeast extract. Dependence for the addition of SAM in the incubation mixture is evident when partially purified yeast tRNA methyltransferase is used (result not shown).

Addition of S-adenosyl-L-homocysteine (SAH) strongly reduces the rate of the enzymatic reaction in the crude extract; the reaction is almost abolished at 0.5×10^{-3} M of SAH (Figure 4a). Thus, yeast contains a methyltransferase able to produce m^1G-37-containing yeast $tRNA^{Phe}$

In the same crude yeast extract there is no production of any trace of X-37 and Y-37 nucleosides. However, when this m^1G-37-containing $tRNA^{Phe}$ is microinjected into the cytoplasm of *X. laevis* oocyte, it becomes almost fully modified into the Y-37 derivative (Figure 4b). This experiment demonstrates that: (i) m^1G-37 in $tRNA^{Phe}$ is the first obligatory intermediate in the Y-37 nucleoside formation; and (ii) the corresponding enzymes in the yeast extract are not functional. This is probably due to the lack of necessary co-factors and/or to the loss of corresponding enzymes during extract preparation.

Chromatographic identification of the modified nucleotides found in position 37 of yeast $tRNA^{Phe}$

Characterization of the different nucleotides found in position 37 of yeast $tRNA^{Phe}$, as revealed in the chromatograms in Figure 3 with chromatographic system I, were further performed with two other chromatographic systems, respectively II and III (see Materials and methods) involving neutral or only weakly acidic solvents (Figure 5).

These controls were necessary in order to ascertain further the identity of the two major compounds found in Figure 3. The compound which clearly migrates in chromatographic system I as m^1G-37 (see Figure 3), with a monophosphate group on the 3′-OH of ribose, did not migrate as such in chromatographic systems II and III (Figure 5, column Aa). The reason is that after RNase T2 digestion of the tRNA, this particular nucleotide usually arises as the alkaline phosphatase resistant 2′-3′ cyclic mononucleotide $m^1G > p$. This compound is quantitatively converted into the corresponding 3′ monophosphate nucleotide only if the chromatographic system involves a strongly acidic solvent such as that of the second dimension in system I; it is only partially converted into m^1Gp (3′) if longer incubation with RNase T2 (20 h instead of 2 h) is performed (compare columns Aa and Ab in Figure 5).

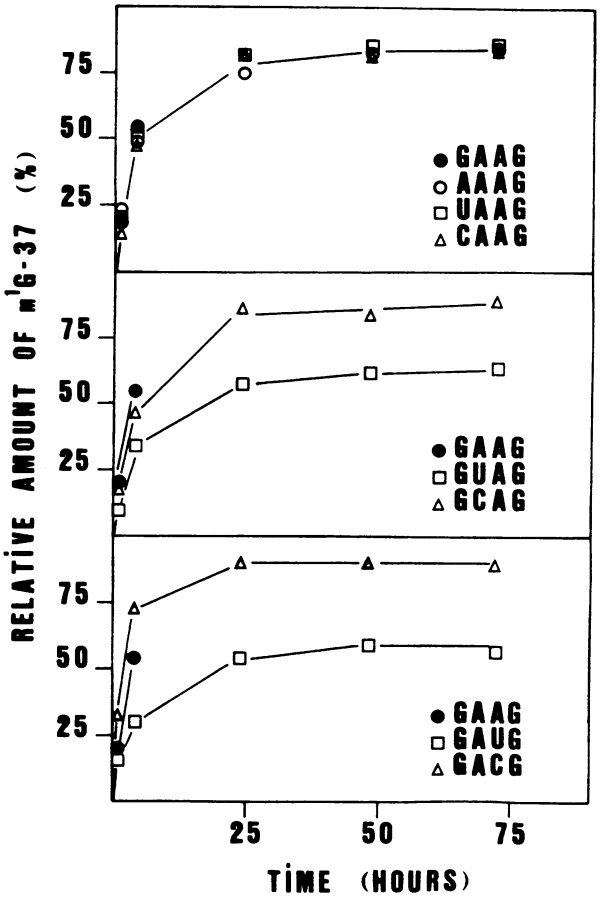

Fig. 6. Comparison of guanosine-37 methylation in reconstructed yeast $tRNA^{Phe}$ microinjected into *X. laevis* oocytes. Experimental conditions and data treatments were identical as those in Figure 3b. The oligonucleotides used to restructure the tRNA are indicated with the corresponding symbols. The initial formation of m^1G-37 in tRNA reconstructed with the oligonucleotide GAAG (see in Figure 3b) is also indicated.

It is well known that, under the conditions we use for the hydrolysis of tRNA by RNase T2, a dinucleotide diphosphate YpAp is produced (Maelicke *et al.*, 1975); also the glycosidic bond of Y nucleoside is highly unstable, even under mildly acidic conditions (Thiebe and Zachau, 1968). Therefore, the spot we named 'Y' in Figure 3 most probably corresponds to a dinucleotide having lost the Y base. The intact YpAp most probably corresponds to spot 'Z' (see Figure 3) in which the Y base is progressively liberated during the chromatography with acidic solvent (second dimension of system I or first dimension of system III) giving rise to the characteristic wide-spread spot 'Y' (see Figure 5, column B).

Using neutral solvents of system II, only one well-characterized spot corresponding to YpAp is evident (see Figure 5, column B). Its chromatographic behaviour is affected by alkaline phosphatase treatment without loss of the internal ^{32}P-label. In addition, this compound strongly adsorbs to a BD-cellulose column, requiring the presence of organic solvent (ethanol) for elution (results not shown), indicating the presence of the Y base in this dinucleotide.

Enzymatic synthesis of m^1G or Y nucleoside depends on different structural parameters of the yeast $tRNA^{Phe}$

In order to obtain information on the specificity of the different enzymes involved in the biosynthesis of m^1G-37 and Y-37 nucleosides, a series of chimeric yeast $tRNAs^{Phe}$ was constructed in which one of the three nucleosides of the GAA anticodon is replaced by another nucleoside (see step 7 in Figure 2). These chimeric $tRNAs^{Phe}$ have a guanosine-37 and differ by only one base in the anticodon. Each of these tRNAs is microinjected into *X. laevis* oocytes. After incubation at 19°C, they are processed as above (see Figure 3) to detect the presence of modified guanosine-37. Our results indicate that none of the variants of yeast $tRNA^{Phe}$ is a substrate for the enzyme involved in the biosynthesis of X-37 and Y-37 nucleosides; only the activity of the enzyme leading to the synthesis of m^1G-37 is evident (Figure 5). In this latter case, clearly none of the three bases in the anticodon is essential for the action of this modification enzyme; this is particularly evident in the case of the *wobble* base (position 34).

Discussion

Y nucleosides found 3′ adjacent to the anticodon of several eukaryotic $tRNA^{Phe}$ are the most complex nucleosides characterized so far. We may expect that their biosynthetic pathways are similarly complex and their study has indeed been a challenge for a long time. The lack of an appropriate unmodified tRNA substrate, the ignorance of the co-factors required to develop an appropriate *in vitro* assay system and the possible instability of the corresponding enzymes *in vitro* have made the problem particularly intractable.

To overcome these difficulties, we have constructed a yeast $tRNA^{Phe}$ in which the naturally occurring Y-37 nucleoside is replaced enzymatically *in vitro* by a guanosine-37. During the anticodon loop restructuring, a ^{32}P-label is introduced between nucleosides 37 and 38. This ^{32}P-labelled tRNA substrate is then microinjected into the cytoplasm of *X. laevis* oocytes. From our results, it is clear that such reconstructed yeast $tRNA^{Phe}$ with G-37 instead of Y-37 is, under the physiological conditions of a living cell, an efficient substrate for the several oocyte enzymes involved in the Y-37 nucleoside biosynthesis. Since no transfer of mature tRNA from the cytoplasm to the nucleus seems to occur (De Robertis *et al.*, 1982), our results indicate that these modification enzymes are present in the cytoplasm of *X. laevis* oocytes.

The most interesting aspect of our work is the identification of a tRNA-carrying m^1G-37 as the first intermediate which accumulates before a substantial amount of Y-37 containing $tRNA^{Phe}$ appears in the *X. laevis* oocyte (see Figure 3). Small amounts of at least one other intermediate (called 'X') were also detected but this compound does not accumulate, thereby indicating that it is processed rapidly by the enzymes leading to Y-37. These observations do not exclude the possibility that intermediates other than X-37 exist between m^1G-37 and Y-37.

It is interesting to note that the m^1G-37 containing $tRNA^{Phe}$, as produced in a yeast extract, is an efficient substrate for the oocyte enzymes. Here again, the formation of X-37-containing $tRNA^{Phe}$ clearly preceeds the formation of Y-37-containing $tRNA^{Phe}$. The fact that partially purified tRNA methyltransferase from yeast requires the presence of SAM in the reaction mixture in order to modify the G-37 into m^1G-37 and that the enzymatic reaction is strongly inhibited by SAH favours idea that this reaction is catalysed by the SAM-dependent tRNA (guanosine-37 N^1)methyltransferase already purified from yeast by Smolar *et al.* (1975) (see also Sindhuphak *et al.*, 1985). The same tRNA (guanosine-37 N^1)methyltransferase seems to exist in rabbit reticulocyte as revealed by the experiments of Bruce *et al.* (1982).

Several years ago, studies on $tRNA^{Phe}$ extracted from tumour

cells indicated the presence of m^1G-37 in place of the commonly occurring Y nucleoside. This observation led Kuchino *et al.* (1982) to postulate that the presence of m^1G-37 was an impediment for the enzymes leading to G-37 into Y-37. The methylation of G-37 in position N^1 or N^3 was proposed to vary according to the physiological state of the cell. It is well known that the glycosidic bond of m^3G is very unstable even under mild acidic conditions (Itaya *et al.*, 1985). For example, the half-life of this glycosidic bond is 42 s at pH 1 which contrasts with the higher stability of the glycosidic bond of m^1G under similar conditions. Since we observed that the glycosidic bond of the first intermediate in Y nucleoside biosynthesis as detected in our experiments is resistant even to strong acidic treatment, this could certainly not be m^3G but rather must be m^1G. This is in accordance with the results of Smith *et al.* (1985) obtained by n.m.r. studies of ^{13}C-enriched $tRNA^{Phe}$. Initially, they suspected either carbon in position 10 or in position 11 of the third ring of Y base to come from the methyl group of [^{13}C]methylmethionine; more recent work allowed them to claim that the source of carbon 10 is the methyl group of methionine while the source of carbon 11 is still a quandary (P.F.Agris, personal communication). The existence of a m^1G-37 as an intermediate in Y-37 biosynthesis is not compatible with the model of Pergolizzi *et al.* (1979) who proposed that in Vero cells, formation of the third ring and the lateral chain of Y base involved a whole lysine molecule. However, we cannot exclude (although we believe it is improbable) that the biosynthetic pathway for Y-37 biosynthesis in Vero cells is different from that in yeast and in *X. laevis* oocytes.

We should point out that the enzymatic formation of m^1G-37 is observed with all batches of *X. laevis* oocytes we have microinjected so far. However, for unknown reasons, the subsequent enzymatic formation of Y-37 does not always occur. These observations suggest that the activity of the enzyme leading to m^1G-37 in $tRNA^{Phe}$ is regulated independently of the enzymes leading to Y-37.

Concerning the specificity of the tRNA recognition by the various enzymes involved in Y-37 nucleoside biosynthesis, it is remarkable that on the one hand the tRNA (guanosine-37 N^1)methyltransferase does not require any particular anticodon sequence while, on the other hand, the m^1G-37 into Y-37-transforming enzymes are functional only on a yeast $tRNA^{Phe}$ containing the normal GAA anticodon.

Similar situations were found for other enzymatic nucleoside modifications in the anticodon loop of tRNA; for example, the enzymatic conversion of G-34 into the 2′-*O*-methyl derivative Gm-34 in yeast $tRNA^{Phe}$ was not dependent on the anticodon sequence (Droogmans *et al.*, 1986) while the enzymatic conversion of G-34 into Q-34 (Carbon *et al.*, 1983) and of A-34 into I-34 (Haumont *et al.*, 1984) were clearly dependent on the anticodon sequence. The interest of the present situation is that the same nucleoside G-37 in yeast $tRNA^{Phe}$ is subjected to the actions of distinct maturation enzymes with clearly different specificities. A similar situation exists for the enzymatic conversion of G-34 into glycosyl Q-34 in yeast $tRNA^{Arg}$ microinjected into *X. laevis* oocyte (E.Haumont, personal communication).

One aspect of tRNA recognition by these modification enzymes that we have not yet solved concerns the possible dependence on structural parameters outside the anticodon. One approach would be to replace the anticodon and 3′ adjacent nucleotide in various tRNAs by the GAAG sequence and check whether or not G-37 in such restructured tRNAs becomes modified after microinjection into *X. laevis* oocytes (work in progress). A first indication that such dependence may exist comes from the observation that pure *E. coli* tRNA (guanosine-37 N^1)methyltransferase (Hjalmarsson *et al.*, 1983) is unable to methylate the G-37 in reconstructed yeast $tRNA^{Phe}$ (results not shown).

In summary, our present working hypothesis for Y-37 biosynthesis in $tRNA^{Phe}$ is as follows: in a first step, G-37 is modified to m^1G-37 by the action of a SAM-dependent tRNA methyltransferase. This methylating enzyme (specific for guanosine-37 but not for the kind of anticodon) could be the same as the one which modifies several tRNAs specific for different amino acids (e.g. leucine, histidine, proline, tryptophan and aspartic acid). In subsequent steps, m^1G-37 is further modified into Y-37 by several other enzymes. We have established the existence of at least one intermediate (compound 'X') which does not accumulate in the process of Y-37 biosynthesis. One explanation may be that these enzymes are organized in a multienzymatic complex for which only $tRNA^{Phe}$ with a GAA anticodon can be a substrate.

Materials and methods

Enzymes and chemicals

All enzymes and chemicals are the same as those described in a previous paper (Droogmans *et al.*, 1986). Pure *E. coli* tRNA (guanosine-37 N^1)methyltransferase, prepared as described in Hjalmarsson *et al.* (1983) and tRNA extracted from the *E. coli* trm D mutant were generous gifts of Drs P.M.Wikström, A.S.Byström and G.R.Björk (Umeå, Sweden). The enzymatic synthesis of tetranucleotides is described in Droogmans *et al.* (1986).

In vitro enzymatic restructuring of tRNA

The nine-step procedure for restructuring yeast $tRNA^{Phe}$ is described in Figure 2. Step 1 consists of the excision of the anticodon and its 3′ adjacent nucleotide as described by Bruce and Uhlenbeck (1982). All further enzymatic reactions were performed essentially as described in previous papers (Carbon *et al.*, 1982; Fournier *et al.*, 1983). Step 2 involves dephosphorylation by treatment of the two associated half tRNA molecules by alkaline phosphatase. The resulting dephosphorylated 3′ half molecule was purified by electrophoresis on a denaturing 15% polyacrylamide gel. Step 5 consists of ^{32}P-labelling of the 5′ extremity of the 3′ fragment using [γ-^{32}P]ATP and T4-polynucleotide kinase. We observed that the ligation of a tetranucleotide on the 3′ fragment (step 7) can be accomplished only if this fragment is annealed with a 5′ fragment (step 6). It is essential that the 3′ extremity of the 5′ half be phosphorylated to avoid the ligation between the two half tRNAs. This fragment is obtained (step 3) from the product of step 1 by denaturing 15% polyacrylamide gel electrophoresis. The extended 3′ fragment resulting from step 7 was purified by denaturing 15% polyacrylamide gel electrophoresis and annealed (step 8) with a pure 3′ dephosphorylated 5′ fragment obtained in step 4. The final step (9) consists in phosphorylating the 5′ end of the extended 3′ fragment using non-radioactive ATP and T4-polynucleotide kinase. Sealing of the anticodon loop is then performed by addition of T4-RNA ligase in this latter reaction mixture. The restructured yeast $tRNA^{Phe}$, ^{32}P-labelled on phosphorus 3′ adjacent to G-37, is purified by denaturing 15% polyacrylamide gel electrophoresis.

Preparation of crude yeast extract

The yeast strain D 261 was grown overnight at 30°C until the late exponential phase in 1 l of medium containing 1% yeast extract, 0.1% KH_2PO_4 and 2% ethanol. Cells are harvested by centrifugation at 5000 *g* for 5 min and washed with ice-cold distilled water. All further operations were performed at 0–4°C. The pellet was suspended in 10 ml buffer containing 20 mM Tris pH 7.6, 20 mM $Mg(Ac)_2$, 5 mM β-mercaptoethanol and 1 mM EDTA. The suspension was mixed with 10 g of glass beads (0.5 mm in diameter) and the cells disrupted in a Braun homogenizer for 1 min. After pelleting the glass beads and cell debris by low speed centrifugation, the supernatant was centrifuged for 1 h at 100 000 *g*. To the resulting supernatant, glycerol was added to a final concentration of 50%. The final extract at 0.5 mg protein/ml can be stored at −20°C with no loss of tRNA (guanosine-37 N^1)methyltransferase activity for at least 2 weeks.

Assay for tRNA (guanosine 37 N^1)methyltransferase in a crude yeast extract

The incubation mixture (final volume 100 μl) consisted of 100 mM Tris pH 8, 1 mM DTE, 0.1 mM EDTA, 10 mM $MgSO_4$, 20 mM NH_4Cl, 10^6 c.p.m. ^{32}P-Labelled tRNA and 25 μl of crude yeast extract. Incubation took place at 25°C. After different periods of time, 15-μl aliquots were taken, diluted with water up to 100 μl and immediately phenol extracted. The tRNA in the aqueous phase was ethanol precipitated at −80°C and further purified by denaturing 15% polyacrylamide gel electrophoresis. Modification of G-37 into m^1G-37 was detected

after hydrolysis of the ^{32}P-labelled $tRNA^{Phe}$ by RNase T2 followed by cellulose t.l.c. of the resulting nucleotides. The procedure is identical to that described by Carbon *et al.* (1982) for G-34 to Q-34 formation except that chromatographic system II was used (see below).

Chromatographic methods

Two-dimensional t.l.c. of nucleotides was performed on cellulose thin layer plates (10 × 10 cm, 0.1 mm; Macherey-Nagel) using different solvent systems.

System I (Nishimura, 1979): first dimension developed with isobutyric acid/conc. NH_4OH/water (66:1:33, v/v/v); second dimension developed with conc. HCl/isopropanol/water (17.6:68:14.4; v/v/v).

System II (Silberklang *et al.*, 1979): first dimension developed with isobutyric acid/conc. NH_4OH/water (66:1:33; v/v/v); second dimension developed with 0.1 M sodium phosphate pH 6.8/solid $(NH_4)_2SO_4$/n-propanol (100:60:2; v/w/v).

System III (Feldmann and Falter, 1971): first dimension developed with 5% ammonium acetate pH 3.5/isopropanol (25:60; v/v); second dimension developed with saturated $(NH_4)_2SO_4$/1 M sodium acetate pH 5.5/isopropanol (40:9:1; v/v/v).

Benzoylated DEAE-cellulose column chromatography was performed essentially according to Gillam *et al.* (1967).

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