Identity elements for N²-dimethylation of guanosine-26 in yeast tRNAs

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ABSTRACT

N²,N²-dimethylguanosine (m²G) is a characteristic nucleoside that is found in the bend between the dihydro-uridine (D) stem and the anticodon (AC) stem in over 80% of the eukaryotic tRNA species having guanosine at position 26 (G26). However, since a few eukaryotic tRNAs have an unmodified G in that position, G26 is a necessary but not a sufficient condition for dimethylation. In yeast tRNA* G26 is unmodified. We have successively changed the near surroundings of G26 in this tRNA until G26 became modified to m²G by a tRNA(m²G26)methyltransferase in Xenopus laevis oocytes. In this way we have identified the two D-stem basepairs C11-G24, G10-C25 immediately preceding G26 as major identity elements for the dimethylating enzyme modifying G26. Furthermore, increasing the extra loop in tRNA* from four to the more usual five bases influenced the global structure of the tRNA such that the m²G26 formation was drastically decreased even if the near region of G26 had the two consensus basepairs. We conclude that not only are the two consensus base pairs in the D-stem a prerequisite for G26 modification, but also is any part of the tRNA molecule that influence the 3D-structure important for the recognition between nuclear coded tRNAs and the tRNA(m²G26)methyltransferase.

INTRODUCTION

In the series of events in the cell that ultimately lead to a mature tRNA molecule is the enzymatic modification of bases and ribose moieties, occurring at the polynucleotide level, unique in several respects (reviewed in 1). The modifying enzymes are nucleic acid specific and site specific. Each enzyme catalyses the formation of one unique type of modified nucleoside at its specific site in a large variety of tRNA species, each of them with a different primary sequence. Besides the identity of the base and the position of the base in the primary sequence, it is tempting to believe that only a limited number of common identity elements should exist for each specific modifying enzyme and that these elements have to be recognized by the respective enzyme within a characteristic three-dimensional tRNA structure.

Few studies have been carried out to systematically identify how tRNAs are recognized by a modifying enzyme, and it is evident that no simple general rules exist (2, 3). It was earlier generally accepted that modifications seemed to require an almost full length tRNA polynucleotide as substrate. Recently, however, Gu and Santi (4) showed that only the T-loop-and-stem fragment from tRNA was needed for recognition by bacterial tRNA(m⁵U54)methyltransferase. Some of the enzymes that modify bases in the anticodon (AC) loop recognize sequences immediately surrounding the nucleoside to be modified; this is valid for formation of Q34, Y37 and tA37 (3) and for ²35 (5), while others, such as the Gm34- and m¹G37-forming enzymes, depend on identity elements that are more scattered within the tRNA structure (3, 6). In certain eukaryotic tRNAs the formation of ²35 (7, 8, 9) and of m⁵C34 (10) are dependent on the presence of an intron in the pre-tRNAs.

In yeasts as in other eukaryotes, the majority of the different modifications in nuclear coded tRNAs are formed already within the nucleus (reviewed in 11). One of these nuclear localized (12) enzymes is the tRNA(m²G26)methyltransferase (13) that methylates G26 in the bend between the D-stem and the AC-stem to dimethylguanosine (m²G).

Yeast tRNA* is one of the few eukaryotic tRNA species in which the G26 is not modified (14). We have made synthetic variants of yeast tRNA and analyzed the formation of modified nucleosides after injection of the tRNAs into the cytoplasm of X. laevis oocytes. The oocytes have earlier been shown to efficiently modify in vitro transcribed, completely unmodified tRNAs at all available sites (15). We used yeast tRNA* as substrate because of its lack of inherent modification of G26 and because of the now available detailed information about its 3D-structure (16, 17). This heterologous in vivo system with its complete modification capacity was chosen to allow a first mode of identification of important parameters for the recognition between tRNAs and G26 modifying enzymes.

In this paper we demonstrate that wild type yeast tRNA* is recognized by the G26 monomethylating activity present in X. laevis oocytes in a reaction that is characteristic for many higher
eukaryotes but not found in yeast. In order to reach the dimethylation state of G26 it was necessary to exchange several bases in the vicinity of G26 in yeast tRNA^ before it became fully methylated to m^2G26. Thus, by using the X. laevis tRNA(m^2G26)methyltransferase, an enzyme known to efficiently modify also heterologous tRNAs, we have identified those structural elements in yeast tRNAs that are necessary for determining the enzymatic modification of G26 to m^2G26 via the intermediate m^G26.

MATERIALS AND METHODS

Bacteria and plasmids

In the plasmid pTFMa (a generous gift from R. Giegé, France) the T7 RNA polymerase promoter is followed by a synthetic tRNA^ gene coding also for the CCA terminal and with the double mutation G1-C72. The plasmid is a pUC18 derivative with a F1 replication origin (18). Corresponding single-stranded DNA was isolated from E. coli TG1( supE, hsdR5, thi, Δ(lac-proAB), F'(traD36, proAB+, lacY, lacZAM15)) according to standard procedures (19). E. coli DH5α (supE44, ΔlacU169Δ80 lacZAM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was used as recipient for plasmid transformations. Plasmid p67YF0 (20) was derived from pSP65 and carried a synthetic wild type tRNA^ gene. Plasmid p67YF26 is a C25 to T25 mutant of the tRNA^ gene carried in a pUC18 derivative downstream of a T7 promoter (21).

DNA preparations and site-directed mutagenesis

Large-scale and small-scale plasmid preparations were done by standard procedures (19). Preparations of single-stranded DNA and mutagenesis were according to the booklet ‘Oligonucleotide-directed in vitro mutagenesis, version 2′ from Amersham, UK. Mutations were controlled by dyeoxy sequencing with M13 universal primer and with Klenow or T7 DNA polymerase (22). The different tRNA variants were constructed using the following universal primer and with Klenow or T7 DNA polymerase (22). In the plasmid pTFMa (a generous gift from R. Giegé, France) the T7 RNA polymerase promoter is followed by a synthetic tRNA^ gene coding also for the CCA terminal and with the double mutation G1-C72. The plasmid is a pUC18 derivative with a F1 replication origin (18). Corresponding single-stranded DNA was isolated from E. coli TG1( supE, hsdR5, thi, Δ(lac-proAB), F'(traD36, proAB+, lacY, lacZAM15)) according to standard procedures (19). E. coli DH5α (supE44, ΔlacU169Δ80 lacZAM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1) was used as recipient for plasmid transformations. Plasmid p67YF0 (20) was derived from pSP65 and carried a synthetic wild type tRNA^ gene. Plasmid p67YF26 is a C25 to T25 mutant of the tRNA^ gene carried in a pUC18 derivative downstream of a T7 promoter (21).

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Preparation of radiolabelled tRNA transcripts

The procedure was in essence as described in (15). In the incubation tube 20 μl of α^32P GTP (10 mCi/ml, 3000 Ci/mmol, Amersham, UK) was lyophilized together with 6 μl of 100 μM GTP before the following was added: 1 μl of water, 2 μl of 5X transcription buffer (200 mM Tris–HCl pH 7.5, 30 mM MgCl2, 10 mM spermidine, 50 mM NaCl), 1 μl of 100 mM DTT, 0.5 μl of RNAin (40 U/μl, Promega), 2 μl of ATP, CTP, UTP 2.5 mM each, 2 μl of linearized plasmid template (1 μg/μl) and 1.5 μl of T7 RNA polymerase (Biolabs 20U/μl). Transcription mixtures were incubated for 3 h at 37°C. The tRNA transcripts were purified by 10% PAGE–8 M urea. The 32P-labelled tRNA was eluted over night, precipitated twice by ethanol in the presence of 3 μg of polyGU and dissolved in 10 μl of water yielding in general 2×10^7 cts/min of TCA-precipitable material, i.e. about 1.2 pmole recovery of tRNA.

Oocyte microinjections and recovery of microinjected tRNA

32P-labelled tRNA was injected into the cytoplasm of X. laevis oocytes in stages 5 and 6 (mainly as in 23) using 50 nl of tRNA (about 1×10^5 cts/min, i.e. about 6 fmols) per oocyte. Each tRNA variant was injected into 40–50 oocytes, which were incubated at 19°C in Barth solution. Samples of 6–10 oocytes were withdrawn after 3–48 h, frozen in liquid nitrogen and kept at −80°C until tRNA was extracted (15). Total RNA was recovered from oocytes after quick homogenisation in 0.2 ml of 0.2 M Na-acetate pH 6, 10 mM MgCl2, 1 mM EDTA, 2% SDS followed by two phenol/chloroform extractions, one ethanol precipitation and finally purified on 10% PAGE–8 M Urea.

Analysis of modified nucleotides

Samples of 32P-labelled tRNA isolated from oocytes were degraded completely either into 5'-mononucleotides with nuclease P1, or into 3'-mononucleotides with RNase T2. Conditions for nuclease P1 degradation (Boehringer Mannheim, Germany) were: to 4 μl of tRNA in water was added 4 μl of 0.1 M Na-acetate, pH 5.2 and 2 μl of nuclease P1 (2 μg/μl, 0.3 μ/l in 30 mM Na-acetate buffer pH 5.2) and for RNase T2 (Sigma, USA) were: to 4 μl of tRNA in water was added 4 μl of 0.1 M Na-acetate pH 4.6 and 2 μl of RNase T2 (1 U/μl in 30 mM Na-acetate pH 4.6). After incubation for 3 h at 37°C the digested tRNA products (10 μl) were loaded and dried onto 20×20 cm TLC plates (Polygram Cel 300, 0.1 mm of cellulose, Macherey-Nagel, Düren, Germany or Eastman Chromagram sheet 13255 cellulose, Kodak). Separation in 1st dimension was in isobutyric acid:NH40H:water 66:1:33, (v:v:v) and in 2nd dimension in isopropanol:HCl:water, 68:17.6:14.4 (v:v:v) (24, also giving map positions for the different nucleotides). The TLC plates were exposed to Fuji Rx Safety films at −80° for 7 h to 1 week. Quantification of identified spots was done by scratching the spot from the plates and counting the radioactivity by liquid scintillation techniques. Using the amount of label in the GMP spots and with the knowledge of the number of GMPs present in the whole tRNA transcript, the molar ratio of modified nucleotides per mole tRNA was calculated (15).

RESULTS

A hypothetical consensus sequence for modification of G26 in tRNAs to dimethyl-Guanosine26

Comparison of the 130 sequenced eukaryotic, nuclear coded tRNAs with G in position 26 (25) show that 105 of them have m^G at this position, 17 others have m^G and only 8 have a completely modified G26 (Table I). Yeast tRNA^M^P^ belongs to this last category. Primary sequences downstream of m^G26 vary considerably, while upstream in the D-stem the m^G26 is to this last category. Primary sequences downstream of m^G26 vary considerably, while upstream in the D-stem the m^G26 is...
Table I. Comparison of 130 eukaryotic, nuclear coded tRNAs with G at position 26 (25)

<table>
<thead>
<tr>
<th>tRNA sequence at positions</th>
<th>Frequency of G26 modification</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-10-5'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C G</td>
<td>101</td>
<td>19</td>
</tr>
<tr>
<td>G C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C G</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>G U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U G</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>A C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U G</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>A U</td>
<td></td>
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<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G G</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figures within parenthesis refer to 22 yeast S. cerevisiae tRNAs. Bold underlined: dominant effects.

The G26 modification in wildtype and mutants of tRNA^asp and tRNA^phe

When the wild-type like tRNA^asp (variant a) was injected into oocytes, G26 did not become modified to m^2G (Figures 2 and 3). Instead 2 moles of m^2G/mole tRNA were formed (Table II); one of them at position G6. This G6 modification has been found in several tRNAs from higher eukaryotes but does not occur naturally in yeast tRNAs (25). The other mole of m^2G was formed at position 26 (T2 data). Thus the oocytes contain an enzyme activity that in contrast to the yeast cells is able to modify G26 in wt-like yeast tRNA^asp to N^2-monomethyl-G26. However, this monomethylation was abolished if an extra C
Table II. Levels of modifications in mutants of yeast tRNA<sup>Asp</sup> and tRNA<sup>Phe</sup> after microinjection of tRNAs into <i>X. laevis</i> oocytes

<table>
<thead>
<tr>
<th>Yeast tRNA variants</th>
<th>Mutations introduced</th>
<th>Modified guanosines formed (mole/mole tRNA)&lt;sup&gt;a&lt;/sup&gt; (Pos. 6 + 10 + 26)</th>
<th>m&lt;sup&gt;2&lt;/sup&gt;G&lt;sup&gt;b&lt;/sup&gt; (Pos. 26)</th>
<th>m&lt;sup&gt;3&lt;/sup&gt;G&lt;sup&gt;b&lt;/sup&gt; (Pos. 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>U1→G1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.0</td>
<td>0.9</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>A72→C72&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a209</td>
<td>+C47</td>
<td>1.1</td>
<td>0.1</td>
<td>0.0</td>
</tr>
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<td>U25→C25</td>
<td>1.8</td>
<td>0.8</td>
<td>0.1</td>
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<td>U25→C25</td>
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<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>+U47</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>G10→A10</td>
<td>1.4</td>
<td>0.4</td>
<td>0.0</td>
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<tr>
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<td>U11→C11</td>
<td>1.4</td>
<td>0.2</td>
<td>1.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>A24→G24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>U25→C25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a214</td>
<td>U11→C11</td>
<td>1.5</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>A24→G24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>U25→C25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+U47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a215</td>
<td>U11→C11</td>
<td>0.9</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>A24→G24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>U25→C25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+C47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Phe&lt;/sup&gt;</td>
<td></td>
<td>m&lt;sup&gt;2&lt;/sup&gt;G&lt;sup&gt;b&lt;/sup&gt; (Pos. 10 + 26) (Pos. 26)</td>
<td>m&lt;sup&gt;3&lt;/sup&gt;G&lt;sup&gt;b&lt;/sup&gt; (Pos. 26)</td>
<td></td>
</tr>
<tr>
<td>YFR0</td>
<td>wild type</td>
<td>0.8</td>
<td>n.d.</td>
<td>0.7</td>
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<tr>
<td>YF26</td>
<td>C25→U25</td>
<td>1.6</td>
<td>n.d.</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Accuracy ± 0.1. Duration of incubation in oocytes was 48 h at 19°C.
<sup>b</sup>Degradation with Nuclease P1.
<sup>c</sup>Degradation with RNase T2.
<sup>d</sup>All tRNA<sup>Asp</sup> variants carry these mutations.
<sup>e</sup>Variant a207 degraded by RNase T2 gave 0.8 m<sup>3</sup>G26 per mole tRNA, n.d.: not determined.

Residue was inserted into the variable loop (Table II, variant a209) and G26 was again essentially unmodified.

Changing U25 to C25 or G10 to A10 and in this way creating a Watson—Crick base pair in the D-stem did not lead to the recognition of G26 by the m<sup>2</sup>G26 methyltransferase for dimethylation (Table II, variants a200 and a204). The shift U25 to C25 in variant a200 introduced the hypothetical consensus base at position 25 (Table II), but this is not enough for creating even a partial recognition for the dimethylation enzyme.

When an extra uridine was inserted into the variable loop of mutant tRNA<sup>Asp</sup>a200, as in variant a210, the formation of m<sup>2</sup>G26 decreased from 0.8 to 0.5 moles/mole tRNA. This decrease was somewhat less drastic compared to when C was inserted in wt-like tRNA<sup>Asp</sup> (a209) in which only 0.1 mole m<sup>2</sup>G26/mole tRNA was formed (cf above and Table II).

Only when both base pairs 10—25 and 11—24 were exchanged into the hypothetical consensus sequence, as in mutant a207, did G26 become fully modified to dimethyl-G26. Furthermore, besides at position G6, this tRNA has now got an additional position for m<sup>2</sup>G-formation (1.4 moles/mole tRNA) presumably at position G10. Even after creating a 'consensus sequence' in the D-stem upstream of G26, as in tRNA<sup>Asp</sup>a207, the insertion of an additional nucleoside at position 47, U or C, in the extra loop, drastically reduced the potential of G26 to become dimethylated.

Yeast tRNA<sup>Phe</sup> contains the consensus sequence as we have defined it above and a change in this sequence should result in a decrease in the level of m<sup>2</sup>G26. When the m<sup>2</sup>G26 formation in wt tRNA<sup>Phe</sup> was measured it gave 0.7 mole m<sup>2</sup>G26 and 0.8 mole m<sup>2</sup>G per mole tRNA (Figure 3 and Table II). Already the exchange of one base, C25 for U25 (mutant YF26), in the consensus sequence of tRNA<sup>Phe</sup> resulted in a significantly less efficient dimethylation of G26. Instead the level of m<sup>2</sup>G doubled to 1.6 moles/mole tRNA forming m<sup>2</sup>G10 and m<sup>2</sup>G26 (Figures 2 and 3 and Table II).

**Kinetics of mono- and dimethyl-G26 formation in tRNA mutants**

Figure 4 shows TLC-analyses of RNase T2 degraded <sup>32</sup>P-GMP labelled tRNA from variant a207, a tRNA having the hypothetical D-stem consensus sequence and a 4-membered extra loop. The tRNA was recovered from oocytes after various intervals of time. Quantitation of radioactivity in m<sup>2</sup>G and m<sup>3</sup>G spots, arising exclusively from G26, revealed a two-step reaction mechanism with a fast initial monomethylation step followed by a slower formation of the final, dimethylated G26 product giving almost 1 mole m<sup>3</sup>G per mole tRNA in 24 hours. The nuclease P1 data in Figure 3 confirm the two-step reaction mechanism for dimethylation of G26 in both Asp- and Phe-specific tRNAs and that N<sup>2</sup>-monomethylation of other sites can occur independently and simultaneously to the mono- and dimethylation reactions on G26.

Besides N<sup>2</sup>-monomethylation at positions G6 and G26, the a207 variant exposed an additional site for N<sup>2</sup>-monomethylation of G. This site, presumably position G10, appeared in the kinetic experiments (Figure 3) as being slowly catalyzed and not yet fully modified even after 48 h.

**DISCUSSION**

Two D-stem base pairs are the only major sequence identity elements for the recognition between tRNA and oocyte dimethyl-G26-methyltransferase

Here we have provided evidence that the two bases pairs directly upstream of G26 in the D-stem in tRNAs are positive identity elements for the dimethyl-G26-methyltransferase present in the...
cytoplasm of X. laevis oocytes. Our conclusions on tRNA\textsuperscript{Asp} mutants and oocyte enzymes are valid only if the size of the extra loop is four nucleotides as in the original nuclear coded tRNA\textsuperscript{Asp}. It is the identity of the two basepairs C11-G24 and G10-C25 and not only the presence of Watson-Crick base pairs that are important for the m\textsubscript{2}G formation. All these four nucleotides must be present in the mutant yeast tRNA\textsuperscript{Asp} for this tRNA to function as substrate for the dimethylating oocyte enzyme; intermediate changes in the direction towards the consensus sequence does not lead to recognition for dimethylation. It is thus logical that a single exchange at position 25 in the tRNA\textsuperscript{Phe} caused a drastic decrease in the level of dimethylated G26 in this tRNA.

Insertion of a U or a C in position 47, but keeping the consensus sequence C11-G24, G10-C25 as in tRNA\textsuperscript{Phe} (see Figure 1 and Table II) completely abolished the potential of G26 to become dimethylated but also to an appreciable extent to become monomethylated. That the type and location of the nucleotides in the extra loop has a general effect on the 3D-structure of the tRNA molecule is supported by our data but is also based on the fact that they take part in several tertiary interactions (16, 17, 27), and that the insertion of U47 clearly alters the reactivity towards Pb\textsuperscript{2+}-catalyzed cleavages in the extra loop and the AC loop of tRNA\textsuperscript{Asp} (28). Thus efficient dimethylation of G26 is not only dependent on the presence of a set of identity nucleotides, but also on a precise conformation of the tRNA.

This conclusion is reminiscent of the one that is now prevailing for the recognition between tRNAs and most of the aminoacyl-tRNA-synthetases (20, 28, 29). Interestingly enough, while the insertion of U47 had almost no effect on the rate of asparylation \textit{in vitro} (28), the replacement of G10 by A10 considerably reduced the acceptor capacity of the tRNA\textsuperscript{Asp} (30).

Spatial arrangement of the identity elements is also important

A closer look at the differences in the conformation may explain the need for the G10-C25 and C11-G24 base pairs in order for G26 to become modified. We have compared the spatial arrangements of nucleotides 10, 11, 24, 25 and 26 in the crystal structure of tRNA\textsuperscript{Asp}, lacking the consensus sequence, with those in tRNA\textsuperscript{Phe}, that has the consensus sequence. Computer analyses (performed by E. Westhof, CNRS, Strasbourg, France) revealed that the 11-24 basepair and nucleotide 25 in the tRNA\textsuperscript{Asp} are easily superimposed on the 11-24 basepair and nucleotide 25 in the tRNA\textsuperscript{Phe} while G10 is not (cf Figure 1). In yeast tRNA\textsuperscript{Phe}, G10 is monomethylated and basepaired with C25. This m\textsubscript{2}G10 is better stacked on m\textsubscript{5}G26 than G10 on G26 in tRNA\textsuperscript{Asp}. Because it is opposite to U25, the G10 of tRNA\textsuperscript{Asp} has moved into the minor groove, thereby exposing its 2-amino group towards the solvent (20, 30). Conversely, the 2-amino group of G26 in tRNA\textsuperscript{Asp} is more buried than in yeast tRNA\textsuperscript{Phe}. Thus, the D-stem sequence in tRNA\textsuperscript{Asp} seems to influence the surface around G26 so that, compared to the case in tRNA\textsuperscript{Phe}, G10 has moved towards the exterior and G26 towards the interior, causing the 2-amino group of G26 to be more buried and then presumably less available for dimethylation. The exchange of basepairs in the D-stem of tRNA\textsuperscript{Asp} into the consensus ones should then lead to exposure of the 2-amino group of G26, expected to be manifested in the ability to form m\textsubscript{5}G26, an effect we have shown to occur.

Moreover, in tRNA\textsuperscript{Phe} the 2'-hydroxyl group of A9 possibly makes two hydrogen bonds; it receives one from N4 in C11 and gives one to N7 in G10. In yeast tRNA\textsuperscript{Asp} the same 2'-hydroxyl group of A9 can make only one hydrogen bond either to OH in U11 or to N7 of G10; on the basis of distances the latter one is the most probable one. Such complex stabilizations as well as the local geometry of the interplaying nucleosides within the consensus sequence might influence both the recognition between the tRNA and the methylating enzyme as well as how efficiently the methyl groups are transferred to the 2-amino group of G26.

In yeast as in other eukaryotic cells, N\textsubscript{2}-monomethylated G10, present in some tRNAs, is formed by an enzyme distinct from the G26 enzyme (31, 32). In the 3D-structure of yeast tRNA\textsuperscript{Phe} this G10 is very close to G26, giving a surface of the tRNA molecule at which two distinct N\textsubscript{2}-Guanosine-specific tRNA monomethyltransferases have to compete. From the present work
on mutant tRNA<sup>asp</sup> and in (15) it is clear that G10 methylation occurs at a slower rate and possibly after the methylation has occurred at position G26. Furthermore, our data indicate that the introduction of the consensus sequence for m<sub>2</sub>G26 into tRNA<sup>asp</sup> might have created a conformation that also allowed a very slow N<sup>2</sup>-monomethylation of G10, a modification normally not present in this tRNA.

**Mechanisms for G26-dimethylation in tRNAs**

In agreement with in vitro data on *Tetrahymena* modifying enzymes (33) our kinetic analyses of m<sub>2</sub>G26 methylation in vivo in *X. laevis* oocytes (cf Figures 3 and 4) show that the formation of dimethyl-G26 occurs in two steps with a fast initial monomethylation to m<sup>2</sup>G26. In our case the addition of the second methyl group, giving the final product m<sub>2</sub>G26, required other recognition parameters than the first step and occurred at a considerably slower rate. This lowered rate for dimethylation of G26 was found both for mutant tRNA<sup>asp</sup> and for wildtype tRNA<sup>pre</sup>, indicating that an inherent property of the oocyte system limited the rate, but not the yield, in the second dimethylation step. These results might be explained either by: (i) different physiological conditions are required for the two methylation steps; (ii) a dissociation step is needed between the two methyl transfers; (iii) two different enzymes are involved.

As in yeast cells the formation of dimethyl-G26 in oocytes (34) normally occurs in the nucleus. The majority of the G26 methylating enzyme molecules might therefore be present in the nucleus, giving a low level in the cytosol, the compartment in which we injected the tRNA. Thus the cytosol is presumably not a fully adequate environment for the tRNA(G26) modification reactions. Therefore the difference in mono- and dimethylation rates could be due to that the physiological conditions in the cytosol might be less favourable for N<sup>2</sup>-dimethylation than for N<sup>2</sup>-monomethylation of G26, although this explanation seems less probable.

The differences in rates between the mono- and the dimethylation reactions of G26 in tRNAs could indicate that the tRNA molecule has to dissociate from the monomethylating activity before performing the dimethylation step. Such a dissociation might be necessary for the complex to exchange the S-adenosylhomocysteine for new substrate molecules of S-adenosylmethionine or ATP. A dissociation might also be necessary for moving the enzyme if the second step requires another domain of the enzyme to be exposed to the tRNA. The rate difference could also imply that the two sequential reactions are catalyzed by two different enzymes.

In the yeast cell the G26 in wild-type nuclear coded tRNA<sup>asp</sup> is not modified at all. When this tRNA was injected into the cytoplasm of *X. laevis* oocytes (this work) or incubated with mouse and rat extracts (35), it became monomethylated to m<sup>2</sup>G26. The reason for this relaxed specificity in the heterologous reactions between yeast tRNA<sup>asp</sup> and enzymes from higher eukaryotes might be that the enzyme(s) of such cells have a N<sup>2</sup>-monomethyl-G26-methyltransferase activity with less or other requirements for recognition than the dimethyating activity. In *Saccharomyces cerevisiae* the *TRM1* gene encodes a 63 kDa protein able to catalyze both the mono- and dimethylation steps when forming m<sub>2</sub>G. This was shown both in vitro (13) and in vivo where the cloned yeast *TRM1* gene gave m<sub>2</sub>G in *E. coli* tRNA, normally completely devoid of this modification (36). The possible presence in higher eukaryotes of a similar enzyme capable to both mono- and dimethylate G26 in tRNAs does not, however, exclude the simultaneous presence of an enzyme that exclusively N<sup>2</sup>-monomethylates G26.

In conclusion the present work has for the first time shown parameters in tRNAs that are important for the correct interaction between tRNAs and G26-methlyating enzymes. This knowledge is the basis for our continued work, aiming at a fuller understanding of this kind of tRNA-protein interactions where one protein is able to recognize a selected set of tRNA species as its substrates.

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