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Structural Elements in Yeast tRNAs Required for Homologous Modification of Guanosine-26 into Dimethylguanosine-26 by the Yeast Trm1 tRNA-Modifying Enzyme†

Johan Edqvist, Kristina Blomqvist, and Kerstin B. Stråby*

Department of Microbiology, University of Umeå, S-90187 Umeå, Sweden

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ABSTRACT: In eukaryotic tRNAs, guanosines in position 26 (G26), located at the junction between the D-stem and the anticodon stem of tRNA, are usually modified to N^2,N^2-dimethylguanosine (m_2^2G). Although G26 is a prerequisite for biosynthesis of m_2^2G26, it is not self-sufficient for the formation of the dimethylated G26, since in exceptional cases eukaryotic tRNAs have an unmodified G26. In the yeast *Saccharomyces cerevisiae* the only tRNA species with an unmodified G26 is $tRNA^{Asp}$. Using *in vitro* transcripts of this tRNA, as well as of yeast $tRNA^{Phe}$, a tRNA containing m_2^2G26 *in vivo*, we have investigated the requirements on tRNA sequences and structures for the formation of m_2^2G26 by the yeast enzyme, i.e. in a homologous *in vitro* system. We have now demonstrated that G26 was efficiently dimethylated *in vitro* also after deletion of the entire anticodon stem and loop. We conclude that the elements necessary for a productive interaction between G26 in nuclear coded yeast tRNAs and the yeast G26 modifying enzyme are located within the core of the tRNA. For modification of G26 to m_2^2G26 via monomethylated G26, important primary and secondary structural elements in the tRNAs are a size of at least five nucleotides in the variable loop together with two G–C base pairs in the D-stem. This is the first case reported where the minimal requirements on nuclear coded tRNAs for a yeast modifying enzyme has been elucidated.

Transfer RNA (tRNA) interacts with a large number of proteins such as processing enzymes, modifying enzymes, aminoacyl-tRNA synthetases, and translation factors. Recently, much progress has been achieved in elucidating the interaction between the tRNAs and their cognate aminoacyl-tRNA synthetases (reviewed in Giegé et al., 1993, and McClain, 1993). In essence, the aminoacyl-tRNA synthetases require a rather small set of specific nucleotides at specific sites in the tRNAs. These identity elements often include the anticodon bases, the discriminator base at position 73 and/or bases in the acceptor stem (Rould et al., 1991; Cavarelli et al., 1993). Furthermore, the tRNA tertiary structure is often required to be intact to allow the correct presentation of the identity elements to the aminoacyl-tRNA synthetase (Sampson et al., 1992; Perret et al., 1992).

At present, less is known about how tRNA is recognized by and interacts with the tRNA-modifying enzymes. Since most tRNA-modifying enzymes catalyze the formation of one specific modified base at its specific site in a large variety of tRNA species, the modifying enzymes must recognize determinants shared by many tRNAs. Thus, besides requiring the specific target base, a modifying enzyme might primarily recognize certain structural features within the tRNA. An intact tRNA tertiary structure is needed for optimal formation of 1-methylguanosine-37 (m^1G37) (Holmes et al., 1992; Edqvist et al., 1993), of queuosine-34 (Q34), of pseudouridylate-40 ($\Psi 40$) (Edqvist et al., 1993), and of inosine-34 (I34) (Achsel & Gross, 1993). The presence and structure of an intron located next to the anticodon are a necessity for the formation of $\Psi 35$ in the middle of the anticodon of the eukaryotic $tRNA^{Tyr}$ (Johnson & Abelson, 1983; Choffat et al., 1988; van Tol & Beier, 1988; Szweykowska-Kulinska & Beier, 1992). On the other hand, fragments of tRNAs can also function as substrates for some modifying enzymes as reported for the bacterial tRNA-guanine transglycosylase (Curnow et al., 1993) and tRNA(m^5U54)methyltransferase (Gu & Santi, 1991). Evidently the principles behind the recognition between tRNA and different modifying enzymes are very diverse.

In eukaryotes, the modified base N^2,N^2-dimethylguanosine (m_2^2G) is found in almost all tRNAs having a guanosine at position 26 (G26) at the junction between the D-stem and the anticodon stem. The *TRM1* gene, encoding the tRNA-modifying enzyme N^2,N^2-dimethylG26-methyltransferase (Trm1p), has been cloned from the yeast *Saccharomyces cerevisiae* (Ellis et al., 1987). The Trm1p catalyzes the transfer of both methyl groups to G26 during the synthesis of m_2^2G26, shown by expressing *TRM1* in *Escherichia coli*, normally devoid of m_2^2G26 in its tRNA (Ellis et al., 1986). *TRM1* is responsible for the modification of G26 to m_2^2G26 in both nuclear and mitochondrial coded tRNAs in yeast (Hopper et al., 1982), and the Trm1p is known to be transported into the yeast nucleus as well as into the yeast mitochondria (Rose et al., 1992).

The ability of nucleosides at different positions in synthetic yeast tRNAs to be modified can be tested by microinjection of the tRNAs into *Xenopus laevis* oocytes. This technique has been successfully used for elucidating both processing of pre-tRNAs (Melton et al., 1980) and for heterologous modification of yeast tRNAs (Droogmans & Grosjean, 1987; Grosjean et al., 1990). In this cellularly intact system with all required cofactors present, it was shown that G26, normally unmodified in yeast $tRNA^{Asp}$, becomes monomethylated to N^2-methylguanosine (m^2G26) (Edqvist et al., 1992). For adding the second methyl group to the monomethylated G26 forming m_2^2G26, the enzyme present in *X. laevis* oocytes

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* Corresponding author. Tel: +46-90-102485; Fax: +46-90-112630.

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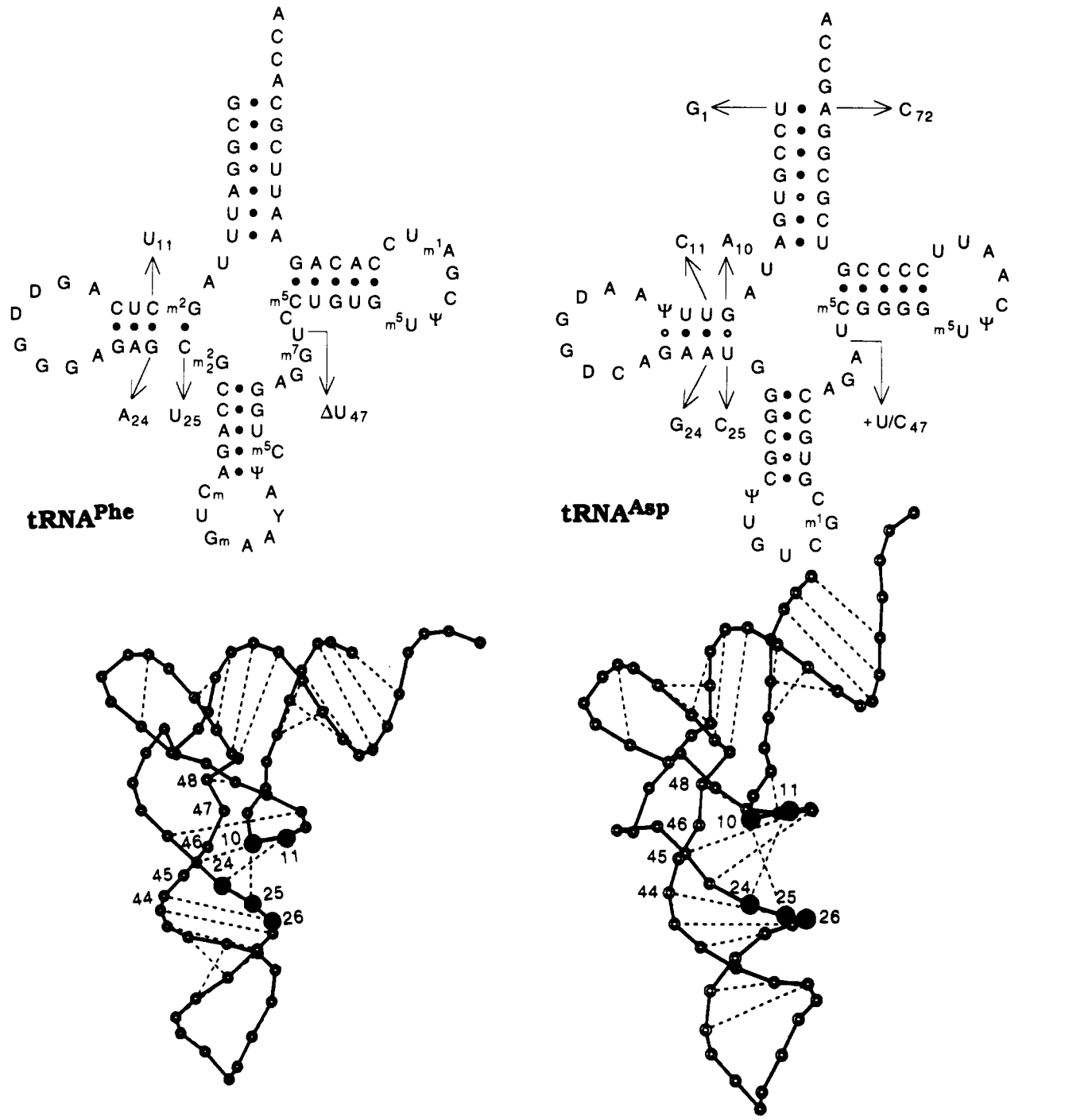

FIGURE 1: Cloverleaf models with modified nucleosides and tertiary structure models of yeast $tRNA^{Asp}$ (Gangloff et al., 1971) and yeast $tRNA^{Phe}$ (Holbrook et al., 1978). Arrows indicate mutated positions.

requires that G26 is preceded by two G–C pairs in the D-stem (Edqvist et al., 1992). Since these studies clearly indicated a difference between the requirements by modifying enzymes from oocytes and from yeast cells on the tRNA for the methylation of G26, we decided to elucidate what parameters that are of importance in a truly homologous system. Here we present our studies on yeast tRNAs and their interactions with the yeast tRNA-modifying enzyme N^2,N^2-dimethylG26-methyltransferase (Trm1p).

For both yeast $tRNA^{Asp}$ and the m_2^2G26-containing yeast $tRNA^{Phe}$ (Figure 1) there is a wealth of information concerning their tertiary structures (Holbrook et al., 1978; Westhof et al., 1985; Romby et al., 1987). Their identity elements, ensuring specific aminoacylation, have also been identified (Sampson et al., 1992; Pütz et al., 1993). We have chosen these two yeast tRNAs as substrates in this study, where we for the first time have identified those structural features in nuclear coded yeast tRNAs that are important for the formation of m_2^2G26 by the yeast tRNA-modifying enzyme dimethylG26-methyltransferase.

EXPERIMENTAL PROCEDURES

DNA Preparations, Site-Directed Mutagenesis, and Preparation of Labeled tRNA Transcripts. Twelve new synthetic genes encoding $tRNA^{Asp}$ variants (+U47; C11; C11, +U47; G24; G24, +U47; C11, G24; C11, G24, +U47; A10, +U47; A10, +C47; C11, C25; G24, C25; G24, C25, +U47) were made using site-directed mutagenesis of single-stranded DNA according to the "*Oligonucleotide-directed in vitro mutagenesis system, version 2.1*" from Amersham, UK (P1/

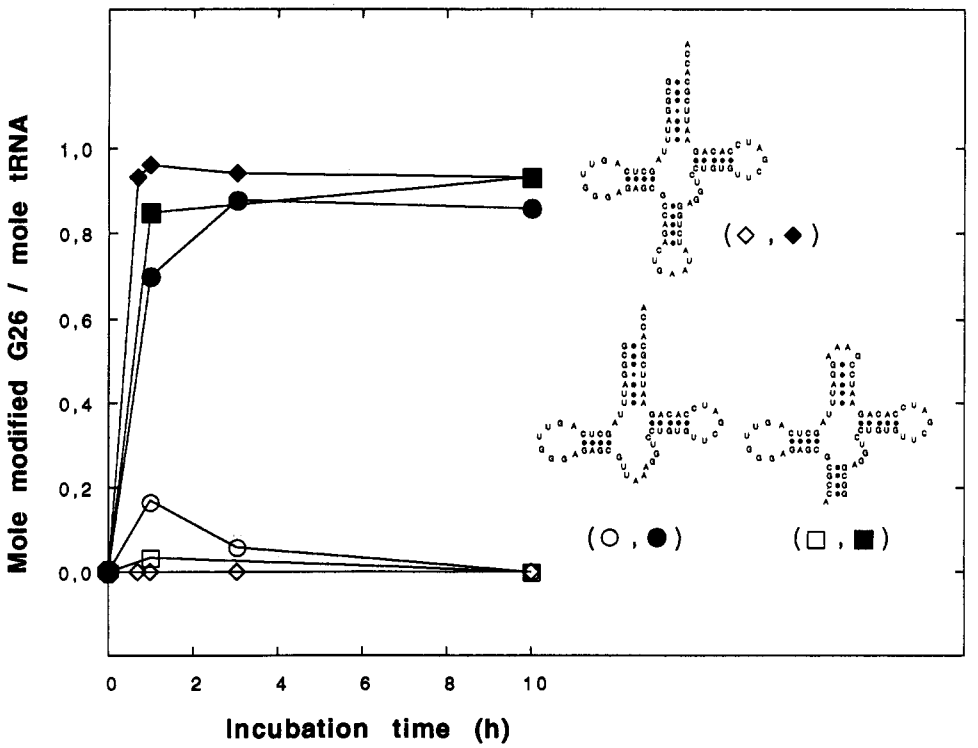

FIGURE 2: Kinetics for the formation of m^2G26 (open symbols) and 26 (filled symbols) in yeast $tRNA^{Phe}$ wt (◇) and in two yeast $tRNA^{Phe}$ variants. In one variant (○) the anticodon hairpin is replaced with a tetraloop (UUAA). The other (□) is circularly permuted with its 5′ and 3′ ends in the anticodon stem and the acceptor stem replaced with a five base pair hairpin with a stable GAAA loop (Dichtl et al., 1993). Monomethylated G26 was not detected in $tRNA^{Phe}$ wt.

290/91/4). In all the $tRNA^{Asp}$ variants used in this study, the base pair U1–A72 has been exchanged for G1–C72 as U1 is known to be an unfavorable start for the T7 RNA polymerase (Perret et al., 1990).

Three new variants of synthetic yeast $tRNA^{Phe}$ genes (U11, A24, U25; ΔU47; U11, A24, U25, ΔU47) were constructed with the Transformer site-directed mutagenesis kit from Clontech, USA, based on the method developed by Deng and Nickoloff (1992). Large-scale and small-scale plasmid preparations were done according to standard procedures (Sambrook et al., 1989). Preparation of ^{32}P-G labeled tRNA transcripts with T7 RNA polymerase has been previously described (Edqvist et al., 1992).

Modification of tRNA and Analysis of Modified Nucleotides. Yeast strain *S. cerevisiae* DB745 (*his*4, *leu*2-3, *ura*3, *ade*2-1) carrying plasmid pGT554, which contains the yeast N^2,N^2-dimethylG26-methyltransferase gene *TRM1* linked behind the inducible *GAL1* promoter (Li et al., 1989), was grown in YPGD (galactose) medium. When cells reached late logarithmic growth phase, cells were harvested by centrifugation and crude extracts were prepared in NET-NP buffer as previously described (Li et al., 1989). The crude extracts were dialyzed overnight in NET-NP buffer. After addition of glycerol, the methylase extracts were stored at −20 °C. Overexpression of the *TRM1* gene was shown not to interfere with the presence and activity of other tRNA-modifying enzymes in the extract.

The methylation of tRNA transcripts was mainly according to Kjellin-Stråby and Boman (1965). A 20 μL reaction mixture containing 4 μL buffer mix (0.5 M Tris-HCl, pH 8.0, 5 mM DTT, 0.5 mM EDTA, 50 mM $MgCl_2$, 0.1 M NH_4Cl), 0.5 μL yeast crude extract, 1 μL *S*-adenosyl-L-(*carboxyl*-^{14}C)methionine (59.5 mCi/mmol, 0.025 mCi/mL, Amersham, UK), and 10^6 cpm ^{32}P-G labeled tRNA transcripts was incubated at 30 °C. The radiolabeled *S*-adenosylmethionine had higher chemical purity and was therefore used as the methyl donor instead of an unlabeled variant. After the incubation was completed, the reaction mixture was phenol extracted and ethanol precipitated. Full-length tRNA was isolated by PAGE, eluted, and then hydrolyzed completely with P1 nuclease (Edqvist et al., 1992). The presence of modified nucleotides was analyzed by 2D-TLC (Nishimura, 1979). The level of modified bases was determined by liquid scintillation techniques or by the use of a phosphor imager (Molecular Dynamics, USA) supplied with the ImageQuant software.

Each tRNA variant was tested in several independent methylation assays. Depending on the yeast extract preparation, the extent of methylation varied in absolute values up to 0.1 mol/mol of tRNA. The data in Figures 2 and 3 are all from experiments done with the same extract preparation.

RESULTS

The base substitutions, insertions, and deletions shown in Figure 1 were introduced into synthetic tRNA genes encoding yeast $tRNA^{Asp}$ and yeast $tRNA^{Phe}$. These genes were transcribed with T7 RNA polymerase to give completely unmodified, ^{32}P-G-labeled transcripts of a length known to function as efficient substrates for modification enzymes (Phillips & Kjellin-Stråby, 1967; Grosjean et al., 1990). The transcripts were incubated in extracts prepared from yeast cells that strongly overexpress the N^2,N^2-dimethylG26-methyltransferase from a *TRM1* gene fused to the inducible *GAL1* promoter on a high copy vector (Li et al., 1989). The formation of tRNA modifications on variants of yeast $tRNA^{Asp}$ and yeast $tRNA^{Phe}$ was analyzed by two-dimensional thin-layer chromatography (2D-TLC) (Nishimura, 1979).

The Anticodon Stem and Loop Is Dispensable for the Dimethylation of G26. In the tRNA, position 26 immediately precedes the anticodon stem and loop which, due to its nearness to G26, might be essential for the formation of m_2^2G26. Two

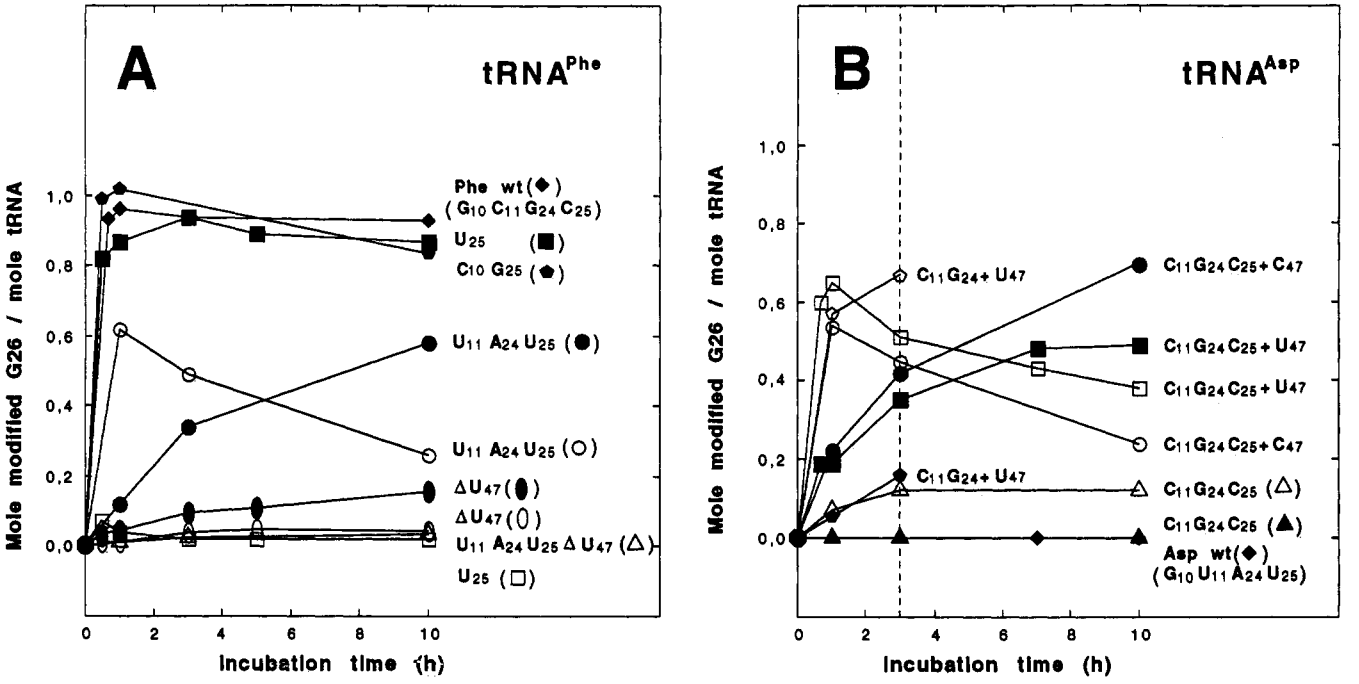

FIGURE 3: Formation of m^2G26 (open symbols) and m_2^2G26 (filled symbols) in variants of $tRNA^{Phe}$ (A) and $tRNA^{Asp}$ (B) with mutations in the D-stem and/or the variable loop. No monomethylated G26 was detected in $tRNA^{Phe}$ wt and in $tRNA^{Phe}$ (C10G25). m_2^2G26 was not detected in $tRNA^{Phe}$ (U11A24U25ΔU47) and in $tRNA^{Asp}$ (C11G24C25). Neither m^2G26 nor m_2^2G26 was detected in $tRNA^{Asp}$ wt. The vertical line indicates the incubation times for the data given in Table 1 (lines 1, 9, 18, 19, and 20).

variants of $tRNA^{Phe}$ that lack either the anticodon loop or the entire anticodon stem and loop (Figure 2) are known to fold properly in the core as shown by the tertiary structure specific Pb^{2+} cleavage in the D-loop (Dichtl et al., 1993). These tRNAs were therefore suitable substrates for testing if the anticodon hairpin comprises any determinants for dimethylation of G26.

Using the *in vitro* transcript corresponding to the wild-type (wt) yeast $tRNA^{Phe}$ as substrate in our *in vitro* system, the dimethylation was efficient and after 30 min, G26 was fully modified to m_2^2G26 without any detectable trace of the intermediate m^2G26 (Figure 2). The two short $tRNA^{Phe}$ variants were also efficiently dimethylated at G26 (Figure 2), although the rates of dimethylation were somewhat reduced compared to wt $tRNA^{Phe}$. This rate reduction was also evident by the demonstration of the transient appearance of the intermediate m^2G26. Thus, interestingly and quite unexpectedly, the anticodon hairpin is not required for correct presentation of G26 to the N^2,N^2-dimethyl-G26-methyltransferase, and the productive interaction between the tRNA and the Trm1p does not depend on an intact tRNA. The determinants for the N^2,N^2-dimethylG26-methyltransferase must therefore be located within the tRNA core.

The Presence of G–C Base Pairs in the D-Stem Is Not Sufficient for G26 Modification into m_2^2G26. In order to interfere with the efficient dimethylation of wt $tRNA^{Phe}$, the D-stem base pair G10–C25, which is the closest neighbor to G26, was inverted to C10–G25. This inversion did not interfere with either the level or the rate of dimethylation of G26 (Figure 3A). After substituting C25 with U25 to obtain a G10–U25 base pair as found in $tRNA^{Asp}$, m_2^2G26 was still formed but at a slightly reduced rate. This was shown by the transient appearance of low amounts of the intermediate m^2G26 (Figure 3A). An additional mutation was also introduced in the D-stem of the G10–U25 mutant by exchanging the neighboring C11–G24 base pair for U11–A24. In this $tRNA^{Phe}$ variant (U11A24U25) 7 out of 8 bases in the D-stem were identical to those present in $tRNA^{Asp}$, in which G26 is not modified. Even this tRNA variant was recognized as a substrate by the yeast N^2,N^2-dimethylG26-methyltransferase, although the rate of dimethylation was significantly lower compared to the wild-type case (Figure 3A). In summary, the results show that the base pairs C11–G24 and G10–C25 present in yeast $tRNA^{Phe}$, when exchanged for other base pairs, still allow G26 to be modified, although both the rate and the level of methylation were influenced.

In wt yeast $tRNA^{Asp}$, G26 was, as expected, not methylated in our assay using yeast modifying enzymes (Figure 3B and line 1 in Table 1). Several mutations were introduced into the D-stem of $tRNA^{Asp}$ (Figure 1, Table 1) in order to investigate what mutations that would create a tRNA with recognition for G26-dimethylation within the $tRNA^{Asp}$ frame. Small amounts of m^2G26 (0.03–0.1 mol/mol of tRNA) but no m_2^2G26 was formed when one G–C base pair was introduced in the D-stem either at positions 10–25 (lines 13 and 16 in Table 1) or m_2^2G26 was 11–24 (line 8 in Table 1). The simultaneous introduction of G–C base pairs in both positions 10–25 and 11–24 in the D-stem did not significantly change the low level of modification of G26 (Figure 3B and line 18 in Table 1). Thus, in sharp contrast to previous results from the *X. laevis* system, the introduction of two G–C base pairs in the D-stem in yeast $tRNA^{Asp}$ is not sufficient for the modification of G26 to m_2^2G26 by the yeast N^2,N^2-dimethylG26-methyltransferase. Thus, the yeast Trm1p has additional requirements on the tRNA besides the two G–C base pairs in the D-stem.

Dimethylation of G26 Requires More Than Four Bases in the Variable Loop. In $tRNA^{Asp}$, there are only four bases in the variable loop, while in $tRNA^{Phe}$ there are five. The effect of the size of this loop on the methylation of G26 was investigated by combining the earlier introduced D-stem mutations with mutations in the variable loop.

In $tRNA^{Asp}$, the variable loop was increased to the size present in wt $tRNA^{Phe}$ by inserting a U or C at position 47 (Figure 1, Table 1). G26 was not modified when a five-

Table 1: Levels of m^2G26 and m_2^2G26 (mol/mol of tRNA) in Yeast $tRNA^{Asp}$ Variants after 3 h of Incubation in Yeast Extracts

D-stem mutations	variable loop mutation	m^2G26	m_2^2G26	line
	U11–A24 and G10–U25			
wt	wt	<0.01	<0.01	1
	+U47	<0.01	<0.01	2
	+C47	<0.01	<0.01	3
	C11–A24 and G10–U25			
C11		<0.01	<0.01	4
C11	+U47	<0.01	<0.01	5
	U11–G24 and G10–U25			
G24		0.03	<0.01	6
G2	+U47	<0.01	<0.01	7
	C11–G24 and G10–U25			
C11, G24		0.1	<0.01	8
C11, G24	+U47	0.7	0.2	9
	U11–A24 and A10–U25			
A10		<0.01	<0.01	10
A10	+U47	<0.01	<0.01	11
A10	+C47	<0.01	<0.01	12
	U11–A24 and G10–C25			
C25		0.04	<0.01	13
C25	+U47	0.1	<0.01	14
	C11–A24 and G10–C25			
C11, C25		<0.01	<0.01	15
	U11–G24 and G10–C25			
G24, C25		0.1	<0.01	16
G24, C25	+U47	0.1	<0.01	17
	C11–G24 and G10–C25			
C11, G24, C25		0.1	<0.01	18
C11, G24, C25	+U47	0.4	0.4	19
C11, G24, C25	+C47	0.4	0.4	20

membered variable loop was combined with either the original D-stem of $tRNA^{Asp}$ (lines 2 and 3 in Table 1), a D-stem with G10–U25 changed into A10–U25 (lines 11 and 12 in Table 1), or a D-stem containing a C11–A24 mismatch (line 5 in Table 1) whereas slight modification to m^2G26 was seen when combined with a G10–C25 base pair (lines 14 and 17 in Table 1). However, when the insertion in the variable loop was combined with C11–G24 in the D-stem (Figure 3B and line 9 in Table 1), most of the Gs in position 26 were modified to m^2G26 after 3 h of incubation, while the end product m_2^2G26 appeared only slowly. When G10–U25 was changed into a G–C pair (Figure 3B and line 19 in Table 1), giving two G–C base pairs in the D-stem, the rate of dimethylation was improved. Insertion of C47 (Figure 3B and line 20 in Table 1) instead of U47 further improved both the level and the rate of dimethylation so that the sum of dimethylated and monomethylated G26 was close to 1 mol/mol of tRNA already after 1 h of incubation. Thus, the elements that need to be introduced into $tRNA^{Asp}$ to obtain dimethylation of G26 are two G–C base pairs in the D-stem in combination with an insertion of an extra base, preferentially a C, at position 47 in the variable loop.

To test the importance of the loop size for the dimethylation of G26 also within the $tRNA^{Phe}$ frame, one of the five bases in the variable loop in $tRNA^{Phe}$ was deleted. We chose to delete U47, because this base is the only one of the five bases in the variable loop that is not involved in tertiary interactions. In this mutant (ΔU47), m_2^2G26 was formed at a drastically reduced rate and after 10 h of incubation only 0.2 mol of m_2^2G26/mol of tRNA had been formed (Figure 3A). The deletion of U47 in the variable loop of $tRNA^{Phe}$ was also combined with several D-stem mutations. In the most $tRNA^{Asp}$-like mutant (U11A24U25ΔU47), no m_2^2G26 was detected and only trace amounts of the intermediate m^2G26 were present (Figure 3A). In conclusion, data from experiments with $tRNA^{Phe}$ as well as with $tRNA^{Asp}$ clearly show that the size of the variable loop and the D-stem sequence are the elements within yeast tRNA molecules that are the most important requirements for dimethylation of G26 catalyzed by the yeast tRNA-modifying enzyme N^2,N^2-dimethylG26-methyltransferase.

DISCUSSION

In eukaryotic tRNAs, G26 is, with few exceptions, modified to m_2^2G26. The only yeast tRNA known to lack this G26-modification is $tRNA^{Asp}$, which then has an unmodified G26 (Sprinzl et al., 1991). In this study, we have shown that mutations had to be introduced in both the D-stem and the variable loop of $tRNA^{Asp}$ to obtain dimethylation of the normally unmodified G26 by the yeast N^2,N^2-dimethylG26-methyltransferase. By stepwise changing the 10–25 and 11–24 base pairs in the D-stem and the size of the variable loop, the level of modification of G26 was successively increased. In the most efficiently dimethylated $tRNA^{Asp}$-variants the base pairs G10–U25 and U11–A24 had been changed into G–C base pairs and a U or a C had been inserted in the variable loop at position 47. In $tRNA^{Phe}$, where G26 normally is modified, an exchange of its two corresponding G–C base pairs together with a decrease of the variable loop to four bases consequently leads to that G26 becoming inaccessible for methylation. We therefore conclude that these two base pairs together with the size of the variable loop are very important for the ability of the yeast Trm1p to interact with its substrate.

In $tRNA^{Asp}$, all four bases in the variable loop are involved in tertiary interactions. Changing the size of this loop by inserting U47 affects the tertiary structure of this tRNA, as shown by an altered cleavage by Pb^{2+} (Perret et al., 1992). In our case mutations in the D-stem of $tRNA^{Asp}$ had to be combined with insertions in the variable loop to influence the methylation of G26, indicating that our results are most likely also due to a change in the tRNA tertiary structure rather than to a changed identity of specific bases in the tRNA sequence. Moreover, the N^2,N^2-dimethylG26-methyltransferase does not seem to make base-specific contacts with the D-stem base pair G10–C25, since the dimethylation of G26 in $tRNA^{Phe}$ was insensitive to the inversion of G10–C25 to C10–G25. The target for the methylation, the 2-amino group of G26, is known to be more exposed in $tRNA^{Phe}$ than in $tRNA^{Asp}$ (Edqvist et al., 1992). Thus, mutations introduced in the D-stem and in the variable loop of $tRNA^{Asp}$ have presumably led to an altered orientation of the 2-amino group of G26 and thereby influenced the susceptibility of this group for dimethylation. We conclude that the N^2,N^2-dimethylG26-methyltransferase does not act via base-specific contacts either with the inserted U/C47 or with the D-stem base pairs, but that the mutations in the D-stem and in the variable loop of $tRNA^{Asp}$, in a cooperative manner, alter the tRNA structure and thereby improve the interaction between $tRNA^{Asp}$ and the N^2,N^2-dimethylG26-methyltransferase.

All known nuclear coded yeast tRNAs with dimethylated G26 have G–C base pairs in positions 10–25 and 11–24 in the D-stem as well as at least five bases in the variable loop (cf. Edqvist et al., 1992; Sprinzl et al., 1991). This does not, however, exclude that the structural requirements for dimethylation of G26 might be fulfilled by other combinations of bases than those we have identified here. Such an assumption gains support from the sequence of the yeast mitochondrial $tRNA^{Gly}$ that has a dimethylated G26, although

it lacks two G–C base pairs in the D-stem and has only four bases in the variable loop (Sibler et al., 1986). To our knowledge this is the only clear exception to the requirement we have identified for G26-dimethylation of yeast tRNAs. Structural studies of the yeast mitochondrial $tRNA^{Gly}$ might reveal whether the orientation of the 2-amino group in this tRNA resembles the orientation seen in other yeast tRNAs with a dimethylated G26, such as $tRNA^{Phe}$.

As shown, both the D-stem and the variable loop of yeast $tRNA^{Asp}$ had to be changed to obtain dimethylation of G26 using a yeast enzyme. This is an important finding when considering that both these regions are very characteristic and functionally significant for the $tRNA^{Asp}$. The aspartyl-tRNA synthetase (AspRS) interacts with the ribose and the phosphate groups of U11 and U12 in the D-stem (Cavarelli et al., 1993) and the D-stem base pair G10–U25 is a necessary identity element in the aspartylation reaction (Pütz et al., 1991), although this base pair does not make direct contact with AspRS (Cavarelli et al., 1993). Furthermore, G10–U25 is involved in a tertiary interaction with G45 in the variable loop. This triple interaction, or possibly the G–U pair itself, may govern the conformation of the tRNA to permit the optimal presentation of the other identity elements in the anticodon and acceptor stem needed for aminoacylation (Pütz et al., 1993; Cavarelli et al., 1993). Thus, the D-stem so characteristic for $tRNA^{Asp}$ has been well exploited by the aspartyl-tRNA synthetase.

To ensure specificity in the aminoacylation reaction, the interactions between a tRNA and its cognate aminoacyl-tRNA synthetase focus on the uniqueness of the tRNA. The tRNA-modifying enzymes, on the other hand, that catalyze the formation of modified bases such as m_2^2G26, found in several different tRNAs, must by necessity recognize elements shared by many tRNAs. Hence, properties required for the unique function of one specific tRNA might prevent this tRNA from being recognized by a modification enzyme. When $tRNA^{Asp}$ became recognized as a substrate for the modification enzyme N^2,N^2-dimethylG26-methyltransferase, as a result of the introduction of G–C base pairs in the D-stem and of an extra base in the variable loop, the tRNA at the same time lost unique structural properties required for the aspartylation reaction (Pütz et al., 1991). Seemingly, the necessity to create and preserve the $tRNA^{Asp}$ identity in the aminoacylation reaction is not compatible with the property of having a modified G26. The chemical and biological effects of this natural lack of a modified G26 in $tRNA^{Asp}$ have to await further investigations aiming at understanding the need and function of m_2^2G26.

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