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Structural requirements for enzymatic formation of threonylcarbamoyladenosine (t^6A) in tRNA: An in vivo study with *Xenopus laevis* oocytes

ANNIE MORIN,[1] SYLVIE AUXILIEN,[1,4] BRUNO SENGER,[2,5] RAVINDRA TEWARI,[3] and HENRI GROSJEAN[1]

[1]Laboratoire d'Enzymologie et Biochimie Structurales, C.N.R.S., 1, av. de la Terrasse, F-91198 Gif-sur-Yvette, France
[2]Institut de Biologie Moléculaire et Cellulaire, Université de Strasbourg, F-67084, France
[3]Physical Chemistry Division, National Chemical Laboratory (C.S.I.R.), Pune 411008, India

ABSTRACT

We have investigated the specificity of the eukaryotic enzymatic machinery that transforms adenosine at position 37 (3′ adjacent to anticodon) of several tRNAs into threonylcarbamoyladenosine (t^6A_{37}). To this end, 28 variants of yeast initiator $tRNA^{Met}$ and yeast $tRNA^{Val}$, devoid of modified nucleotide, were produced by in vitro transcription with T_7 polymerase of the corresponding synthetic tRNA genes and microinjected into the cytoplasm of *Xenopus laevis* oocytes. Threonylcarbamoyl incorporation was analyzed in tRNA transcripts mutated in the anticodon loop by substitution, deletion, or insertion of nucleotides, or in the overall 3D structure of the tRNA by altering critical tertiary interactions. Specifically, we tested the effects of altering ribonucleotides in the anticodon loop, changes of the loop size, perturbations of the overall tRNA 3D structure due to mutations disruptive of the tertiary base pairs, and truncated tRNAs. The results indicate that, in addition to the targeted A_{37}, only U_{36} was absolutely required. However, A_{38} in the anticodon loop considerably facilitates the quantitative conversion of A_{37} into t^6A_{37} catalyzed by the enzymes present in *X. laevis*. The anticodon positions 34 and 35 were absolutely "neutral" and can accept any of the four canonical nucleotides A, U, C, or G. The anticodon loop size may vary from six to eight nucleotides, and the anticodon stem may have one mismatch pair of the type A∗C or G∗U at location 30–40 without affecting the efficiency of t^6A_{37} formation and still t^6A_{37} is efficiently formed. Although threonylcarbamoylation of A_{37} occurred with tRNA having limited perturbations of 3D structure, the overall L-shaped architecture of the tRNA substrate was required for efficient enzymatic conversion of A_{37} to t^6A_{37}. These results favor the idea that unique enzymatic machinery located in the oocyte cytoplasm catalyzes the formation of t^6A_{37} in all $U_{36}A_{37}$-containing tRNAs (anticodon NNU).

Microinjection of the yeast $tRNA^{Meti}$ into the cytoplasm of *X. laevis* oocytes also revealed the enzymatic activities for several other nucleotide modifications, respectively m^1G_9, m^2G_{10}, $m^2_2G_{26}$, m^7G_{46}, D_{47}, $m^5C_{48/49}$, and m^1A_{58}.

Keywords: anticodon; identity elements; initiator $tRNA^{Met}$; maturation; microinjection; modifying enzymes; threonylcarbamoyl-adenosine; *Xenopus laevis*

INTRODUCTION

Upon transcription, the primary transcripts are subjected to a series of posttranscriptional maturation steps, such as trimming of 3′ and 5′ ends, addition of the CCA 3′-end, modifications of several ribonucleotides at the level of the base and/or the 2′-hydroxyl of ribose, possible base conversion (editing), and intron splicing. These are catalyzed by a battery of so-called maturation enzymes. In eukaryotic cells, most of these enzymatic events occur in the nucleus and few of the final maturation steps take place in the cytoplasm and/or in mitochondria (for reviews see Hopper & Martin, 1992; Martin, 1995; Westaway, 1995).

Among the large variety of modified nucleotides that are formed during this complex multienzymatic maturation process (see in Limbach et al., 1994; Grosjean et al., 1995), formation of N^6-threonylcarbamoyl-adenosine (t^6A) is of special interest. Indeed, this hyper-

Reprint requests to: Annie Morin, Laboratoire d'Enzymologie et Biochimie Structurales, C.N.R.S., 1, av. de la Terrasse, F-91198 Gif-sur-Yvette, France; e-mail: morin@lebs.cnrs-gif.fr.

[4]Present address: Ecole Normale Supérieure de Lyon, Unité de Virologie Humaine, F-69364 Lyon, France.

[5]Present address: Biochemie Zentrum Heidelberg (BZH), im Neuenheimer Feld 328, D-69120 Heidelberg, Germany.

Abbreviations: TLC, thin-layer chromatography; N, any of the four canonical nucleotides; cpm, counts per minute. Symbols and common names of modified nucleotides from tRNA are those of Limbach et al. (1994). In particular, t^6A corresponds to *N*-[*N*-(9-(β-D-ribofuranosylpurin-6-yl)carbamoyl]L-threonine. The universal numbering system for tRNA positions corresponds to that of Sprinzl et al. (1996).

modified adenosine derivative is always located at position 37, adjacent to the 3′ side of anticodon, in almost every tRNA reading ANN codons (N being one of the four canonical nucleotides A, C, G, or U) (reviewed in Tsang et al., 1983; Björk, 1995a). In a few tRNAs, t^6A_{37} is further methylated on the N^6-carbamoyl group into N^6,N^6 methylthreonylcarbamoyl adenosine ($m^6t^6A_{37}$) and it may also contain a methylthio group in position 2 of the purine ring ($ms^2t^6A_{37}$) or a methyl group on the threonyl moiety (hnt^6A). Sometimes a glycyl group can be found in place of the threonyl group (g^6A_{37}) (see Fig. 1). Together with N^1-methylguanosine (m^1G), also found next to the 3′ end of the anticodon, t^6A (or its derivatives) is the only modified nucleotide that exists in tRNAs of all kingdoms: eubacteria, eukaryotic organisms, and archaebacteria; also in mycoplasma, mitochondria, and chloroplasts (Grosjean et al., 1995). Enigmatic exceptions are the initiator $tRNA^{Met}$(anticodon CAU) of prokaryotes, mitochondria, chloroplasts, and archaeabacteria harboring an unmodified A_{37} or m^1G_{37}, whereas the cytoplasmic initiator $tRNA^{Met}$ of eukaryotes and plants always contain t^6A_{37} (see in Sprinzl et al. However, when microinjected into the cytoplasm of *Xenopus laevis* oocyte, naturally unmodified A_{37} in *Escherichia coli* initiator $tRNA^{Met}$ became rapidly fully modified into t^6A_{37} (Grosjean et al., 1987). This suggests that a peculiarity of the enzymatic machinery, rather than the intrinsic structural property of the tRNA molecule itself, like a particular rigidity of the G-C rich anticodon stem (Schweisguth & Moore, 1997), should explain the lack of t^6A_{37} in naturally occurring prokaryotic, mitochondrial, and archaeal initiator $tRNA^{Met}$. The omnipresence of t^6A_{37} in most tRNAs harboring the anticodon NNU in all living cells suggests that this particular hypermodified nucleotide may play an essential role in tRNA functions (discussed in Weissenbach & Grosjean, 1981; Grosjean & Houssier, 1990; Björk, 1995b).

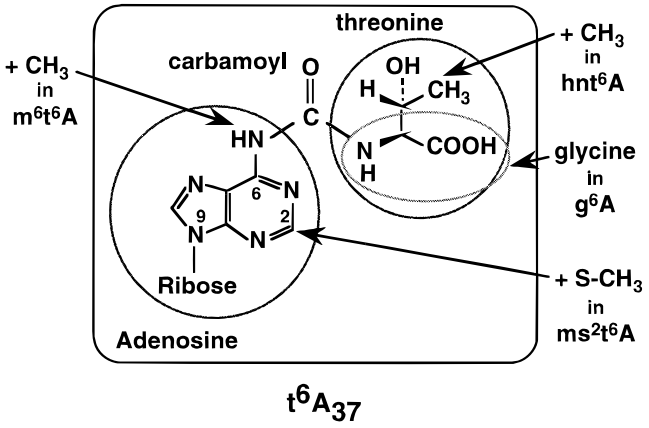

FIGURE 1. Schematic representation of N^6-threonylcarbamoyladenosine (t^6A) structure. This hypermodified nucleotide is always located at position 37 in almost every tRNA reading ANN codons (N = A, C, G, or U). In few tRNAs, t^6A_{37} is further methylated on the N^6-carbamoyl group to N^6,N^6 methylthreonylcarbamoyl adenosine ($m^6t^6A_{37}$). It may also contain a methylthio group in position 2 of the purine ring ($ms^2t^6A_{37}$) or a methyl group on the threonyl moiety (hnt^6A). Sometimes a glycyl group can be found in place of the threonyl group (g^6A_{37}) (see Sprinzl et al., 1996).

The biosynthesis of t^6A_{37} was initially studied in vivo (Chheda et al., 1972; Powers & Peterkofsky, 1972), showing that the side chain of t^6A is derived from L-threonine (Fig. 1). For in vitro studies, t^6A-deficient matured *E. coli* tRNAs were obtained from a threonine-starved culture of an *E. coli* strain (Elkins & Keller, 1974; Körner & Söll, 1974), whereas precursor tRNAs lacking all modified nucleotides are now currently obtained by in vitro transcription of natural tRNA genes (Zeevi & Daniel, 1976; Koski & Clarkson, 1982) or even by total chemical synthesis (Perreault et al., 1989; Bratty et al., 1990). These natural or synthetic tRNAs have been used as substrates for the threonylcarbamoyl-A_{37}-forming enzymes present in extracts of *E. coli*, yeast, or *X. laevis* kidney cells. However, the extent of t^6A_{37} formation in such in vitro reactions is generally low. One reason may be the instability of the tRNA modification machinery in vitro (Elkins & Keller, 1974). Inadequate experimental in vitro conditions may also contribute to the low efficiency. Indeed, the enzymatic reactions involve a bicarbonate-dependent incorporation of L-threonine, ATP, and Mg^{2+} (Elkins & Keller, 1974; Körner & Söll, 1974), attesting for a yet unidentified activated intermediate. However, the possibility that carbamyl-phosphate is one of the required intermediates has been ruled out (Powers & Peterkofsky, 1972). Also, several enzymes are most probably involved in catalyzing this complex modification in tRNAs. Finally, the type of tRNA substrate used in the incubation mixture may also be a problem. Using transcripts of a gene with two *E. coli* tRNAs in tandem ($tRNA^{Thr}$–$tRNA^{Gly}$—both harboring an NNU type of anticodon) and dialyzed S100 *E. coli* cell extract supplemented with various cofactors, allowing the simultaneous formation of different types of modified nucleotides, Zeevi and Daniel (1976) demonstrated that t^6A_{37} was formed in the tandem precursor tRNA substrates together with a few other modifications (m^7G_{46}, T_{54}, and Ψ_{55}). On the other hand, using transcripts of *X. laevis* initiator $tRNA^{Met}$ gene and S100 cell-free extracts prepared from cultured *X. laevis* kidney cells, Koski and Clarkson (1982) reported that threonylcarbamoylation was introduced only after the maturation of the 5′ and 3′ termini and after the formation of several other modified nucleotides in the primary transcript (m^1G_9, m^2G_{10}, m^2G_{26}, m^7G_{46}, D_{47}, and m^1A_{58}). This observation fits with those of Harada et al. (1984), showing that two naturally occurring tRNA precursors, isolated from HeLa cells (pre-$tRNA^{Met}$ and pre-$tRNA^{Leu}$) still containing 5′ and 3′ unprocessed ends, were undermodified compared to the fully matured corresponding tRNAs. Among the missing modified nucleotides

was t^6A. Therefore, it might be that enzymatic formation of t^6A_{37} is dependent on the presence of other modified nucleotides in tRNA and/or on the integrity of the tRNA 3D architecture to which the presence of certain modified nucleotides may contribute (discussed in: Björk, 1995a; Steinberg & Cedergren, 1995; Grosjean et al., 1996). Alternatively, it may be that t^6A_{37} formation is one of the latest modifications that occurs in the cytoplasm after the almost maturated eukaryotic tRNA has crossed the nuclear pore.

To overcome most of the in vitro problems, we decided to study the enzymatic formation of t^6A_{37} by microinjecting tRNA transcripts into *X. laevis* oocytes. This in vivo system has the advantage of preserving the integrity of the tRNA modification machinery as well as containing all the necessary cofactors needed to catalyze the formation of t^6A_{37} and all the other modified nucleotides that are normally present in a given tRNA molecule (reviewed in Grosjean & Kubli, 1986; Grosjean et al., 1990, 1996). However, it should not be forgotten that the oocytes are programmed to fulfill a function of their own, which is not to serve just as an in vivo test tube for biochemists, but to prepare fertilization and embryogenesis. Therefore, the results might also reflect in part some constraints of the biological system.

In this work, we studied the effects of various structural mutations in microinjected yeast initiator $tRNA^{Met}$ and yeast $tRNA^{Val}$ on the formation of t^6A_{37} catalyzed by the modification enzymes present in the cytoplasm of *X. laevis* oocytes. The results indicate that the identity of nucleotides at positions 36 and 37, and to a lesser extent at position 38, within the anticodon loop sequence of tRNAs, are important for efficient formation of t^6A_{37}. The anticodon loop structure and the overall architecture of the tRNA substrate are also important, although limited structural changes in tRNA 3D structure do not dramatically affect the enzymatic formation of t^6A_{37}.

RESULTS

Modification pattern of wild-type yeast $tRNA^{Meti}$ microinjected into *X. laevis* oocytes

Yeast initiator $tRNA^{Met}$ naturally contains 11 modified nucleotides (Fig. 2A) (Simsek & RajBhandary, 1972), whereas *X. laevis* initiator $tRNA^{Met}$, which differs from yeast $tRNA^{Meti}$ in 18 positions (76% homology), contains 8 modified residues (Fig. 2B) (Wegnez et al., 1975). Out of these modified nucleotides, seven are common to both tRNAs (m^1G_9, m^2G_{10}, t^6A_{37}, m^7G_{46}, D_{47}, m^5C_{48}, and m^1A_{58}). As indicated by arrows in Figure 2A and B, yeast $tRNA^{Meti}$ contains D_{16} instead of unmodified C_{16} in *X. laevis* $tRNA^{Meti}$, $m^2_2G_{26}$ instead of m^2G_{26}, m^5C_{49} instead of unmodified G_{49}, and a characteristic *O*-ribosyl-5′-phosphate purine at position 64 (Arp_{64}) that is present only in cytoplasmic $tRNA^{Meti}$ of yeast (Keith et al., 1990) and plants (Kiesewetter et al., 1990).

To test whether the *X. laevis* modification machinery was able to catalyze the formation of each individual modified nucleotide normally present in yeast $tRNA^{Meti}$, we prepared four sets of runoff transcripts of yeast synthetic wild-type $tRNA^{Meti}$ gene, each radiolabeled with one of the four [α-^{32}P]-triphosphate nucleotides. They were microinjected into the cytoplasm of *X. laevis* oocytes. These microinjected tRNA transcripts were fairly stable in the *X. laevis* oocyte because more than 90% of the amount of [^{32}P]$tRNA^{Meti}$ initially injected was routinely recovered as the main radioactive band on a polyacrylamide gel after electrophoresis (data not shown). The recovered full-length $tRNA^{Meti}$ was extracted from the gel and then hydrolyzed into 5′-mononucleotides with nuclease P1 and into 3′-mononucleotides with RNase T2. Each hydrolyzate was analyzed by 2D chromatography on thin-layer cellulose plates, using two types of solvent systems, as indicated in Materials and Methods.

Figure 3a, b, c, d, e, and f shows six of the most representative autoradiograms of the 16 independent analyses that were performed. The radioactive modified nucleotide in each spot on the TLC was identified by comparison to reference maps (see Keith, 1995). Information about their location in the precursor molecule was deduced from 3′-nearest neighbor labeling analysis. Only m^2G from position 10 and m^2G resulting from incompletely modified m^2_2G at position 26 could not be evaluated independently. Indeed, the 3′ neighbors of G_{10} and G_{26} are C in both cases (see Fig. 2A).

Table 1 displays the yields obtained for nucleotide modifications at nine distinct positions in the microinjected yeast $tRNA^{Meti}$ after 48 h incubation. Except for $m^2_2G_{26}$, they all occurred at high yield (0.7–1.0 mol/mol of tRNA). Figure 4A and B shows the time courses for each of them in microinjected transcripts. Remarkably, the enzymatic formation of m^1A_{58} in the TΨ loop and t^6A_{37} in the anticodon loop were the most efficient ones. The kinetics of m^2G formation corresponds to the methylation of guanosine at both positions 10 and 26. It first increased up to 1.3 mol/mol of tRNA after 20–40 h incubation and then decreased because of the conversion of the fraction of m^2G_{26} into $m^2_2G_{26}$. A similar observation was made earlier for the $m^2_2G_{26}$ formation in microinjected yeast $tRNA^{Phe}$ (Grosjean et al., 1990) and yeast $tRNA^{Asp}$ transcripts (Edqvist et al., 1995). The lack of modification at position 64 was expected because the corresponding enzyme is only present in the cytoplasm of yeasts and plants (Keith et al., 1990; Kiesewetter et al., 1990). Only the absence of D_{16} cannot be explained for the moment. Because the enzymatic formation of m^1G_9, $m^2_2G_{26}$, m^7G_{46}, and m^5C_{49} are significantly slower than formation of t^6A_{37}, one can suggest that threonylcarbamoylation of A_{37} is

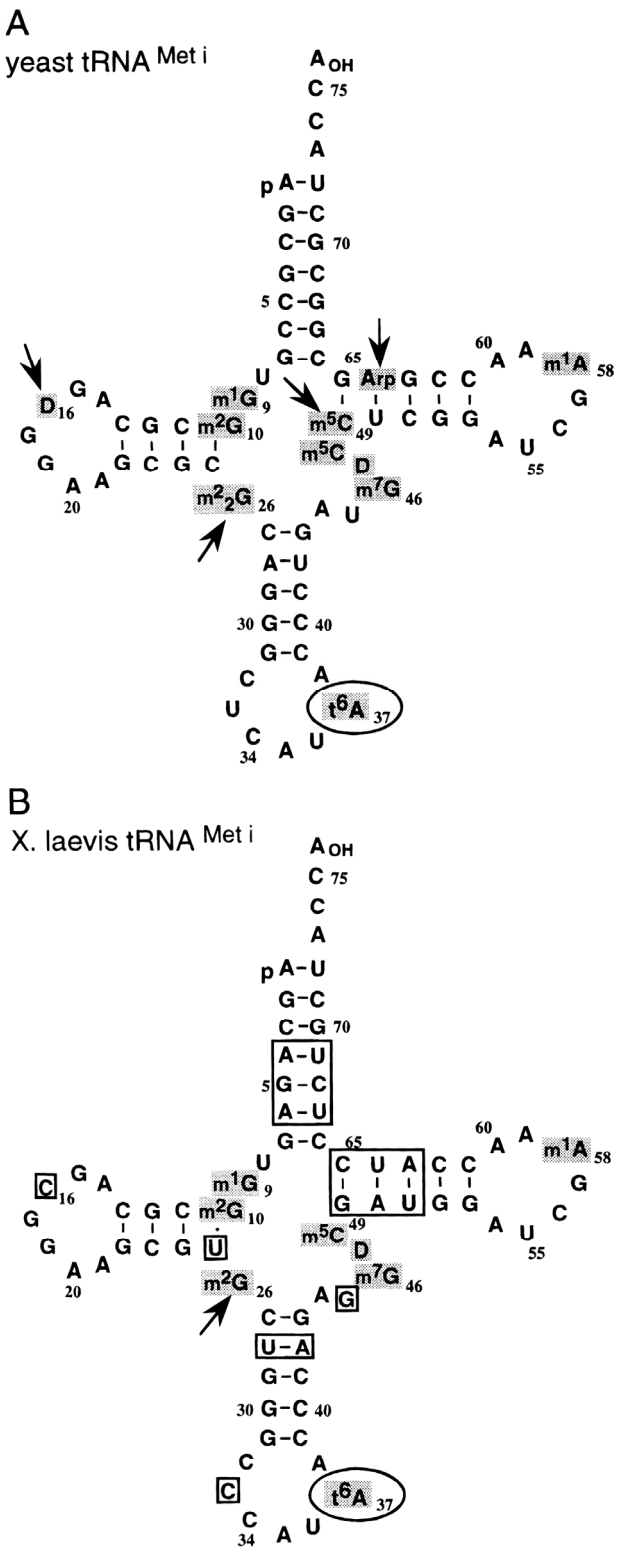

FIGURE 2. Cloverleaf representation of yeast and *X. laevis* $tRNA^{Meti}$. **A:** The 11 modified nucleotides that naturally occur in yeast $tRNA^{Meti}$. **B:** The eight modified nucleotides that naturally occur in *X. laevis* $tRNA^{Meti}$ (gray boxes). In both cases, t^6A_{37} is circled. Boxed nucleotides correspond to the 18 nt that are different in the two tRNA sequences. Arrows indicate where the modified nucleotides differ. Symbols of modified nucleotides are those of Limbach et al. (1994). Universal numbering system for tRNA positions are used (Sprinzl et al., 1996).

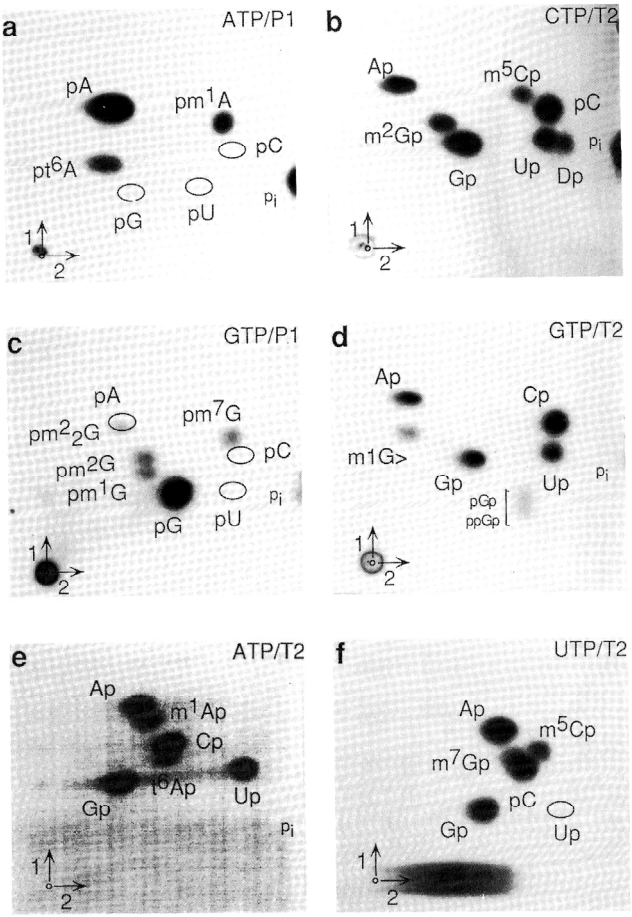

FIGURE 3. Autoradiograms of thin-layer cellulose plates obtained after 2D chromatography of tRNA hydrolyzates. Transcripts of $tRNA^{Meti}$ gene (mut#1: A1G, see Table 2) were ^{32}P-labeled with ATP, CTP, GTP, or UTP (as indicated). They were microinjected into *X. laevis* oocytes and incubated for 48 h. Total RNA was phenol-extracted, gel-purified, and submitted to total hydrolysis with nuclease P1 or RNase T2 (as indicated). Each hydrolyzate was chromatographed using either the "N/R" solvent system (**a–d**) or the "N/N" solvent system (**e**, **f**), as described in Materials and Methods. Arrows indicate the direction of migration in the first and second dimension, respectively. Identification of modified nucleotides was made by comparison with reference maps as in Keith (1995). Open circles correspond to unlabeled 5′- or 3′-mononucleotide AMP (pA), CMP (pC), UMP (pU or Up), and GMP (pG) as visualized under UV light. Free inorganic phosphate is referred to as Pi.

not much dependent on prior nucleotide modification at these particular locations of the tRNA molecule. Because D_{47}, m^5C_{48}, and m^1A_{58} are almost simultaneously synthesized, we cannot exclude that these latter nucleotide modifications facilitate t^6A_{37} formation.

Analysis of yeast tRNA database

In the latest compilation of tRNA sequences, there are 11 cytoplasmic t^6A_{37}-containing tRNAs (anticodon NNU) sequenced so far from yeast (Sprinzl et al., 1996). These are tRNA specific for Met (anticodon CAU, initiator, and elongator), Ile (IAU and ΨAΨ), Thr (IGU and U?GU, with an unknown U_{34}), Asn (GUU),

TABLE 1. Type and location of modified nucleotides in tRNA.[a]

	Modified nucleotides			
	Naturally occurring $tRNA^{Met}$ initiator from		Yeast $tRNA^{Met}$ transcripts microinjected into oocytes	
Positions	*X. laevis*	Yeast	Type	Yield
9	m^1G	m^1G	$G \rightarrow m^1G$	0.9 ± 0.1
16	C	D	U	No modification
10+26	m^2G	m^2G	$G \rightarrow m^2G$	1.3 ± 0.1
26	m^2G	m^2_2G	$m^2G \rightarrow m^2_2G$	0.4 ± 0.1
37	t^6A	t^6A	$A \rightarrow t^6A$	1.1 ± 0.1
46	m^7G	m^7G	$G \rightarrow m^7G$	0.9 ± 0.2
47	D	D	$U \rightarrow D$	0.8 ± 0.1
48	m^5C	m^5C	$C \rightarrow m^5C$	0.7 ± 0.1
49	G	m^5C	$C \rightarrow m^5C$	0.7 ± 0.1
58	m^1A	m^1A	$A \rightarrow m^1A$	1.0 ± 0.1
64	U	Arp	A	No modification

[a]On the left is the information from sequence analysis in naturally occurring $tRNA^{Meti}$ from *X. laevis* (Wegnez et al., 1975) and yeast, respectively (Simsek & RajBhandary, 1972; see also Fig. 2). On the right are molar yields of modified nucleotides (expressed in mol/mol of tRNA) formed in microinjected transcript of yeast $tRNA^{Meti}$ (mut#1) after 48 h incubation at 20 °C. Each value (mean ± SD) was obtained from at least three kinetic experiments as shown in Figure 4. Symbols and numbering rules are as in legend of Figure 2.

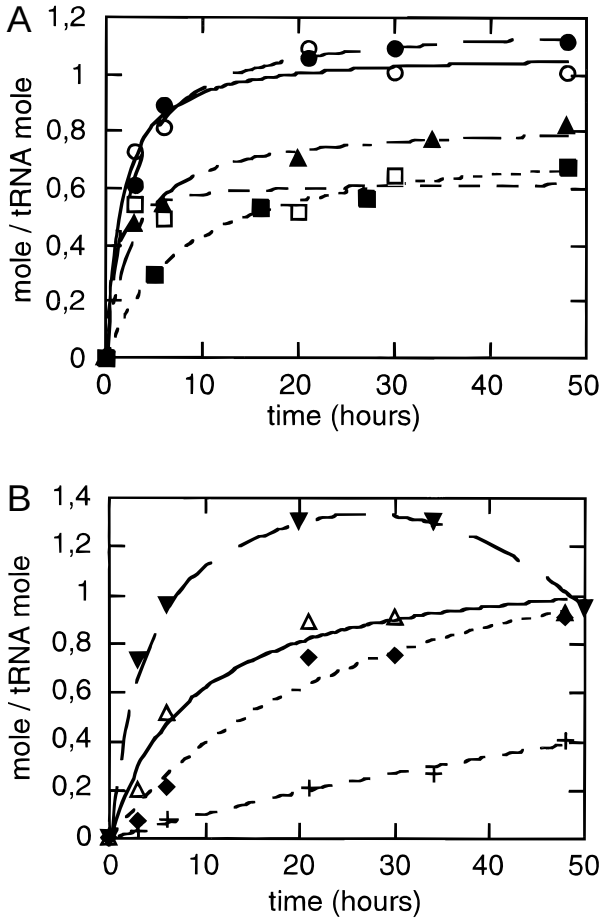

FIGURE 4. Time courses for the formation of the various modified nucleotides in microinjected transcripts of yeast $tRNA^{Meti}$ (mut#1: A1G, see Table 2). After the incubation, tRNAs [^{32}P]-labeled with either ATP, CTP, GTP, or UTP were gel purified and completely degraded with either nuclease P1 or T2 into mononucleotides. Each hydrolyzate was analyzed by 2D-TLC as described in Materials and Methods. Estimation of the radioactivity in each spot, as detected in Figure 3 for different incubation times, allowed us to determine the molar ratio of each modified nucleotide versus unmodified ones. **A:** Corresponds to the formation of t^6A_{37} (○), D_{47} (▲), m^5C_{48} (□), m^5C_{49} (■), and m^1A_{58} (●). **B:** Corresponds to the formation of m^1G_9 (△), m^2G_{10+26} (▼), $m^2_2G_{26}$ (+), and m^7G_{46} (♦).

Lys (CUU and mcm^5s^2UUU), Ser (GCU), and Arg (mcm^5UCU). From the recent identification of all tRNA genes in the yeast genome (El-Mabrouk & Lisacek, 1996), these tRNAs correspond to the complete set of t^6A-containing tRNA isoacceptors. Sequence comparison of these tRNAs revealed, as already noticed by others (Yarus, 1982; Tsang et al., 1983), an invariable U_{36} on the 5′ side and an A_{38} on the 3′ side of the target A_{37}, in addition to the expected invariant and semi-invariant nucleotides that are present in all tRNAs (Grosjean et al., 1982). Assuming that only one enzymatic machinery exists in yeast to convert A_{37} into t^6A_{37}, the characteristic trinucleotide sequence $U_{36}A_{37}A_{38}$, which is strictly conserved in all cytoplasmic t^6A-containing tRNAs from all kinds of organisms, might therefore constitute a universal primary identity determinant for the t^6A_{37}-forming enzymes. However, because tRNAs are involved in so many different biochemical processes, it could well be that the above consensus is not only involved in the interaction with a given modification enzyme.

Construction of yeast tRNA variants and test for their ability to form t^6A_{37} after microinjection into *X. laevis* oocytes

The 26 $tRNA^{Meti}$ variants we have constructed to solve the problem of tRNA recognition by the t^6A_{37}-forming enzymes present in the cytoplasm of *X. laevis* oocytes are illustrated in Figure 5A, B, C, and D. To analyze the importance of the conserved nucleotides present in the anticodon loop for the formation of hypermodified t^6A_{37}, we designed by point-mutation 10 variants of yeast $tRNA^{Meti}$ for which one of the nucleotides at positions 34, 35, 36, and 38 was mutated to one of the other three canonical nucleotides. Three additional variants were designed to test the effect of a mismatch as well as of an A-U base pair at positions 30–40 in the anticodon stem. To test the importance of the anticodon loop size, we have deleted U_{33} or inserted an additional C between C_{32} and U_{33} in the backbone of yeast $tRNA^{Meti}$, thus generating an anticodon loop of six or eight nucleotides, respectively. The outcome of local or global perturbations in the yeast $tRNA^{Meti}$ conformation was analyzed using eight additional variants holding mutations that disrupt and thereafter restore tertiary interactions involved in the tRNA 3D

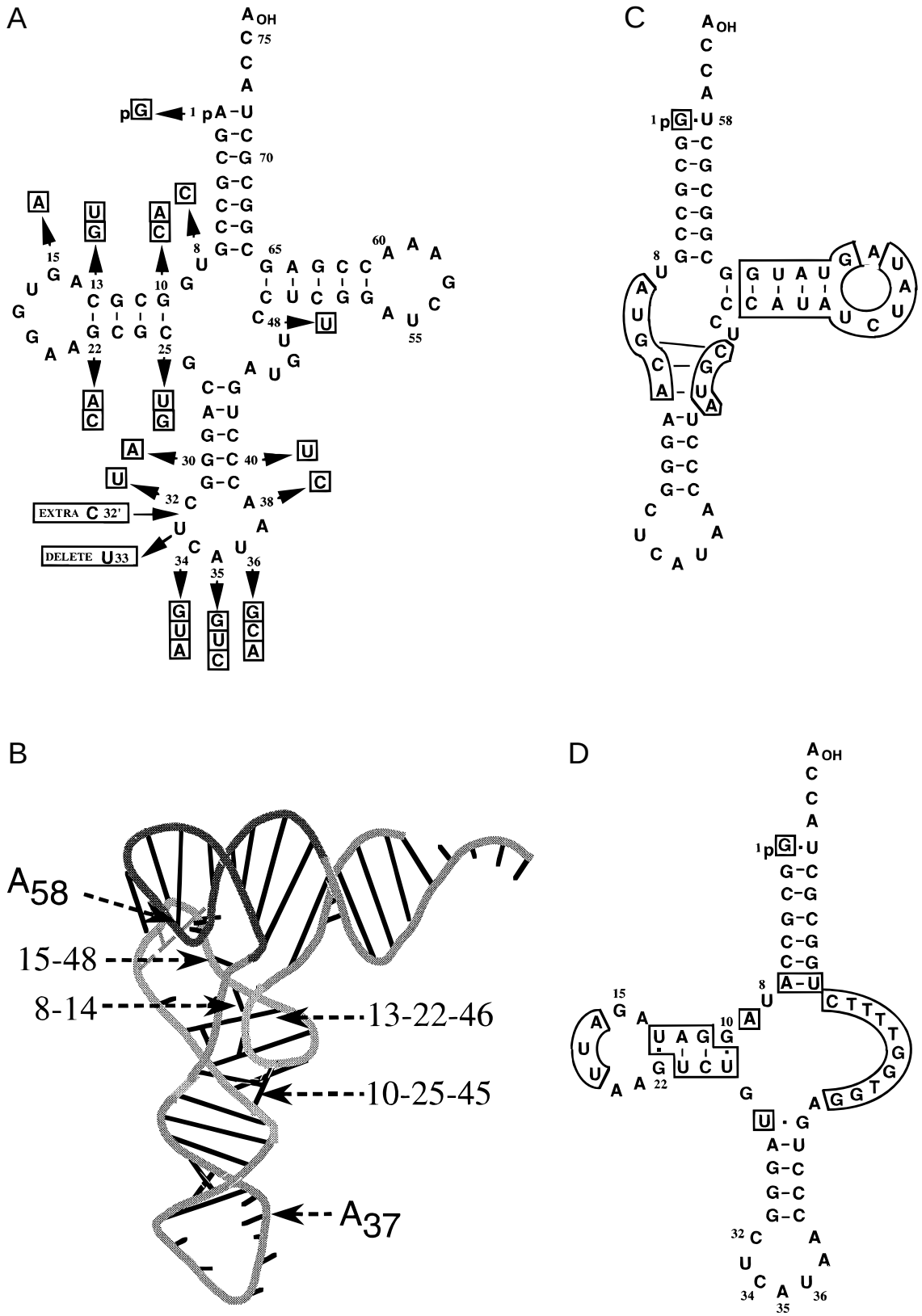

FIGURE 5. Designing of the yeast $tRNA^{Meti}$ variants to analyze structural requirements for the enzymatic formation of t^6A_{37}. All the variants tested were derived from yeast $tRNA^{Meti}$ mut#1 (with mutation A1G, see Table 2). **A:** Mutations of conserved nucleotides in the anticodon loop (mut#2–mut#11), mutations affecting the loop size (mut#12 and mut#13), mutations in the anticodon stem (mut#14–mut#16), and mutations affecting the tertiary interactions in the tRNA (mut#17–mut#24). **B:** Schematic drawing of the yeast $tRNA^{Meti}$ showing the tertiary interactions (dashed lines) that have been mutated in the 3D core. **C:** Truncated chimerical $tRNA^{Meti}$ lacking D arm (mut#25). **D:** Truncated chimerical $tRNA^{Meti}$ lacking T arm (mut#26). In both cases, nucleotides that differ from the wild-type sequence are boxed.

core structure (Fig. 5B). The ability of two truncated yeast $tRNA^{Meti}$, of which either the D-stem-loop or the T-stem-loop has been replaced by shorter oligonucleotides, were also tested. The first truncated $tRNA^{Meti}$ (Met-deltaD, Fig. 5C) was obtained by replacing the D-arm, T-arm, and variable region of the core sequence of yeast $tRNA^{Meti}$ by that found in bovine mitochondrial $tRNA^{Ser}$ (Anderson et al., 1981). The second minimalist tRNA-Met_i (Met-deltaT, Fig. 5D) was obtained by replacing the core sequence of yeast $tRNA^{Meti}$ by that of the nematode worm *Caenorhabditis elegans* mitochondrial $tRNA^{Meti}$ (Wolstenholme et al., 1987). The conformations of these two minimalist yeast $tRNA^{Meti}$ were probed by RNAse (Senger et al., 1995). This analysis revealed a folding into an L-shaped tertiary structure similar to the ones already described for certain mitochondrial tRNAs (de Bruijn & Klug, 1983; Watanabe et al., 1994; reviewed in Steinberg et al., 1994).

To test whether A_{37} in these tRNA variants can be modified to t^6A_{37}, [α-^{32}P] ATP-radiolabeled T_7-runoff transcripts of the corresponding tRNA genes were microinjected into the cytoplasm of *X. laevis*. After incubation at 19 °C from 3 to 48 h, full-length transcripts were recovered by gel electrophoresis and analyzed as described above using nuclease P1. Under these conditions, the formation of both t^6A_{37} and m^1A_{58} can be determined in the same analysis, except for the fragmented tRNA lacking A_{58} (mut#25–mut#26). From independent work, we knew that m^1A_{58} formation catalyzed by the oocyte methylase was independent of the tRNA architecture (Grosjean et al., 1996). Therefore, the specific effects of mutations on t^6A_{37} formation was referred to those on m^1A_{58} formation used as an internal indicator. Figure 6a, b, c, and d shows autoradiographies of a few representative TLC plates. Figure 7a, b, c, d, e, f, g, and h illustrates the time courses obtained for the enzymatic formation of t^6A_{37} and m^1A_{58} in the most interesting $tRNA^{Meti}$ variants tested. Table 2 reports all data obtained in this work, not only with variants of $tRNA^{Meti}$, but also with few other tRNAs (see below).

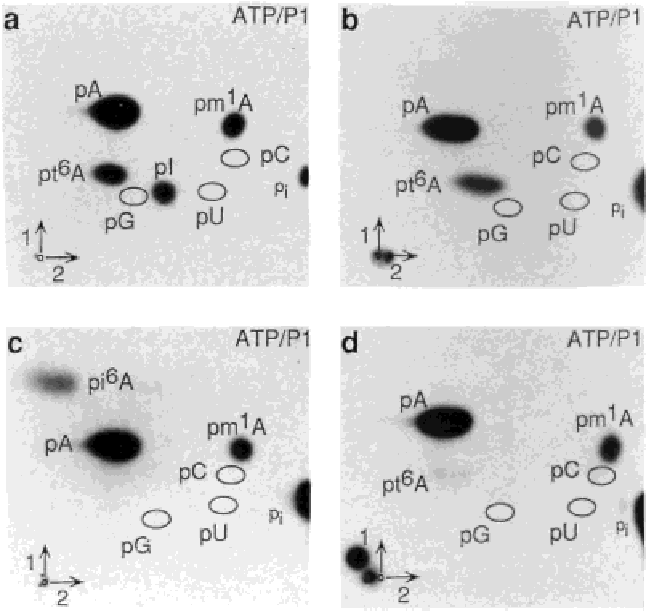

FIGURE 6. Autoradiograms of thin-layer cellulose plates obtained after 2D chromatography of tRNA hydrolyzates of selected microinjected $tRNA^{Meti}$ variants. Transcripts of $tRNA^{Meti}$ gene were ^{32}P-labeled with ATP. They were incubated for 48 h at 20 °C in the oocytes. Total oocyte RNA was phenol-extracted, gel-purified, and submitted to complete hydrolysis with nuclease P1. Each hydrolyzate was chromatographed using the "N/R" solvent system (**a–d**) as described in Materials and Methods. Arrows indicate the direction of migration in the first and second dimensions, respectively. **a:** Formation of t^6A_{37}, m^1A_{58}, and I_{34} in tRNA mut#2 (C34A). **b:** Formation of t^6A_{37} and m^1A_{58} in tRNA mut#5 (A35G). **c:** Formation of i^6A_{37} and m^1A_{58} in tRNA mut#8 (U36A). **d:** Formation of m^1A_{58} and trace amount of t^6A_{37} in tRNA mut#11 (C32A38 → U32C38). Open circles correspond to unlabeled 5′ mononucleotide CMP (pC), UMP (pU), and GMP (pG) as visualized under UV light. Free inorganic phosphate is referred to as Pi.

U_{36} within the anticodon loop is a major determinant for t^6A_{37} formation

Base substitution at either position 34 or 35 of the anticodon has no effect of the enzymatic formation of t^6A_{37} (mut#2–mut#7 in Table 2; Fig. 6a,b). Interestingly, when C_{34} was mutated to A_{34} (mut#2), this adenosine was slowly but quantitatively modified into inosine (I_{34} in Fig. 7a; see also Auxilien et al., 1996). When the so-called cardinal nucleotide U_{36} (Yarus, 1982) was mutated to A (mut#8), C (mut#9), or G (mut#10), no t^6A_{37} was formed (Fig. 6c). In the case of U_{36} mutated into A_{36} (mut#8), the adenosine-37 was now slowly modified to the N^6-isopentenyl derivative (i^6A in Fig. 7b), the degree of modification after 48 h incubation reaching only 0.5 mol/mol tRNA. A low yield of t^6A_{37} was detected in a microinjected yeast $tRNA^{Meti}$ variant in which both C_{32} and A_{38} were mutated, respectively, to U_{32} and C_{38} (mut#11, in Fig. 6d, 7c; Table 2). Thus, clearly t^6A_{37} occurs in yeast $tRNA^{Meti}$ only when target A_{37} is flanked with 5′-U_{36}. Any of the four canonical nucleotides can be present in positions 34 and/or 35 of the anticodon loop. The presence of an A_{38} enhanced considerably the rate of the reaction.

Size of the anticodon loop and base pairs in the proximal anticodon stem can be altered

The size of the anticodon loop and therefore its precise conformation seems not to be an essential parameter for tRNA recognition by *X. laevis* t^6A-forming enzymes. Indeed, the deletion of U_{33} (mut#12) or the addition of an extra C between C_{32} and U_{33} (mut#13), leading to tRNA variants displaying mutated anticodon loop of six or eight nucleotides, respectively,

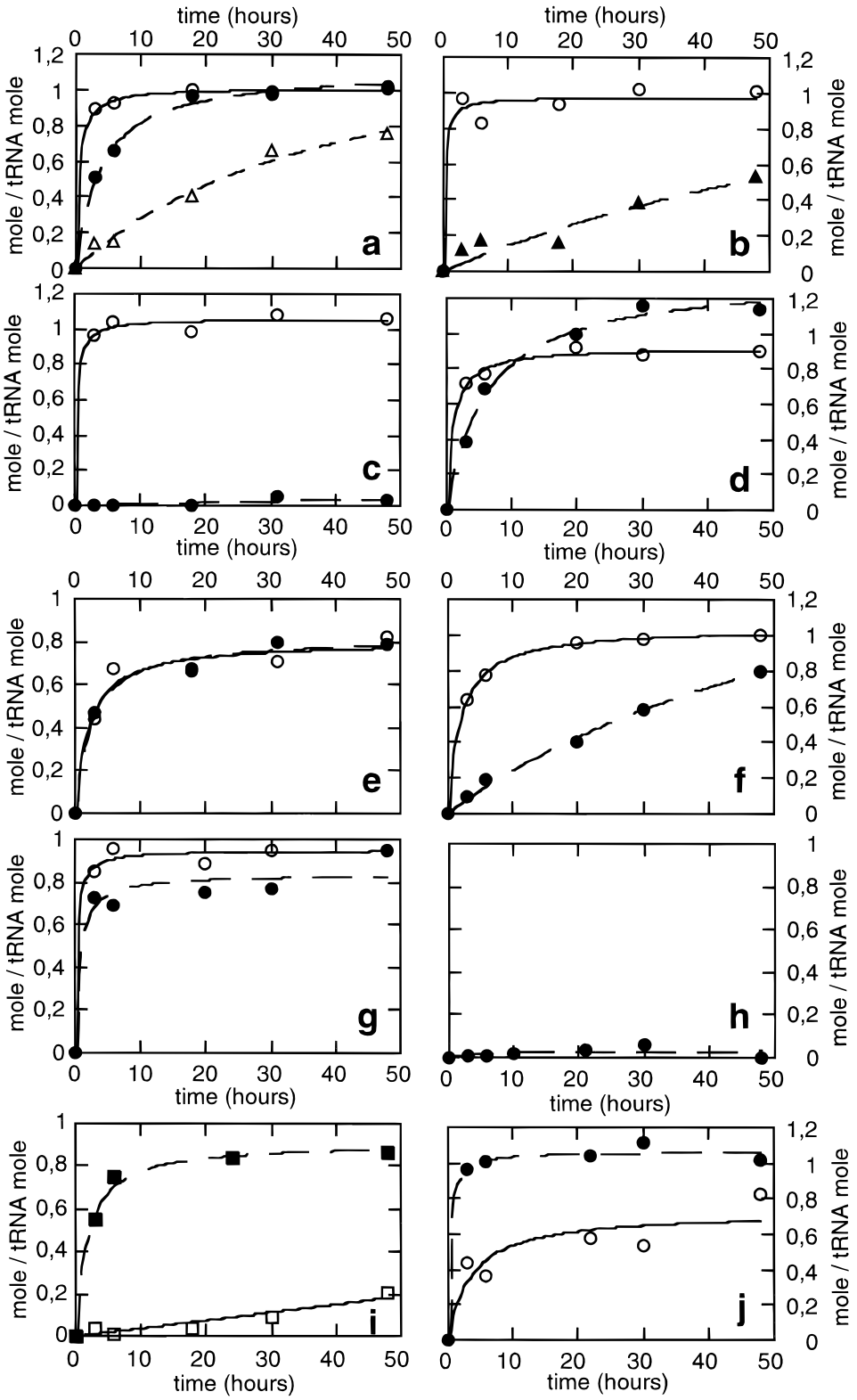

FIGURE 7. Time courses for the formation of modified adenosines in microinjected variants of yeast $tRNA^{Meti}$ (**a–h**), variants of yeast $tRNA^{Val}$ (**i**), and *E. coli* $tRNA^{Thr}$ (**j**). All transcripts were [^{32}P]-labeled with ATP. All other experimental conditions were as in Figure 4. In all panels, except panel **i**, the same symbols are used for t^6A_{37} (●) and m^1A_{58} (○). In panel **i**, (□) and (■) both correspond to t^6A_{37}, but in different tRNA (mut#Val-1 and mut#Val-2, respectively). Formation of I_{34} (△) in $tRNA^{Meti}$ mut#2 (C34A) and of i^6A_{37} (▲) in $tRNA^{Meti}$ mut#8 (U36A) are shown in **a** and **b**, respectively. The other panels correspond to $tRNA^{Meti}$ mut#11 (C32A38 → U32C38; **c**), $tRNA^{Meti}$ mut#14 (G30A, **d**), $tRNA^{Meti}$ mut#12 (deletion of U33, **e**), $tRNA^{Meti}$ mut#17 (U8A14 → C8A14, **f**), $tRNA^{Meti}$ mut#21 (G10C25U45 → C10G25U45, **g**), $tRNA^{Meti}$ mut#25 (D-stem/loop deleted, **h**) and *E. coli* $tRNA^{Thr4/2}$ in **j** (see also Fig. 8).

TABLE 2. Effects of mutations in various tRNAs on their capacity to become substrates for the t^6A_{37} and m^1A_{58} modification machinery of *X. laevis* oocytes.[a]

Mutations		Extent of modifications	
Name	Type	t^6A_{37}	m^1A_{58}
Yeast tRNA initiator			
$tRNA^{Meti}$	Wild-type	ND	ND
mut#1	A1 → G1	1.0 ± 0.1	1.0 ± 0.1
Mutations in the anticodon loop			
mut#2	C34 → A34	1.0 ± 0.2	1.0 ± 0.1
mut#3	C34 → G34	1.1 ± 0.2	0.9 ± 0.1
mut#4	C34 → U34	1.2 ± 0.2	0.9 ± 0.1
mut#5	A35 → G35	1.1 ± 0.2	1.0 ± 0.1
mut#6	A35 → U35	1.2 ± 0.2	1.0 ± 0.1
mut#7	A35 → C35	1.0 ± 0.1	1.0 ± 0.1
mut#8	U36 → A36	0 ± 0*	1.0 ± 0.1
mut#9	U36 → C36	0 ± 0	1.0 ± 0.1
mut#10	U36 → G36	0 ± 0	0.8 ± 0.1
mut#11	C32 A38 → U32 C38	0.04 ± 0.01	1.0 ± 0.1
Mutations affecting loop structure			
mut#12	Deletion of U33	0.8 ± 0.1	0.9 ± 0.1
mut#13	Addition of C32′	0.8 ± 0.1	0.8 ± 0.1
Mutations in the anticodon stem			
mut#14	G30 → A30	1.2 ± 0.2	0.5 ± 0.1
mut#15	C40 → U40	1.1 ± 0.2	0.4 ± 0.1
mut#16	G30 C40 → A30 U40	1.2 ± 0.2	0.6 ± 0.1
Mutations affecting tertiary interactions in the tRNA core			
mut#17	U8 A14 → C8 A14	0.8 ± 0.1	1.0 ± 0.1
mut#18	G15 C48 → A15 C48	1.1 ± 0.2	1.0 ± 0.1
mut#19	G15 C48 → G15 U48	1.1 ± 0.2	1.0 ± 0.1
mut#20	G15 C48 → A15 U48	0.9 ± 0.3	0.9 ± 0.1
mut#21	G10 C25 U45 → C10 G25 U45	0.9 ± 0.2	0.9 ± 0.1
mut#22	G10 C25 U45 → A10 U25 U45	0.9 ± 0.3	0.9 ± 0.3
mut#23	C13 G22 G46 → G13 C22 G46	1.0 ± 0.3	1.0 ± 0.2
mut#24	C13 G22 G46 → U13 A22 G46	1.0 ± 0.3	1.0 ± 0.1
Fragmented tRNAs			
mut#25	D-stem/loop deleted	0.02 ± 0.01	NA
mut#26	T-stem/loop deleted	0.01 ± 0.01	NA
Other tRNA transcripts			
yeast#Val-1	U34 C36 → C34 U36	0.2 ± 0.1	0.7 ± 0.1
yeast#Val-2	U32 U34 C36 C38 → C32 C34 U36 A38	1.0 ± 0.2	1.0 ± 0.1
yeast $tRNA^{Ile}$	Wild-type	1.0 ± 0.1	0.7 ± 0.1
E. coli $tRNA^{Thr}$	U34 → C34	1.0 ± 0.1	0.8 ± 0.1

[a]Modifications are expressed as moles of modified nucleotides per tRNA mole after 48 h incubation at 20 °C. Each value (mean ± SD) was obtained from at least three kinetic experiments as illustrated in Figure 7. ND, not determined; NA, not applicable. Type and position of each mutation were detailed in the text. Asterisk indicates the formation of i^6A (0.5 mol/mol of tRNA), instead of t^6A, at position 37 in $tRNA^{Meti}$ mut#8.

did not reduce substantially the potentiality of yeast $tRNA^{Meti}$ to become modified to t^6A_{37} (Fig. 7e; Table 2).

Moreover, tRNA variants that include a mismatch base pair A_{30}–C_{40} (mut#14), or a wobble pair G_{30}–U_{40} (mut#15) or a Watson–Crick base pair A_{30}–U_{40} [mut#16—that was never found in these positions in any cytoplasmic tRNA sequenced so far (Grosjean et al., 1982; Sprinzl et al., 1996)], displayed t^6A_{37} modification as in wild-type yeast $tRNA^{Meti}$ (Fig. 7d; Table 2). These results are at variance with the earlier prediction based on sequence comparison of t^6A-containing tRNAs because none of these tRNAs displayed a mismatched or wobble base pair in the anticodon stem (Yarus, 1982; Tsang et al., 1983).

Only local perturbations of the 3D architecture are allowed

The crystal structure of yeast $tRNA^{Meti}$ is known at 3 Å resolution (Basavappa & Sigler, 1991). We have investigated whether point mutations that perturb tertiary interactions (see Fig. 5B) can affect the potentiality of yeast $tRNA^{Meti}$ to be modified to t^6A at position 37 in

the anticodon loop, m^1A_{58} being taken as internal control.

Change of U_8 into C_8 (mut#17), disrupting the crucial U_8*A_{14} tertiary hydrogen bonding interaction (Major et al., 1993), affected significantly the rate, but not the final yield of t^6A_{37} formation, whereas the rate and the yield of m^1A_{58} formation were the same as for the wild-type $tRNA^{Meti}$ (Fig. 7f; Table 2). Disrupting the so called Levitt purine-15*pyrimidine-48 base pair by changing G_{15} into A_{15} (mut#18) or C_{48} into U_{48} (mut#19) or restoring it by a double mutation (mut#20 containing a canonical A_{15}*U_{48} bond) had no significant effect on the rate (kinetics not shown) and the final yield of both t^6A_{37} and m^1A_{58} (Table 2). Other 3D mutations that disrupted tertiary interactions G_{10}*C_{25}*U_{45} (mut#21 and mut#22) or C_{13}*G_{22}*G_{46} (mut#23 and mut#24) also did not change the modification efficiency of these tRNA variants (Fig. 7g; Table 2).

To determine whether more drastic 3D perturbations have an effect of t^6A_{37} formation, we tested two truncated $tRNA^{Meti}$ (Fig. 5C,D). The first chimerical $tRNA^{Meti}$ lacks a D-stem and loop (mut#25), the second minimalist $tRNA^{Meti}$ lacks a T-stem and loop (mut#26). As shown in Figure 7h and Table 2, both minimalist $tRNA^{Meti}$ displayed very low levels of t^6A_{37}.

Thus, whereas local perturbations are acceptable to the t^6A-forming enzymes, the overall cloverleaf structure of the cytoplasmic tRNA substrate has to be maintained. In other words, the integrity of the cloverleaf architecture is an important parameter for tRNA recognition by the *X. laevis* t^6A-forming enzymes.

t^6A_{37} can occur in non-natural substrates only after few mutations in the anticodon loop

In order to further investigate the role of nucleotides in the anticodon loop on enzymatic formation of t^6A_{37}, two variants of yeast $tRNA^{Val}$ (anticodon UAC) were constructed (Fig. 8, only the anticodon stem/loop sequences are shown). This tRNA naturally is not a substrate for the t^6A-forming enzymes and has an unmodified A_{37}. The first $tRNA^{Val}$ mutant (mut#Val-1) possesses only the methionine CAU anticodon instead of the valine UAC anticodon. The second yeast $tRNA^{Val}$ mutant (mut#Val-2) exhibits an anticodon loop that is now strictly identical with that of wild-type yeast $tRNA^{Meti}$ (Fig. 2A).

When microinjected into the cytoplasm of *X. laevis* oocytes, in contrast with the mutant of yeast $tRNA^{Meti}$ bearing C_{38} (mut#11, in Fig. 7c; Table 2), tRNA mut#Val-1, which contains the $tRNA^{Met}$ anticodon CAU together with C_{38}, displayed a detectable but rather low level of t^6A_{37} modification (Fig. 7i; Table 2). Results with tRNA mut#Val-2 displayed the same level of t^6A_{37} modification (Fig. 7i; Table 2) as yeast wild-type yeast $tRNA^{Meti}$. Thus, clearly the whole architecture of an anticodon loop containing the consensus sequence

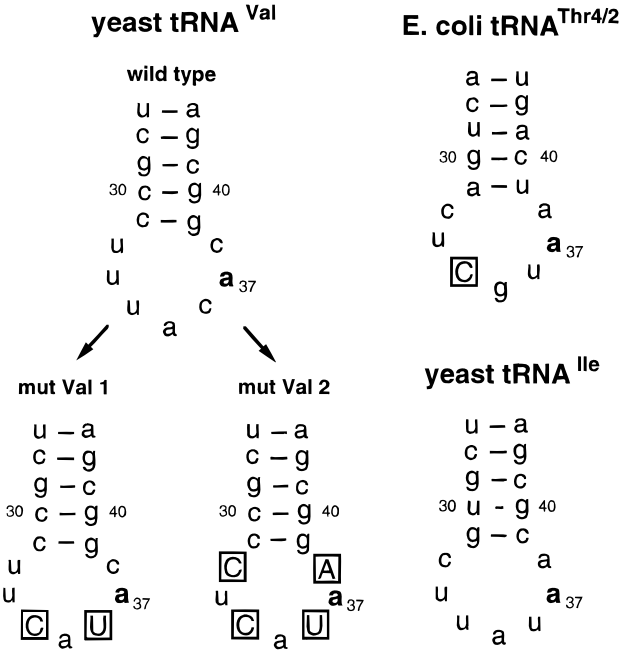

FIGURE 8. Anticodon-stem loop sequences of the yeast $tRNA^{Val}$ variants, of *E. coli* $tRNA^{Thr4/2}$, and of yeast $tRNA^{Ile}$ used in this study. Mutations are indicated by boxed nucleotides. Yeast $tRNA^{Val}$ variants were designed to contain either the $tRNA^{Meti}$ anticodon sequence $C_{34}A_{35}U_{36}$ in mut#Val-1 or the whole $tRNA^{Meti}$ anticodon loop sequence $C_{32}U_{33}C_{34}A_{35}U_{36}A_{37}A_{38}$ in mut#Val-2. Both *E. coli* $tRNA^{Thr4/2}$ and yeast $tRNA^{Ile}$ to contain the consensus sequence $C_{32}U_{33}N_{34}N_{35}U_{36}A_{37}A_{38}$.

$C_{32}U_{33}N_{34}N_{35}U_{36}A_{37}A_{38}$ is the best identity element for t^6A_{37} modification by the *X. laevis* machinery. This was further verified using transcripts of two additional tRNAs, namely yeast $tRNA^{Ile}$ (anticodon UAU) and *E. coli* $tRNA^{Thr4/2}$ (anticodon CGU) (see Fig. 8). When microinjected into the cytoplasm of *X. laevis* oocytes, these two tRNAs were excellent substrates for the t^6A_{37}-forming enzymes (Table 2). Figure 7j illustrates the time course obtained with microinjected *E. coli* $tRNA^{Thr4/2}$.

DISCUSSION

In this study, we have introduced runoff T7 transcripts of yeast $tRNA^{Meti}$ and yeast $tRNA^{Val}$ genes into the cytoplasm of *X. laevis* oocytes. This in vivo system is endowed with all of the modification enzymes and cofactors required to achieve various kinds of nucleotide modifications that are normally present in a eukaryotic cell at that stage of development. It has been used previously to detect the enzymatic formation of several modified nucleotides in various microinjected tRNAs (recombinant tRNAs or tRNA transcripts; see, for examples, Haumont et al., 1984, 1987; Droogmans et al., 1986; Droogmans & Grosjean, 1987; Grosjean et al., 1990, 1996).

Our results indicate that microinjected yeast $tRNA^{Meti}$ transcript (mut#1) became modified at nine distinct

positions that correspond to the sites of tRNA that are normally modified in the yeast cell. No additional modified nucleotides were formed in microinjected yeast tRNA. Base modifications located at positions 37 (t^6A), 47 (D), 48 (m^5C), and 58 (m^1A) were among the most efficient ones. The time courses of m^2G and m^2_2G formation reflects the expected two-step formation of dimethyl-G_{26} in which the mono-methyl-G_{26} occurs rapidly, followed by a slower formation of dimethyl-G_{26} (Edqvist et al., 1995). Our observation that t^6A_{37} formation in the end-matured $tRNA^{Meti}$ is very efficient, together with the earlier results of Koski and Clarkson (1982) showing that t^6A_{37} modification cannot occur in *X. laevis* $tRNA^{Meti}$ precursors bearing their 5′- and 3′-extension ends, suggest that the threonylation machinery in the *X. laevis* oocyte is sensitive to early steps of tRNA processing. Also, no $m^6t^6A_{37}$, as in several eukaryotic $tRNA^{Thr}$ (anticodon IGU), nor $ms^2t^6A_{37}$, as in most eukaryotic $tRNA^{Lys}$ (anticodon mcm^5s^2UUU), was formed in any of the microinjected yeast $tRNA^{Meti}$ variants we tested. Thus, distinct structural identity elements, other than the ones present in our yeast $tRNA^{Meti}$ variants, are required by the enzymes catalyzing the formation of such complex hypermodified nucleotides. A precedent of this kind exists for the enzymatic formation of the wybutosine-37 (Y_{37}) that is unique to yeast $tRNA^{Phe}$ (anticodon GmAA), whereas the tRNA:guanine-1 methylase, which catalyzes the formation of m^1G_{37} in yeast $tRNA^{Phe}$ as the first step of Y_{37} biosynthesis, works on several tRNAs (Droogmans & Grosjean, 1987; Grosjean et al., 1990).

Here, we show that the major identity elements for the recognition of tRNA by t^6A-forming enzymes are essentially located within the anticodon loop. The required specific feature is the sequence $U_{36}A_{37}$, together with a strong preference for A_{38}. This conclusion fits well with earlier works (Roberts & Carbon, 1975; Murgola et al., 1984) showing that a single change of C_{36} to U_{36} in *E. coli* $tRNA^{Gly}$ (anticodon UCC) induced t^6A_{37} formation in *E. coli*. Conversely, the consequence of a mutation of U_{36} to C_{36} in *E. coli* $tRNA^{Lys}$ (anticodon UUU) is the complete lack of t^6A_{37} in $tRNA^{Lys}$ (discussed in Murgola, 1995). The anticodon loop size need not be restricted to seven nucleotides and may vary between six and eight nucleotides at least when the nucleotide addition or deletion has been made near the "kinking" site of U_{33}. In the first two anticodon positions (34 and 35), any of the usual four nucleotides may occur. Moreover, the indicated large permissible range for the stem sequences, including the tolerance of even the mismatch base pair at the 30–40 location, agrees with the universal occurrence of t^6A_{37} in anticodon NNU-containing tRNAs of all the phylogenetic kingdoms (see Björk, 1995a). An enigmatic exception is the initiator $tRNA^{Meti}$ of bacteria, but not the cytoplasmic initiator $tRNA^{Meti}$ of eukaryotes. However, when naturally occurring *E. coli* $tRNA^{Meti}$ was microinjected into the *X. laevis* oocytes, A_{37} became rapidly and quantitatively modified into t^6A_{37} (Grosjean et al., 1987). Thus, there seems no intrinsic structural reason for this particular tRNA not to be modified at A_{37}. It might be that the eubacterial modifying machinery is more stringent in its action than the eukaryotic one. Alternatively, an interfering bacterial factor with no counterpart in eukaryotic cells may hinder the selective modification of A_{37} in only $tRNA^{Meti}$.

Interestingly, a few selected point mutations that affect 3D interactions have no drastic influence on the formation of both t^6A_{37} and m^1A_{58}. The perturbation of the overall 3D structure of these tRNA mutants maintains the cloverleaf structure (or 2D), suggesting that the integrity of the latter is sufficient for the recognition by the t^6A_{37}-forming enzymes. Indeed, truncated tRNAs, containing deletion of either the TΨ-arm or the D-arm, were not substrates for the oocyte threonylation machinery. It is important to point out that the truncated tRNA lacking the T-arm possesses the central core of *C. elegans* mitochondrial $tRNA^{Meti}$, for which an L-shaped structure has been proposed (Steinberg & Cedergren, 1994; Watanabe et al., 1994). Moreover, this $tRNA^{Meti}$ variant is able to be aminoacylated by yeast cytoplasmic methionyl-tRNA synthetase to nearly wild-type levels, leading to the conclusion that this hybrid tRNA has an L-shaped functional structure (Senger et al., 1995). Taken together, these results indicate that the enzymatic machinery in the oocytes accountable for t^6A_{37} modification tolerates only local perturbations or some laxity of the tRNA conformation, but requires the integrity of the L-shaped tRNA resulting from a correct folding of a cloverleaf structure. Therefore, the eukaryotic enzymes leading to t^6A_{37} formation clearly belong to what we have called the group II family of tRNA modification enzymes (discussed in Grosjean et al., 1996). Because all rules have their exceptions, it is noteworthy that sequence analysis of the small (3S) $tRNA^{Ser}$ (anticodon GCU) from beef heart mitochondria has revealed the presence of t^6A_{37} despite the lack of the D-arm (Arcari & Brownlee, 1980). It may well be that the tRNA-modifying machinery in mitochondria, as with that of prokaryotes (see above), has different requirements for tRNA recognition.

Analysis of nucleotide sequences of t^6-containing tRNAs in the latest tRNA database (Sprinzl et al., 1996) reveals some interesting peculiarities. Conservation of nucleotides in the proximal anticodon stem, for which Yarus (1982) pointed out some clear correlation with the so-called cardinal nucleotide at position 36 (extended anticodon theory), is not obvious in yeast t^6-containing tRNAs. A preference for a purine–pyrimidine base pair exists at positions 31–39 and for a pyrimidine–purine at positions 27–43. The preference for base pairs G-C or C-G is also evident at positions 30–40. However, almost all tRNAs sequenced so far follow this rule (Grosjean et al., 1982; Sprinzl et al., 1996),

leading to the conclusion that this feature, although important for some structural or functional aspects of the tRNA molecule, may not be relevant to t^6A_{37} formation. It is noteworthy that an unpaired Ψ_{31}–Ψ_{39} combination naturally exists in the anticodon proximal stem of yeast elongator $tRNA^{Met}$ (anticodon CAU) and a U_{30}–G_{40} wobble-pair occurs in yeast $tRNA^{Ile}$ (anticodon IUA), attesting that a strict Watson–Crick base pair in this region of anticodon stem is not essential for recognition by yeast t^6A-forming enzymes. At the subsequent three locations (29–41, 28–42, and 27–43) in the anticodon stem of yeast tRNAs, canonical Watson–Crick base pairs are always seen. In *E. coli* tRNAs, few cases of mispairing were observed only at positions 27–43, 30–40, and 31–39. These observations suggest that structural constraints in the anticodon stem of eukaryotic and prokaryotic tRNAs, and consequently their ability to interact with a modification enzyme, are not the same.

The exclusive occurrence of C at position 32 in eukaryotic t^6A-containing tRNAs is not applicable for *E. coli* t^6A-containing tRNAs in which a C_{32} and, less frequently, a U_{32}, are found. Also, several t^6A-containing eukaryotic initiator $tRNA^{Meti}$ (anticodon CAU), including that of *X. laevis*, have C_{33} instead of the almost universally conserved U_{33} (Wegnez et al., 1975), whereas a few mitochondrial t^6A-containing tRNAs have Ψ, C, or G at position 38 (Sprinzl et al., 1996). Thus, comparison of tRNAs from different organisms bearing t^6A_{37} indicates that semi-invariant nucleotides at positions 32 (pyrimidine), 33 (U), and 38 (purine) do not have the same importance as the major identity determinant U_{36} and the targeted A_{37}. They might control the efficiency of the threonylation reaction as we observed with $tRNA^{Meti}$ and $tRNA^{Val\text{-}1}$ bearing C_{38}.

In contrast to the above conclusions for t^6A_{37} formation, the isopentenylation of A_{37} (i^6A_{37}) in other tRNAs catalyzed by *E. coli* tRNA Δ^2-isopentenyl-pyrophosphate:tRNA Δ^2-isopentenyl transferase obeys distinct rules: strict dependence on the nucleotide sequence $A_{36}A_{37}A_{38}$, strict requirement for a seven-membered anticodon loop, and limited tolerance on mispairing in the anticodon stem (Motorin et al., 1997). Obviously, the major identity elements that are required by two tRNA modification systems acting on the same target A_{37} are different. Hence, one should beware of generalizations.

MATERIALS AND METHODS

Plasmids, enzymes, and chemicals

Most of the variants of synthetic yeast initiator $tRNA^{Met}$ ($ytRNA^{Meti}$) and of synthetic yeast $tRNA^{Val}$ ($ytRNA^{Val}$) used in this work have been described and used elsewhere (Senger et al., 1992, 1995). A few additional variants of yeast $tRNA^{Meti}$ were derived from the above mutants by site-directed mutagenesis according to Kunkel et al. (1991). They were all cloned in the multicloning site of a pUC118 phagemid under the control of an upstream T_7 promoter and a linearizing *Bst*N 1 (*Mva* 1) restriction site downstream of the gene. To facilitate gene transcription by T_7 polymerase, all tRNA variants were derived from a mutant of $ytRNA^{Meti}$ in which adenosine-1 of the amino acid stem was changed in guanosine (mut#1, in Fig. 5A and Table 2). The reasoning and procedures for construction of plasmids containing minimalist $tRNA^{Meti}$ (lacking D-loop and stem or T-loop and stem, but preserving some 3D architecture) are detailed in Senger et al. (1995) and Senger and Fasiolo (1996). The plasmid containing yeast intronless $tRNA^{Ile}$ (anticodon UAU) was described in Szweykowska-Kulinska et al. (1994). The synthetic *E. coli* $tRNA^{Thr4/2}$ gene (anticodon CGU), reconstituted from six synthetic oligomers, was cloned under the T7 promoter in the pEMBL8$^+$ plasmid and this was a kind gift of Dr. J. Caillet (IBPC, Paris). Large-scale plasmid preparations were done according to standard procedures (Sambrook et al., 1989). The [α-^{32}P]-radiolabeled NTP (where N is U, C, A, or G; 400 Ci/mmol) was from Amersham (UK). Bacteriophage T_7 RNA polymerase and RNasin were from Promega Biotech (Madison, Wisconsin), *Mva* 1 restriction enzyme from Boehringer-Mannheim (Germany), *Penicillium citricum* nuclease P1 and ribonuclease T2 from Sigma (St Louis, Missouri), and thin layer cellulose plates from Schleicher & Schuell (Dassel, Germany). DNA T_7-sequencing kit for verifying each sequence of the variant tRNA genes was from Pharmacia P-L Biochemicals (Sweden). All other chemicals were from Merck Biochemicals or Boehringer-Mannheim (Germany).

Preparation of radiolabeled tRNA transcripts

The procedure for producing tRNA substrates is essentially as described earlier (Grosjean et al., 1990) except that 10 mM GMP was added. Briefly, 2 μg of *Mva* 1-linearized DNA template was incubated in 10 μL of transcription buffer containing 50 μCi (100 μCi in the case of GTP) of one of the four [α-^{32}P] NTP (400 Ci/mmol), 0.3 μL of RNasin (40 U/μL), and 1 μL of T_7 RNA polymerase (20 U/μL). Each nucleotide triphosphate in the mixture was at 1 mM final concentration except the radiolabeled [^{32}P]NTP, for which the final concentration was 0.1 mM (0.25 in the case of GTP). The transcription mixture was incubated for 4 h at 37 °C. The runoff tRNA transcripts were then purified by 6% polyacrylamide gel electrophoresis in the presence of 8 M urea. Only full-length transcript, as revealed by autoradiography of the gel, was used for microinjection experiments. It was recovered from the gels by overnight elution with a salt solution containing 0.5 M ammonium acetate buffer, pH 5.0, 10 mM Mg acetate, 0.1 mM EDTA, 0.1% (w/v) SDS at room temperature. The eluted tRNA was ethanol-precipitated at −20 °C, washed twice with cold 70% (v/v) ethanol, dried under vacuum, and redissolved in 5 μL of dust-free and diethylpyrocarbonate pre-treated water. Final clarification of the tRNA solution was always done by quick centrifugation in an Eppendorf centrifuge just before filling the microinjecting micropipettes.

Oocyte microinjections and recovery of tRNAs

Adult female *X. laevis* were from South Africa and purchased in France through the "Centre d'Elevage de Xénopes,"

University of Rennes (F-35042, France). A piece of ovary was surgically removed from non-hormone-stimulated female and placed in a Petri dish containing modified Barth's solution (MBS) as described (Peng, 1991), supplemented with L-glutamine (0.08 g/L), oxaloacetic acid (0.15 g/L), Na pyruvate (0.11 g/L), 1% of MEM vitamins solution (Gibco-BRL), penicillin G (10 μg/L), streptomycin sulphate (10 μg/L), and phenol red (20 μg/L). Individual oocytes were manually separated with watchmaker's forceps and sorted to select fully grown stage VI oocytes (the collagenase–pronase treatment was avoided because of the presence of RNAse contamination). They were stored in the dark overnight at 19–20 °C. Approximately 4–20 fmol of [^{32}P]-labeled tRNA transcripts (corresponding to about 10–50 $\times$ 10^3 Cerenkov cpm) in a volume of 40–50 nL were injected into the cytoplasm of the vegetal yellow hemisphere of each oocyte using homemade microcapillary glass needles and an air pressure microinjection system. Each tRNA variant was injected into a series of 40–60 oocytes. Injected oocytes were kept at 20 °C in fresh MBS solution as described above. Groups of 6–10 "good looking" oocytes, with no white spot on the dark pole, were withdrawn after the indicated duration (3–48 h) and quickly frozen at −80 °C until the nucleic acids were extracted and submitted to electrophoresis on 6% polyacrylamide gel. Only full-length [^{32}P]-labeled tRNAs were recovered from such fractionation and further analyzed for their modified ribonucleotide content as described below.

Analysis of modified nucleotides

Full-length tRNAs isolated from oocytes were digested exhaustively overnight at 37 °C with nuclease P1 (0.3 U/μL) in 20 μL of 50 mM ammonium carbonate buffer at pH 5.3 to produce 5′-ribonucleotide monophosphates or with RNase T2 (0.01 U/μL) in 20 μL of 50 mM ammonium carbonate at pH 4.5 to produce 3′-ribonucleotide monophosphates. Carrier yeast total tRNA (about 1 μg) was routinely added in the mixture. The resulting hydrolyzates were each analyzed by two-dimensional chromatography on thin-layer cellulose plates in two types of solvent system. System "N/R" was isobutyric acid:25% NH_4OH:water (66:1:33, v/v/v) in the first dimension and 0.1 M sodium phosphate at pH 6.8:solid $(NH_4)_2SO_4$:*n*-propanol (100:60:2, v/w/v) in the second dimension. Solvent system "N/N" was the same solvent as above for the first dimension and 2-propanol:HCl 37%:water (68:17.6:14.4, v/w/v) in the second dimension. After autoradiography, identification of radioactive spots was done by comparison with published reference maps (Keith, 1995). Quantification of each radioactive modified nucleotide was performed after scratching the corresponding spots from the plate and counting the radioactivity by liquid scintillation technique or, more recently, by counting the radioactivity directly from the plate with a PhosphorImager (Molecular Dynamics, USA), using ImageQuant software. Knowing the nucleotide composition of each of the tRNA substrates, the molar ratio of each individual modified nucleotide per mole of tRNA formed during incubation in the oocytes was calculated. By comparing the results from different independent experiments, reproducibility was estimated to be $\pm$0.15 mol/tRNA mol (on average, see Tables 1 and 2).

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