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Major Identity Determinants for Enzymatic Formation of Ribothymidine and Pseudouridine in the TΨ-loop of Yeast tRNAs

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Almost all transfer RNA molecules sequenced so far contain two universal modified nucleosides at positions 54 and 55, respectively: ribothymidine (T_{54}) and pseudouridine (Ψ_{55}). To identify the tRNA elements recognized by tRNA:m^{5}uridine-54 methyltransferase and tRNA:pseudouridine-55 synthase from the yeast *Saccharomyces cerevisiae*, a set of 43 yeast tRNA^{Asp} mutants were used. Some variants contained point mutations, while the others included progressive reductions in size down to a tRNA minisubstrate consisting of the TΨ-loop with only one G·C base-pair as stem (9-mer). All substrates (full-sized tRNA^{Asp} and various minihelices) were produced *in vitro* by T7 transcription and tested using yeast extract (S100) as a source of enzymatic activities and *S*-adenosyl-L-methionine as a methyl donor.

The results indicate that the minimal substrate for enzymatic formation of Ψ_{55} is a stem/loop structure with only four G·C base-pairs in the stem, while a longer stem is required for efficient T_{54} formation. None of the conserved nucleotides (G_{53}, C_{56}, A_{58} and C_{61}) and U_{54} for Ψ_{55} or U_{55} for T_{54} formation can be replaced by any of the other three canonical nucleotides. Yeast tRNA:m^{5}uridine-54 methyltransferase additionally requires the presence of a pyrimidine-60 in the loop. Interestingly, in a tRNA^{Asp} variant in which the TΨ-loop was permuted with the anticodon-loop, the new U_{32} and U_{33} residues derived from the TΨ-loop were quantitatively converted to T_{32} and Ψ_{33}, respectively. Structural mapping of this variant with ethylnitrosourea confirmed that the intrinsic characteristic structure of the TΨ-loop was conserved upon permutation and that the displaced anticodon-loop did not acquire a TΨ-loop structure. These results demonstrate that a local conformation rather than the exact location of the U-U sequence within the tRNA architecture is the important identity determinant for recognition by yeast tRNA:m^{5}uridine-54 methyltransferase and tRNA:pseudouridine-55 synthase.

Keywords: tRNA; modification; pseudouridine; ribothymidine; identity

Introduction

To date, the primary sequences of more than 500 tRNA species from a large variety of organisms have been determined (Sprinzl *et al.*, 1996). All of these mature tRNA molecules invariably contain a variety of different modified nucleosides which are synthesized after transcription of the corresponding tRNA genes, during the complex maturation process. Some modifications are unique for a given tRNA species and/or for a tRNA from a given organism, like the Wye-37 (wybutosine) in eukaryotic tRNA^{Phe}, 1-methylinosine-37 in eukaryotic tRNA^{Ala} or archaeosine-15 in tRNA of most archaebacteria. Many other modified nucleosides, like 5-methyluridine (ribothymidine) at position 54 and pseudouridine at position 55 in the tRNA TΨ-loop are present in almost all tRNA species from all organisms examined so far (Grosjean *et al.*, 1995). While T_{54} is not found in tRNAs from *Mycoplasma* (Andachi *et al.*, 1989) or from some archaebacteria

Abbreviations used: Ψ, pseudouridine; T(m^{5}U), ribothymidine; SAM, *S*-adenosyl-L-methionine; DTT, dithiothreitol; DTE, dithioerythritol; ENU, ethylnitrosourea; NTP, nucleoside triphosphate; 2D t.l.c., two-dimensional thin-layer chromatography; 3D, three-dimensional.

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(Gupta, 1984), Ψ_{55} is universally present in most (if not all) tRNAs from every living organism. The only exceptions are tRNAs harbouring a non-conventional sequence in their TΨ-loop (see below).

Concerning tRNA-modifying enzymes, two important questions need to be addressed: (i) at which stage of the complex maturation process does the modification of a given nucleoside occur? and (ii) how do(es) the corresponding enzyme(s) recognize the target nucleoside(s) within the tRNA architecture? In eukaryotic cells, early steps of tRNA maturation obviously occur within the nucleus. By microinjecting a yeast tRNA^{Tyr} gene into the nucleus of *Xenopus* oocytes, Nishikura & De Robertis (1981) were able to show that of the 17 modified nucleosides found in the full-sized matured gene products, T_{54} and Ψ_{55} were among the earliest ones that appeared in a primary transcript (104 nt), still harbouring unprocessed 5′ and 3′ terminal extensions and an intron. Similarly, analysis of the nucleoside composition of various precursor tRNAs, such as pre-tRNA^{Phe} or pre-tRNA^{Tyr} which accumulate at the non-permissive temperature in yeast temperature-sensitive (ts) mutants, reveals the presence of both T_{54} and Ψ_{55}, along with a few other modified nucleosides (Knapp *et al*., 1979; reviewed by Hopper & Martin, 1992). The microinjection of various 3D-structure mutants of yeast tRNA^{Asp} and tRNA^{Asp} fragments demonstrated that these ''early'' modifications correspond to the tRNA-modifying enzymes which are not dependent on overall 3D tRNA structure (Grosjean *et al*., 1996). These findings suggest that the enzymatic machinery leading to formation of T_{54} and Ψ_{55} is not sensitive to the overall 3D architecture of tRNA but rather depends on some local identity elements (a few selected nucleotides and/or a special conformation and/or a particular flexibility) that are characteristic of the tRNA TΨ-stem/loop. The discovery that *Escherichia coli* tRNA:-m^{5}U$_{54}$-methyltransferase (also referred to as RUMT) efficiently recognizes and methylates U_{54} within a synthetic minisubstrate consisting of only the TΨ-stem/loop (17-mer) (Gu & Santi, 1991; Guenther *et al*., 1994; Gu *et al*., 1996) strongly supports this hypothesis. In the latter case, systematic analysis of synthetic minihelix variants led to the conclusion that the specific structure of the TΨ-loop plays the key role in the interaction with *E. coli* RUMT (Gu *et al*., 1996). Moreover, detailed analysis of the catalytic mechanism of *E. coli* RUMT also reveals that some important conformational change in the vicinity of U_{54} should occur in order that the target carbon-5 of U_{54} becomes methylated by the enzyme (Kealey *et al*., 1994). Some analogy may even exist with the well characterized ''flipping out'' of the target base in double-stranded DNA by the cytosine-5 DNA methyltransferases, uracil-DNA glycosylase and several other enzymes (reviewed by Roberts, 1995).

In contrast, almost nothing is known concerning the identity elements that are required for modification by tRNA:Ψ_{55} synthase. This enzyme catalyses the isomerization of U_{55} to Ψ_{55} by the cleavage of the glycosidic bond, rotation of the base (or a conformational change of the enzyme), formation of a new carbon–carbon bond and release of Ψ_{55}-containing tRNA (Kammen *et al*., 1988). However, the detailed mechanism of this reaction, which is probably common to all the RNA:Ψ synthases studied so far, remains unsolved (Cortese *et al*., 1974; Kline & Söll, 1982; Samuelsson, 1991; Ivanetich & Santi, 1992). The tRNA:Ψ_{55} synthase has been purified from *E. coli* and cloned (Nurse *et al*., 1995). The recombinant protein was shown to be highly specific for U_{55} in the TΨ-loop of most tRNAs. Prior formation of T_{54} is clearly not required since T7 tRNA transcripts lacking T_{54} were efficiently modified into Ψ_{55}. Dependence on higher-order structure of the RNA substrate was ruled out based on the fact that efficient pseudouridylation occurred in the absence of Mg^{2+} in the reaction mixture (Nurse *et al*., 1995). However, the only direct evidence that the activity of tRNA:Ψ_{55} synthase, as well as of several other enzymes acting on the TΨ-loop region, indeed does not require the correct folding of tRNA came from microinjection experiments using tRNA^{Asp} fragments (Grosjean *et al*., 1996).

Here we have studied the structural requirements for tRNA recognition by *Saccharomyces cerevisiae* tRNA:m^{5}U$_{54}$-methyltransferase and tRNA:Ψ_{55} synthase using yeast tRNA^{Asp} as a model system. Our results confirm that neither of these enzymes from an homologous yeast extract, requires the complete 3D architecture of the tRNA substrates, since they efficiently recognize and modify various fragments of tRNA as small as stem/loop minihelices. However, whereas tRNA:Ψ_{55} synthase is active on a minisubstrate containing only four base-pairs in the stem, tRNA:-m^{5}U$_{54}$-methyltransferase requires a longer stem and probably some other additional interactions with the substrate to achieve efficient modification.

Results

Consensus sequence for yeast tRNAs containing T and/or Ψ

Inspection of 45 yeast cytoplasmic tRNAs sequenced so far (Sprinzl *et al*., 1996) reveals a remarkable conservation of the nucleotides at six positions in the TΨ-stem/loop, namely G_{53}, T_{54}, Ψ_{55}, C_{56}, A_{58} and C_{61} (Figure 1a). The only exception concerns the yeast cytoplasmic tRNA$_i^{Met}$ where A_{54}, A_{60} and unmodified U_{55} (instead of Ψ_{55}) were found (Figure 1b) (Simsek & RajBhandary, 1972). The same observation was made for most cytoplasmic tRNA$_i^{Met}$ from various eukaryotic origins (*Drosophila*, *Xenopus laevis*, rabbit liver and human placenta; Sprinzl *et al*., 1996). A survey of the entire tRNA bank confirms that the conserved residues listed above (Figure 1a) are found in the vast majority of cytoplasmic elongator tRNAs, irrespec-

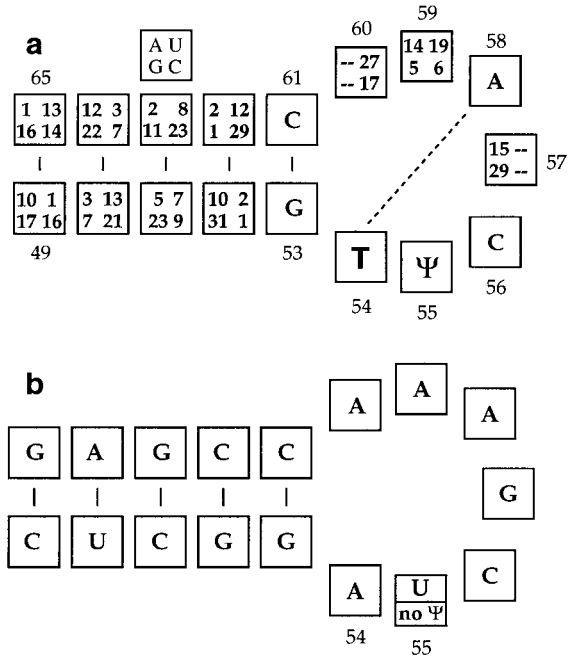

Figure 1. (a) Consensus sequence of the TΨ-stem/loop in all yeast tRNAs modified at positions 54 and 55. (b) Sequence of TΨ-stem/loop of yeast initiator $tRNA_i^{Met}$, unmodified at position 55. Numbers within the squares show the frequency of corresponding nucleotides, in the order indicated in the box shown above the stem. The reverse-Hoogsteen base-pair *in trans* between T_{54} and A_{58} is designated by a broken line.

tive of their origin. However a few interesting exceptions are worth pointing out.

The nucleotide sequences of the TΨ-loop in two cytoplasmic $tRNA^{Ala}$ from *Bombyx mori* are identical to that of yeast $tRNA_i^{Met}$ (Figure 1b), but in those cases U_{55} is modified into Ψ_{55} (Sprague *et al.*, 1977). The same applies to human $tRNA^{Ala}$ in which a guanosine was found at position 60 (Bunn & Mathews, 1987). Although the TΨ-loop of bovine liver $tRNA^{Asp}$ differs from the consensus sequence at position 60 (A_{60}), the U_{54}-U_{55} sequence is still modified to T_{54}-Ψ_{55} (Vakharia & Singhal, 1982). Another deviation from the consensus sequence was found in rat liver $tRNA_1^{Val}$ which has A_{59} and A_{60}. In this tRNA U_{54} remains unmodified, yet Ψ_{55} is still present (Jank *et al.*, 1977). Therefore, assuming that the yeast modifying enzymes behave as the higher eukaryotic ones, the absence of Ψ_{55} in most eukaryotic $tRNA_i^{Met}$ cannot be explained solely by the presence of A_{54} and by A or G at position 60. On the other hand, although the TΨ-loop of a few eukaryotic tRNAs has rigorously the same consensus nucleotide sequence as that presented in Figure 1a, U_{54} was found either unmodified (as in cytoplasmic $tRNA^{His}$ from sheep liver or *Drosophila*), or modified to 2′ *O*-methyl-T_{54} (Tm, in many higher eukaryotic tRNAs) or even to Ψ_{54} (as in human $tRNA^{Asn}$ and in $tRNA^{Gln}$ from human, rat/mouse and bovine livers (Nishikura & De Robertis, 1981; Grosjean *et al.*, 1996; Sprinzl *et al.*, 1996). Thus, identity elements for enzymatic formation of T/Tm or Ψ at position 54 of cytoplasmic tRNAs seems to be distinct from those for the enzymatic formation of Ψ_{55}.

Here, we tried to determine the importance of the 3D architecture as well as of the highly conserved bases in the TΨ-stem/loop for T_{54} and Ψ_{55} formation in yeast tRNAs.

Experimental approach

Our earlier work pointed out that formation of T_{54} and Ψ_{55} is not affected in most 3D mutants of yeast $tRNA^{Asp}$ microinjected in *Xenopus* oocytes. Moreover, among all of the nucleotide modifications, enzymatic formation of Ψ_{55} was by far the least sensitive to tRNA architecture (Grosjean *et al.*, 1996). Based on these findings, we proceeded further to define the minimal substrate still capable of being modified by both tRNA:m^5U_{54}-methyltransferase and tRNA:Ψ_{55} synthase, using an *in vitro* homologous yeast system. To this goal, T_7 transcripts of 41 variants of the synthetic yeast $tRNA^{Asp}$ gene were prepared (Figure 2). They correspond to a progressive reduction in the size of the tRNA molecule (ten variants, Figure 2a and b), mutations in the T-stem region (six variants, Figure 2c), changes in TΨ-loop size (three variants, Figure 2e), and mutations in the seven-membered TΨ-loop (22 variants, Figure 2d and f). The content of modified nucleotides in each substrate was measured after incubation with S100 yeast extract as described in Materials and Methods and as is illustrated in Figure 3 (examples of 2D-t.l.c. analysis), Figure 4 (time-courses of T_{54} and Ψ_{55} formation) and summarized in Table 1. The experiments with yeast S100 extract tested without addition of methyl-donor (SAM) demonstrate the independence of Ψ_{55} formation from parallel T_{54} modification (data not shown). All experiments in this study were performed in the presence of SAM.

Wild-type $tRNA^{Asp}$ contains eight modified bases (Ψ_{13}, D_{16}, D_{20}, Ψ_{32}, m^1G_{37}, m^5C_{48}, T_{54} and Ψ_{55}) and most of them can be easily formed *in vitro* using yeast extract as enzyme source (except D_{16} and D_{20}; Edqvist *et al.*, 1993). However, all of these modifications, except T_{54} and Ψ_{55}, disappear completely in minihelix substrates.

Size-reduction of tRNA has no drastic effect on T_{54} and Ψ_{55} formation

Figure 3 shows the formation of T_{54} and Ψ_{55} in the wild-type yeast $tRNA^{Asp}$ (a) and in the variant of $tRNA^{Asp}$ with deleted D-stem/loop (mut #1) (b) (see also corresponding kinetics in Figure 4a and b). Clearly, in both cases, ΨMP and TMP are formed and each corresponds to about one mole per mole RNA (Table 1). Further deletion of the tRNA anticodon-stem/loop (mut #2) leading to a minihelix structure containing only the acceptor

stem directly attached to the TΨ-stem of tRNA gave qualitatively the same results as for the wild-type $tRNA^{Asp}$ and tRNA mut #1, except that T_{54} formation was slightly less efficient (Table 1 and Figure 4). These results demonstrate that the recognition elements for both modification enzymes are mainly confined to the amino acid acceptor stem prolonged by the TΨ-stem/loop (domain I, see Grosjean *et al.*, 1996).

Stepwise reduction of the size of this domain I (mut #3 to mut #10 as shown in Figure 2) clearly dramatically affects the formation of T_{54} while the formation of Ψ_{55} remains efficient down to a mini-substrate that comprises the TΨ-loop and four

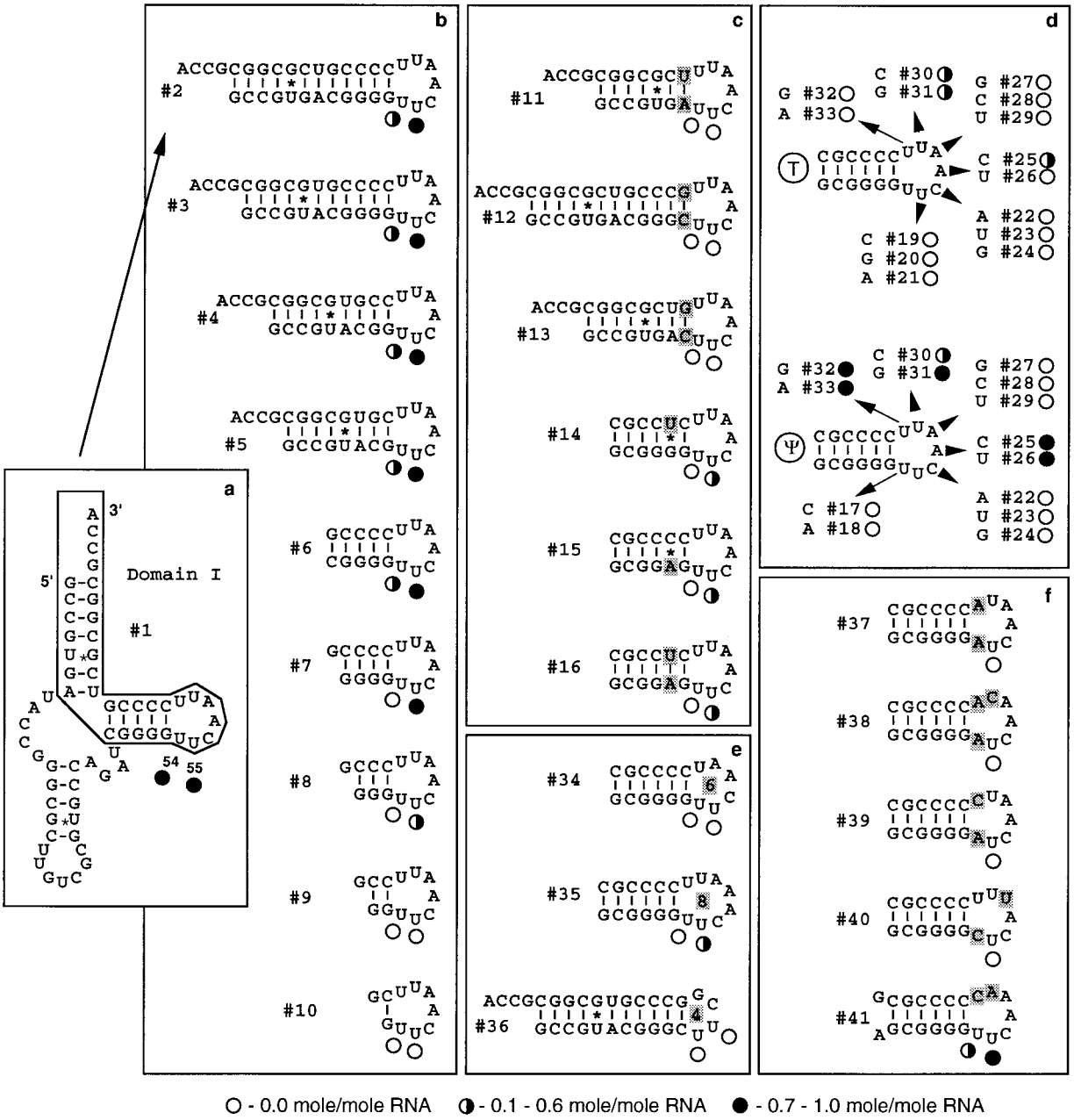

Figure 2. Structures of tRNA mutants and minihelices tested as substrates of tRNA:m^5U_{54}-methyltransferase and tRNA:Ψ_{55} synthase from yeast. $tRNA^{Asp}$(GUC) with a deleted D-stem/loop (a), minihelices of different stem lengths (b), minihelices with mutations in the TΨ-stem (c) and in the TΨ-loop (d). Minihelices with reduced or increased loop sizes and with multiple mutations in the loop are presented in (e) and (f). The part of the $tRNA^{Asp}$ sequence referred to as Domain I and corresponding to minihelix mutant #2 is boxed in (a). Short RNA minihelices (mut #8, #9 and #10) are probably not folded into stem/loop structure at working temperature (30°C). Mutation sites are shown by shaded letters. Modification level for T_{54} and Ψ_{55} after incubation with yeast extract is schematically indicated by circles. Empty circles represent the absence of modification, half-filled circles correspond to 0.1 to 0.6 mole of modified nucleotide/mole RNA and filled circles correspond to 0.7 to 1.0 mole/mole RNA. All substrates are numbered as in Table 1.

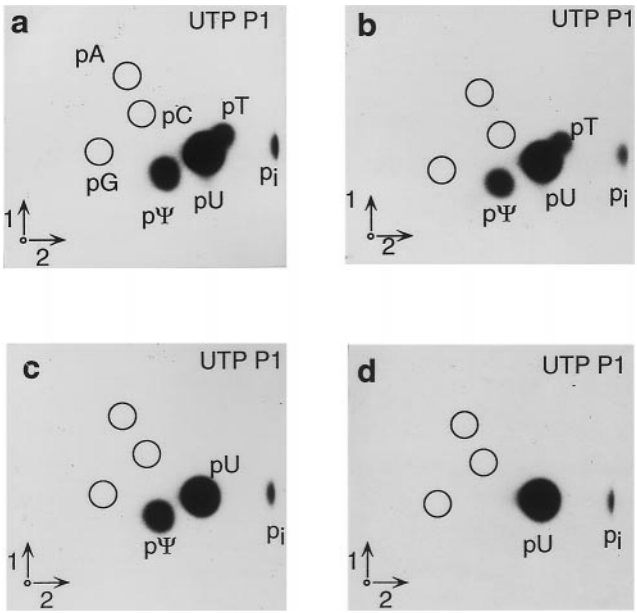

Figure 3. Autoradiography of two-dimensional thin-layer chromatographic resolution of 5′-UMP, 5′-ΨMP and 5′-TMP formed in RNA substrates upon incubation at 30°C in the presence of a S100 yeast extract. All substrates were internally labelled by UTP and digested by nuclease P_1 (labelled UTP P1 on Figure) before 2D t.l.c. separation using the chromatographic system (N/N) described previously (Silberklang *et al.*, 1979). Formation of ribothymidine and pseudouridine in wild-type $tRNA^{Asp}$ (a), $tRNA^{Asp}$ with deleted D-stem/loop (mut #1) (b), minihelix substrate #32 (c) and minihelix substrate #9 (d).

base-pairs in the stem (Table 1 and Figure 4a and b). Only a trace amount of Ψ_{55} is formed in a mini-substrate with three base-pairs in the TΨ-stem, while the minihelices with two or one base-pair (see Figures 3d,4a, b) are no longer modified. These results are most likely explained by inappropriate folding of these minimalist molecules, unable to form a stable loop-structure at 30°C, as used for incubation.

$G_{53}\cdot C_{61}$ and at least two Watson-Crick base-pairs in the proximal TΨ-stem are major determinants for U_{54} and U_{55} modifications

The importance of the stem sequence and, in particular, of the universally conserved $G_{53}\cdot C_{61}$ base-pair, was tested with several RNA variants (mut #11 to 16). The results presented in Table 1 demonstrate that no traces of T_{54} and Ψ_{55} were formed in minisubstrates containing $A_{53}\cdot U_{61}$ (mut #11) or $C_{53}\cdot G_{61}$ (mut #12 and 13). These results emphasize the crucial importance of G_{53} for maintaining a correct local structure of the TΨ-loop by stabilizing the reverse-Hoogsteen base-pairing *in trans* between T or U at positions 54 and A_{58} (Westhof *et al.*, 1983; Romby *et al.*, 1987). Moreover, the introduction of a wobble G*U or an A*C mismatch at positions 52·62 (mut #14 and 15) dramatically decreased, but did not completely abolish, the efficiency of Ψ_{55} formation in minisubstrates. Restoring a Watson-Crick base-pair at position 52·62 (mut #16), instead of a mismatch A*C or wobble G*U, partially reinstated the synthesis of Ψ_{55} by tRNA:Ψ_{55} synthase.

TΨ-loop size critically affects the efficiency of modification

Dependence of pseudouridylation and methylation reactions on the size of the TΨ-loop was tested using three different mutants (mut #34 to 36, Figure 2e), bearing four, six or eight nucleotides in the loop. As shown in Table 1, the formation of T_{54} was completely abolished in all three variants,

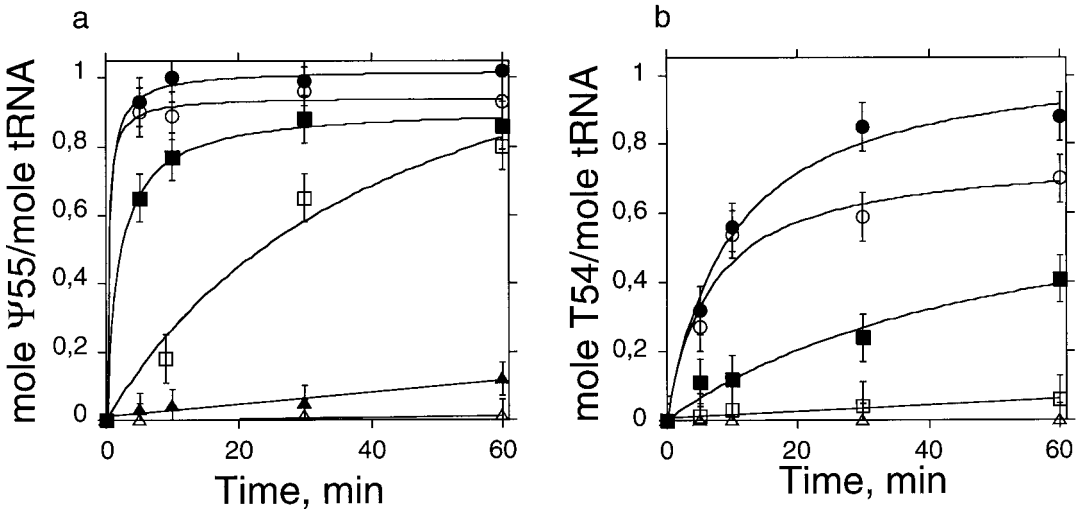

Figure 4. Time course of pseudouridine-55 (a) and ribothymidine-54 (b) formation in wild-type $tRNA^{Asp}$ and selected minihelix variants upon incubation at 30°C in the presence of SAM and yeast S100 extract. Wild-type $tRNA^{Asp}$(●), $tRNA^{Asp}$ variant with deleted D-stem/loop (mut #1) (○), minihelix variant #2 (12 base-pairs) (■), minihelix #7 (four base-pairs) (□), minihelix #8 (three base-pairs) (▲), minihelix #9 (two base-pairs) (△).

while a small but significant amount of Ψ_{55} was detected for the mutant with eight nucleotides in the loop. These results allow us to conclude that, in addition to the conserved $G_{53}\cdot C_{61}$ base-pair in the proximal TΨ-stem, the size of the loop also plays an important role for RNA substrate recognition by tRNA:Ψ_{55} synthase. Comparing the results of mut #36 to those of mut #3 (both variants have the same stem length) brings further support for the importance of the loop size.

All conserved nucleotides in the TΨ-loop are important determinants for recognition

The consensus sequence shown in Figure 1a reveals that four out of the six strictly conserved residues in the TΨ-stem/loop are located in the loop (U_{54}, U_{55}, C_{56} and A_{58}). In order to test the importance of each individual nucleotide of the loop in the efficiency of the pseudouridylation and methylation reactions, we prepared and tested 17 additional variants of minisubstrates with point mutations at positions 54 to 60 (mut #17 to 33, Figure 2d). For three of the strictly conserved nucleotides (positions 55, 56 and 58), all possible substitutions were tested, while for the other positions (54, 57, 59 and 60), we tested only those substitutions that correspond to bases occurring rarely (or not at all; Figure 1a).

The results show that the substitution of any of the strictly conserved residues in positions 54, 55, 56 and 58, abolished completely the formation of both T_{54} and Ψ_{55} (Figure 2d and Table 1). Nucleotide substitutions at other locations in the loop only moderately affected both pseudouridylation and methylation, except at position 60 for which the presence of a pyrimidine was found to be essential only for U_{54} methylation (Table 1, see also Figure 2c). Thus, these results reveal one subtle difference between identity requirements for Ψ_{55} and T_{54} formation within the TΨ-loop.

Multiple point mutations in the TΨ-loop were also tested (Figure 2f). Indeed, inspection of the tRNA data bank reveals the existence of a few eukaryotic tRNA sequences which bear an adenosine at position 54, and yet are still modified to Ψ_{55}, as in the case of *Bombyx mori* or human cytoplasmic $tRNA^{Ala}$ (Sprague *et al.*, 1977; Bunn & Mathews, 1987). These tRNAs with ''abnormal'' consensus sequences in the TΨ-loop have an additional peculiarity, precisely at position 60: purine (A or G) was found instead of the semi-conserved pyrimidine (C or U). Therefore, the possibility was envisaged that a double mutation consisting of A_{54} associated with A or G at position 60 might ''rescue'' the enzymatic formation of Ψ_{55}. The results with RNA variants mut #37, 38 and 39 clearly demonstrated that, at least with the S100 yeast extract, a second mutation at position 60, or two mutations at positions 60 and 61 did not restore Ψ_{55} formation, even after prolonged incubation (Table 1).

Table 1. Effect of mutations in the $tRNA^{Asp}$ TΨ-stem/loop on the extent of modifications upon incubation with yeast S100 extract at 30°C for one hour

RNA	Mutations	T_{54}	Ψ_{55}
Yeast $tRNA^{Asp}$, wt		**0.8**	**1.0**
A. *Truncated $tRNA^{Asp}$*			
Mut #1	Deletion of D-stem/loop	**0.7**	**0.9**
B. *Minisubstrates of different lengths* (TΨ- and acceptor-stem)			
Mut#2	12 bp	**0.4**	**0.9**
Mut#3	11 bp	**0.2**	**1.1**
Mut#4	10 bp	**0.3**	**1.0**
Mut#5	9 bp	**0.2**	**1.0**
Mut#6	5 bp	**0.4**	**0.7**
Mut#7	4 bp	**<0.1**	**0.8**
Mut#8	3 bp	**<0.1**	**0.1**
Mut#9	2 bp	0.0	0.0
Mut#10	1 bp	0.0	0.0
C. *Mutations in TΨ-stem*			
Mut#11	G53·C61 → A53·U61	0.0	0.0
Mut#12	G53·C61 → C53·G61	0.0	0.0
Mut#13	G53·C61 → C53·G61	0.0	0.0
Mut#14	G52·C62 → G52·U62	**<0.1**	**0.2**
Mut#15	G52·C62 → A52·C62	**<0.1**	**0.2**
Mut#16	G52·C62 → A52·U62	**<0.1**	0.5
D. *Mutations in TΨ-loop*			
Mut#17	U54 → C	NA	0.0
Mut#18	U54 → A total tRNA	NA	0.0
Mut#19	U55 → C	0.0	NA
Mut#20	U55 → G	0.0	NA
Mut#21	U55 → A	0.0	NA
Mut#22	C56 → A	0.0	0.0
Mut#23	C56 → U	0.0	0.0
Mut#24	C56 → G	0.0	0.0
Mut#25	A57 → C	**0.1**	**0.9**
Mut#26	A57 → U	**<0.1**	**0.8**
Mut#27	A58 → G	0.0	0.0
Mut#28	A58 → C	0.0	0.0
Mut#29	A58 → U	0.0	0.0
Mut#30	U59 → C	**0.6**	**0.6**
Mut#31	U59 → G	**0.3**	**0.7**
Mut#32	U60 → G	0.0	**0.7**
Mut#33	U60 → A	0.0	**1.1**
E. *TΨ-loops of different size*			
Mut#34	6 nucleotides	0.0	**<0.1**
Mut#35	8 nucleotides	0.0	**0.1**
Mut#36	4 nucleotides	0.0	0.0
F. *Multiple mutations in TΨ-loop*			
Mut#37	U54 → A54 and U60 → A60 U54 ← A54 and U60 → A60	NA	0.0
Mut#38	andU59 → C59	NA	0.0
Mut#39	U54 → A54 and U60 → C60	NA	0.0
Mut#40	U54 → C54 and A58 → U58	NA	0.0
Mut#41	U59 → A59 and U60 → C60	**0.3**	**0.7**
G. *Inversion of TΨ- and anticodon-loops*			
Mut#42	In a Domain I substrate	0.0	0.0
Mut#43	In a truncated tRNA	**0.8**	**0.9**

All values are mole of modified nucleoside per mole RNA, with an accuracy of about 0.1 mole/mole RNA. All time-courses were done for 5, 10, 30 and 60 minutes of incubation at 30°C. Only the data for the 60 minute point are given in the Table.

Taking into account that certain base-pairs, like the unusual C·U interaction *in trans*, might allow the restoration of a correct spatial arrangement of the nucleotides in the TΨ-loop similar to that of the

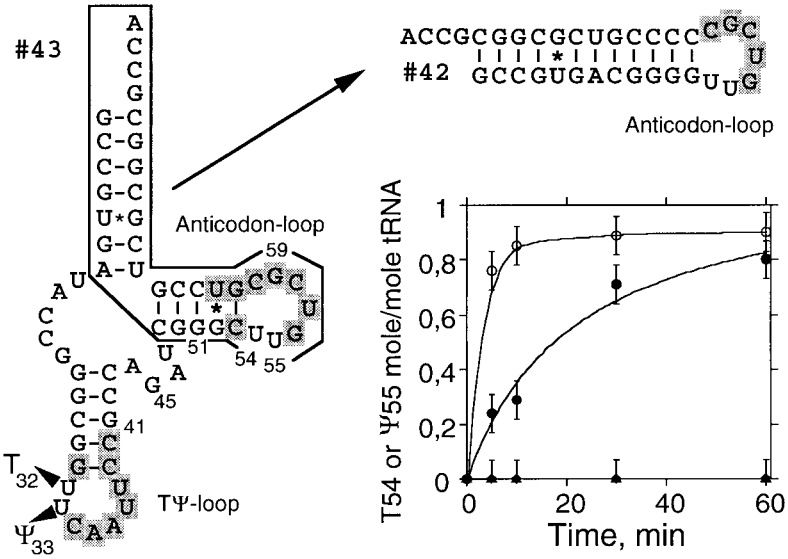

Figure 5. Sequences of $tRNA^{Asp}$ mutants with deleted D-stem/loop and permuted TΨ- and anticodon-loops (#43) and of a minihelix bearing the sequence of the anticodon loop in place of the TΨ-loop (#42). Mutations are shown by shaded letters. The time courses of Ψ_{55} and T_{54} formation are shown on the graph. Minihelix variant #42: formation of T_{54} (▲) and Ψ_{55} (◇); tRNA mutant with permuted TΨ- and anticodon-loops (#43): formation of T_{54} (●) and Ψ_{55} (○).

characteristic $U_{54}\cdot A_{58}$ reverse Hoogsteen base-pair (discussed by Dirheimer *et al.*, 1995), tRNA variant mut #40 was tested. The result (Table 1) shows that no trace of Ψ_{55} was formed, thus suggesting that U_{54} is a major determinant for recognition by the yeast enzyme or that a putative $U_{58}\cdot C_{54}$ interaction *in trans* cannot restore the appropriate local architecture that would allow yeast tRNA:Ψ_{55} synthase to modify U_{55}.

Finally, attempts were made to reduce the number of uridines in the minisubstrate to a single residue. If successful, this would allow the use of photoreactive analogues of UTP (such as s^4UTP) in the T7 transcription reaction to selectively label only U_{54} and/or U_{55}, for application in tRNA-enzyme adduct formation. The mutant #38 containing only one uridine at position 55 was not modified to Ψ55 (Table 1). However, the substrate (mut #41) containing two uridines at positions 54 and 55 was modified to Ψ_{55} to a reasonable extent. This observation reinforces the hypothesis that U_{54} is part of the recognition element that is needed by the yeast tRNA:Ψ_{55} synthase in order to catalyse the isomerization reaction.

The TΨ-loop as such rather than its location within the tRNA molecule is essential for both T_{54} and Ψ_{55} formation

Because $U_{32}U_{33}$ within the seven-membered anticodon loop in a large majority of tRNAs sequenced so far were never found to be modified to $T_{32}\Psi_{33}$, it was questionable whether a transplantation experiment in which the TΨ-loop or TΨ-loop with two adjacent base-pairs of the stem replacing the anticodon loop and *vice versa* would allow $T_{32}\Psi_{33}$ formation. To this end, we constructed the two chimeric tRNA variants mut #42 (replacement of TΨ-loop only) and mut #43 (replacement of TΨ-loop with two adjacent base-pairs of the stem; Figure 5).

RNA mut #42 bears the sequence of the $tRNA^{Asp}$ anticodon loop in the minihelix structure as defined above. As expected, $U_{32}U_{33}$ now in a context similar to the TΨ-loop but lacking the TΨ-loop consensus nucleotides C_{56} and A_{58}, were not modified into TΨ (Figure 5). In contrast, $U_{54}U_{55}$, now located in positions of an anticodon loop (RNA mut #43), but bearing each of the identity elements defined above, was efficiently modified to $T_{54}\Psi_{55}$, which now correspond to what we would conventionally designate as $T_{32}\Psi_{33}$. Interestingly, enzymatic formation of both T and Ψ were as efficient as the formation of T_{54} and Ψ_{55} in the wild-type $tRNA^{Asp}$ or its variant mut #1 (compare Figure 5 with Figure 4a and b). Therefore, this transplantation experiment confirms that the identity elements that were defined above are sufficient for RNA recognition by both the yeast tRNA:Ψ_{55} synthase and tRNA:m^5U_{54}-methyltransferase.

Conformation of TΨ-loop is not dependent on its location within the tRNA molecule

X-ray crystallography and chemical probing of tRNAs in solution have previously shown that the TΨ-loop of tRNA has a rather characteristic conformation, mostly stabilized by the stacking of the $G_{53}\cdot C_{61}$ base-pair over the $T_{54}\cdot A_{58}$ reverse *trans*-Hoogsteen base-pair. These pairings within the loop and the proximal stem induce a characteristic bulged-out conformation of the two pyrimidines at positions 59 and 60 (Westhof *et al.*, 1983; Romby *et al.*, 1987; reviewed by Dirheimer *et al.*, 1995). Data on structural mapping of tRNA in solution with the alkylating reagent ethylnitrosourea strongly suggest that the phosphate at position 60 is hydrogen bonded to residues 61 (O1(P60)···N4(61)) as well as to the ribose of A_{58} (O2(P60)···O2′(A58)) (Romby *et al.*, 1987). This characteristic protection of P_{60} against alkylation led us to probe and compare the structural proper-

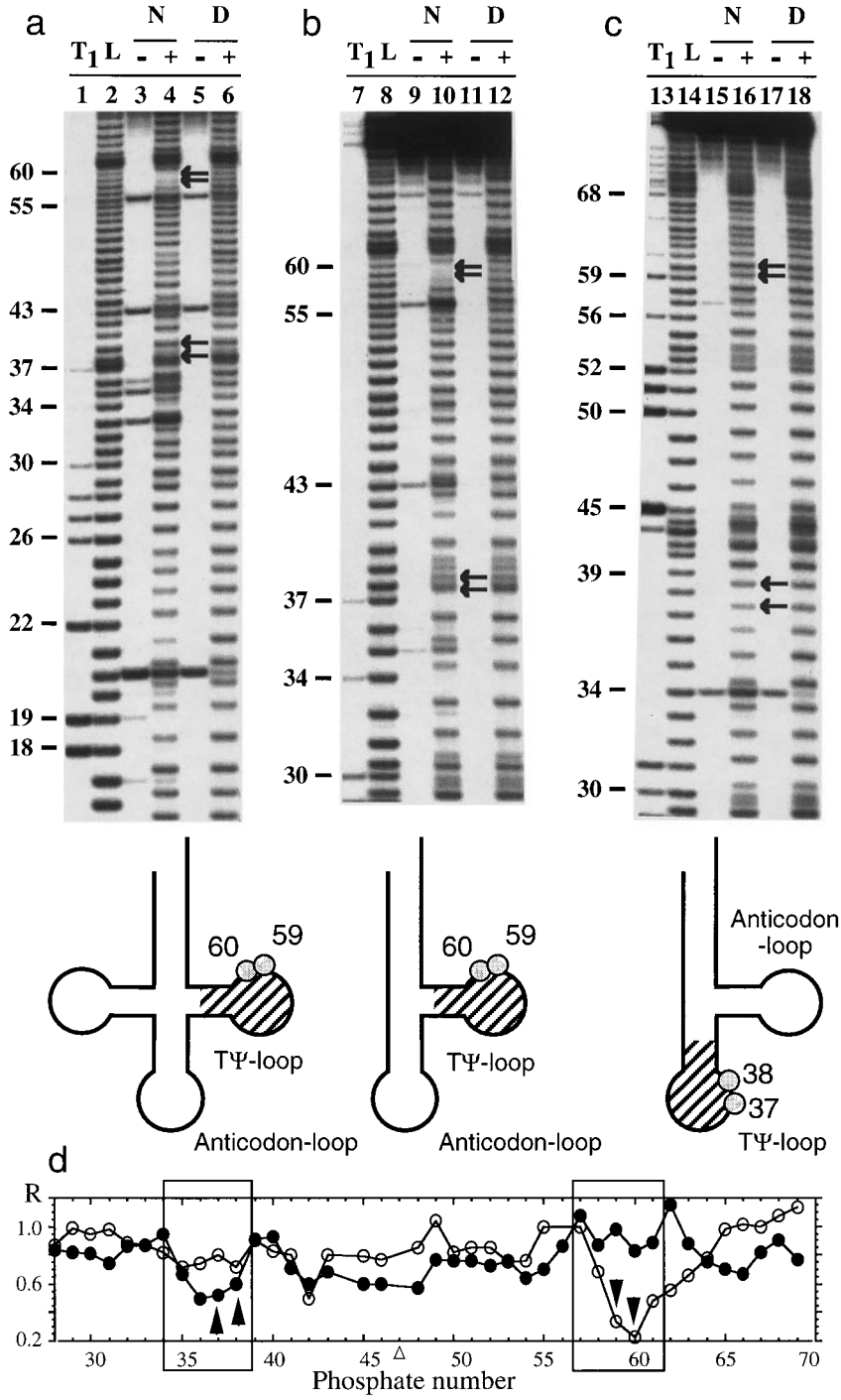

Figure 6. Structural probing with ethylnitrosourea of wild-type $tRNA^{Asp}$(GUC) (a), tRNA variant with deleted D-stem/loop (mut #1) (b), and $tRNA^{Asp}$ mutant with deleted D-stem/loop and permuted TΨ and anticodon loops (#43) (c). Autoradiographs of 12% polyacrylamide/8 M urea gels and location of non-reactive phosphates on the corresponding secondary structures of the transcripts are shown. Lanes 1, 7, 13 and 2, 8, 14 present T_1 and alkaline digests of corresponding transcripts, respectively. Numbers on the left of each panel indicate nucleotide positions within the corresponding tRNA. Ethylnitrosourea treatment was performed in native (lanes 4, 10, 16) and denaturing (lanes 6, 12, 18) conditions. Other lanes correspond to control incubations in the absence of ENU. Bands appearing in controls (lanes 3, 5, 9, 11, 15, 17) correspond to spontaneous degradation of RNA at Py/A sites. Arrows highlight strategic phosphate positions. (d) Patterns of phosphate reactivity towards ethylnitrosourea in two $tRNA^{Asp}$ variants: $tRNA^{Asp}$ with deleted D-stem/loop (mut #1, ○) and $tRNA^{Asp}$ with deleted D-stem/loop and permuted TΨ- and anticodon-loops (mut #43, ●). *R* values are ratios between the intensities of the corresponding electrophoretic bands of the alkylated folded and unfolded RNAs. These intensities were measured by quantification of each band on the gels using a PhosphorImager counter. A ratio $R < 1$ means that the alkylation of a given phosphate is lower in the folded RNA. The open triangle indicates the nucleotide 47 missing in $tRNA^{Asp}$. Arrows point to the phosphates protected against ENU alkylation.

ties of the different loops in two of the $tRNA^{Asp}$ variants, namely tRNA mut #1 and tRNA mut #43, using the wild-type $tRNA^{Asp}$ as control.

Figure 6 shows the results of such chemical probing. The extent of phosphate alkylation, as measured by radioactivity counting of the different bands of the ladder, confirms that the deletion of D-stem/loop in mut #1 (Figure 6b) does not change the accessibility of P_{59} and P_{60} compared to wild-type $tRNA^{Asp}$ (Figure 6a, shown by arrows). These results are consistent with the previously observed general conservation of the ENU-alkylation profile for the 3′-half $tRNA^{Asp}$ molecule and for isolated TΨ-stem/loop (Romby *et al.*, 1987). In the case of tRNA mutant #43, with permuted TΨ- and anticodon-loops, the accessibility of P_{37} and P_{38} (analogues of P_{59} and P_{60} in TΨ-loop) becomes greatly reduced (Figure 6c, shown by arrows). Figure 6d shows the quantitative analysis of the protection pattern for $tRNA^{Asp}$ mut #1 and $tRNA^{Asp}$ mut #43 with inverted anticodon and TΨ-loops. From this experiment it was concluded that the characteristic protection pattern, and consequently the conformation of the TΨ-loop, has been maintained independently from its location within the tRNA molecule. Therefore,

the observed reduction of reactivity of both P_{37} and P_{38} to alkylation correlates well with the appearance of T and Ψ modifications at positions 32 and 33, respectively in tRNA mutant #43 with the permuted loops.

Discussion

Because certain modified nucleosides in tRNA are common to all tRNA species while others are restricted to one or only a few tRNAs, one can expect that the enzymes forming them are sensitive to different elements in the tRNA architecture. The enzymes catalysing the formation of ''unique'' modified nucleotides might depend on a very specific feature present only in selected tRNA substrates, while those catalysing the formation of the frequently found ''common'' modified nucleotides might depend on general gross features of the tRNA structure and/or on only a few local elements that are common to all tRNA molecules.

Here, the enzymatic formation of the two most common modified nucleotides in tRNA, ribothymidine and pseudouridine located, respectively, at positions 54 and 55 in the TΨ-loop, has been studied. The results clearly indicate that a correct L-shaped architecture of the tRNA molecule is dispensable for recognition. Instead, a limited region of tRNA domain I, which includes only a portion of the TΨ-arm (for tRNA:Ψ_{55} synthase) or the TΨ-arm prolonged by the amino acid-acceptor stem (for tRNA:m^{5}U$_{54}$-methyltransferase), plays a major role in the reaction. However, the sequence of nucleotides in the stem, except at positions 53 and 61, which has to be a $G_{53}\cdot C_{61}$ base-pair, has no apparent influence on the efficiency of T_{54} and Ψ_{55} formation. Our results also demonstrate that all of the nucleotides that are almost universally conserved in the TΨ-loop and its proximal stem are essential identity elements for tRNA recognition by yeast tRNA:Ψ_{55} synthase. On the other hand, while minisubstrates remain efficiently modified by tRNA:Ψ_{55} synthase down to four base-pairs in the stem, the tRNA:m^{5}U$_{54}$-methyltransferase clearly requires a more complex RNA structure for efficient recognition and modification. This was already pointed out for the corresponding enzyme from *E. coli*, which is considerably more efficient with entire tRNA molecules than with minihelix substrates and depends also on the 3D-structure stabilizing interactions between tRNA TΨ- and D-loops (Kealey *et al.*, 1994).

Swapping the anticodon-loop and TΨ-loop in yeast tRNA^{Asp} mutant #1 clearly demonstrates that the major identity elements for both tRNA:Ψ_{55} synthase and tRNA:m^{5}U$_{54}$-methyltransferase are located within the TΨ-loop of tRNA. Figure 7a and b illustrates the overall folding of the TΨ-loop of yeast tRNA^{Asp} compared to that of the anticodon loop of the same tRNA molecule. In both cases the loops are formed of seven nucleotides but their spatial arrangement, as determined by X-ray crystallography (Westhof & Sundaralingam, 1986; Westhof *et al.*, 1988) and chemical probing (Romby *et al.*, 1987) is very different. In the structure of the TΨ-loop, the stacking of the $G_{53}\cdot C_{61}$ base-pair over the $U/T_{54}\cdot A_{58}$ reverse-Hoogsteen base-pair *in trans*, leads the two pyrimidines at positions 59 and 60 to be bulged out, while in the anticodon loop, no such interaction *in trans* and particular deformation of the nucleotide chain are observed.

Chemical mapping with ethylnitrosourea revealed that such a characteristic TΨ-loop confor-

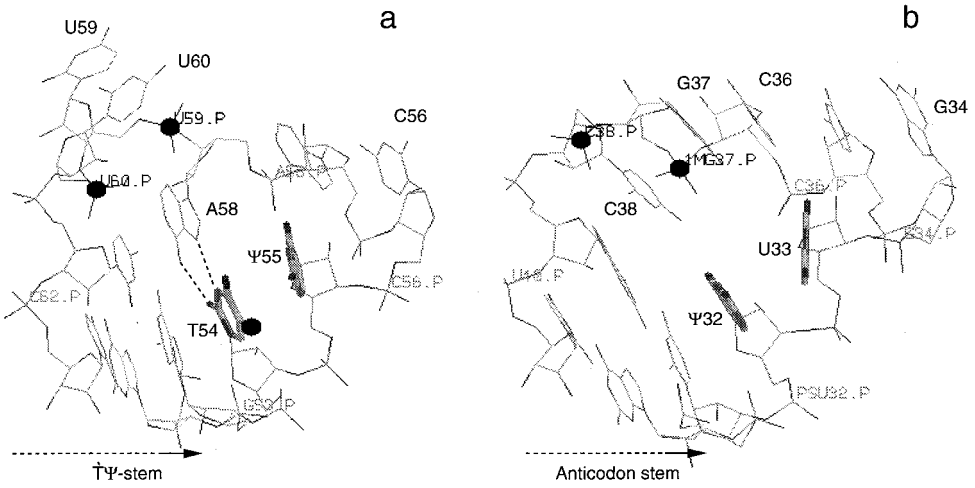

Figure 7. Structural models of tRNA^{Asp}(GUC) TΨ-loop (a) and anticodon-loop (b) presented in similar orientation. The closest two base-pairs of the corresponding stem are designated by broken arrows. Phosphate residues P_{59} and P_{60} and analogous residues P_{37} and P_{38} are presented by dark points (spheres). Bases T_{54}, Ψ_{55}, C_{56}, A_{58}, U_{59}, U_{60} in the TΨ-loop and the corresponding positions (Ψ_{32}, U_{33}, G_{34}, C_{36}, G_{37} and C_{38}) in the anticodon loop are indicated. Hydrogen bonds of reverse *trans*-Hoogsteen base-pair $T_{54}\cdot A_{58}$ in the TΨ-loop structure are shown by broken lines (see also Figure 1). Positions of phosphates P_{53}, P_{56}, P_{58}, P_{62} in the TΨ-loop and P_{32}, P_{34}, P_{36} and P_{40} in the anticodon loop are presented by shadowed letters. Crystallographic coordinates are as given by Westhof *et al.* (1988).

mation is related to the particular loop sequence and does not depend on its location in the tRNA molecule. Since enzymatic formation of both T_{54} and Ψ_{55} ''follows'' the TΨ-loop in the tRNA mut #43 with permuted TΨ and anticodon-loops, one can conclude that it is merely this characteristic TΨ-loop conformation and/or its particular flexibility rather than the location of the U-U sequence within the overall tRNA structure that determines the capability of both tRNA:m$^5U_{54}$-methyltransferase and tRNA:Ψ_{55} synthase to recognize the substrate and effectively catalyse the formation of T_{54} and Ψ_{55}. The crucial importance of base-pair $G_{53}\cdot C_{61}$ for both enzymes confirms this view. It is linked to its role in maintaining a correct local structure of the TΨ-loop by stabilizing the reverse-Hoogsteen base-pairing *in trans* between T or U at position 54 and A_{58}.

Our results on the major identity elements in the TΨ-loop minihelix required for T_{54} formation are different from those obtained for the corresponding enzyme from *E. coli* (Gu & Santi, 1991; Gu *et al*., 1996). Yeast tRNA:m$^5U_{54}$-methyltransferase is more sensitive to the length of the minihelix stem than is the homologous *E. coli* enzyme, and possibly also requires additional minor (yet unidentified) identity elements for efficient methylation of U_{54} into m$^5U_{54}$ (T). Indeed, as shown by Gu & Santi (1991), a low level of U_{54} methylation by *E. coli* enzyme was still detectable for a minihelix with two base-pairs in the stem (11-mer), when tested at 15°C, while in our case no T_{54} formation was detected in a similar minisubstrate incubated at 30°C with yeast extract. A more important deviation was observed with respect to the tolerance of mutations at the first base-pair in the stem. While *E. coli* tRNA:m$^5U_{54}$-methyltransferase appears to be insensitive to nucleotide substitutions at these locations, the yeast counterpart strictly requires the $G_{53}\cdot C_{61}$ base-pair. The size of the loop is also crucial and here both enzymes demonstrate a strong preference for a seven-membered loop. As far as the nucleotide composition of the loop is concerned, the *E. coli* enzyme is clearly more tolerant to nucleotide substitutions within the consensus nucleotides than is the yeast enzyme. Thus the recognition of tRNA by yeast tRNA:m$^5U_{54}$-methyltransferase appears more stringent than in the case of the *E. coli* enzyme and evidently requires additional identity elements outside the TΨ-stem/loop. Alternatively the specificity of the tRNA:m$^5U_{54}$-methyltransferase at low concentration in the complex mixture of enzymes and proteins present in a S100 extract could be different from that observed in the case of a purified recombinant enzyme used at a high ratio of enzyme/substrate. This situation may be analogous to that already pointed out by Yarus (1972) in relation to the specificity of the aminoacylation reaction catalysed by the different aminoacyl-tRNA synthetases. In the latter case, it was demonstrated that misaminoacylation of tRNA by certain amino acids occurs to a hardly detectable level *in vivo* or with a crude extract as a source of enzyme, whereas it rises significantly when assayed *in vitro* with purified tRNA and aminoacyl-tRNA synthetase (Swanson *et al*., 1985; Schulman, 1991).

Minihelices bearing A_{54} in combination with other mutations do not sustain Ψ_{55} formation, whereas in several naturally occurring tRNAs (such as *B. mori* and human tRNA^{Ala}) harbouring those structural features, U_{55} is modified to Ψ_{55} (Sprague *et al*., 1977; Bunn & Mathews, 1987). This may suggest that, in addition to the major identity determinants defined in minisubstrates, certain other combinations of ''compensatory mutations'' within the tRNA architecture (in the D-loop which normally interacts with the TΨ-loop or in the variable loop which provide some flexibility to domain I; Nazarenko & Uhlenbeck, 1995) allow a correct spatial presentation of the TΨ-loop in such A_{54}-containing molecules. A similar hypothesis was proposed by Kealey *et al*. (1994) to explain the differences in recognition patterns between minisubstrates and the whole tRNA molecule by *E. coli* tRNA:m$^5U_{54}$-methyltransferase. Again, an alternative explanation may be that the yeast tRNA:Ψ_{55} synthase has more stringent requirements than the homologous enzyme from higher eukaryotes. Similar situations exist for the heterologous enzymatic formation of m$^2G_{26}$ and m$^1A_{58}$ in yeast tRNA^{Asp}, a tRNA that naturally lacks these two nucleotide modifications (Gangloff *et al*., 1972). As expected, when the corresponding tRNA transcript is incubated with a yeast extract, these two nucleoside modifications do not occur (Edqvist *et al*., 1994; H.G., unpublished results). However, the enzymatic formation of m$^2G_{26}$ and m$^1A_{58}$ occurs at about one mole per mole of tRNA^{Asp} transcript upon incubation with *Xenopus* oocyte extract or on microinjection into the cytoplasm of *Xenopus* oocytes (Edqvist *et al*., 1993; Grosjean *et al*., 1996).

Regarding the observation that modification is not dependent on the tRNA architecture, the present *in vitro* results obtained using a yeast extract fully confirm earlier observations on the recognition of tRNA by *Xenopus* oocyte enzymes *in vivo* (Grosjean *et al*., 1996), except for one notable peculiarity. While the tRNA^{Asp} molecule lacking the D-stem/loop (see mut #1 in Figure 2) was modified to T_{54} and Ψ_{55} by the *Xenopus* enzyme similarly to the yeast enzyme, the minisubstrate consisting of only the domain I of the tRNA molecule (see mut #2 in Figure 2) was efficiently modified to Ψ at both positions 54 and 55 only in the oocytes. In the presence of a yeast extract, the same RNA mut #2 behaved more ''normally'' in the sense that T_{54} and Ψ_{55} were the only products detected at these positions. It is noteworthy that upon microinjection of the gene of yeast tRNA^{Tyr} into the nucleus of *Xenopus laevis* oocytes, it was also noticed that U_{54} was modified to Ψ_{54} (80%) and T_{54} (only 10%) (Nishikura & De Robertis, 1981). It could well be that, for certain tRNAs in higher eukaryotic cells, there is competition

between the site-specific tRNA:m^{5}U$_{54}$-methyltransferase and a yet unidentified RNA:Ψ synthase, distinct from the site-specific tRNA:Ψ$_{55}$ synthase. The analogous location of U$_{54}$ in the TΨ-loop and of U$_{32}$ in the anticodon loop that is also modified to Ψ$_{32}$ in certain tRNAs (Wrzesinski *et al.*, 1995; Grosjean *et al.*, 1996; Sprinzl *et al.*, 1996) suggests that Ψ$_{54}$ (but not Ψ$_{55}$) and Ψ$_{32}$ may be formed by the same enzyme.

Here, the use of RNA minisubstrates obtained by T$_7$ transcription of synthetic oligodeoxynucleotides allowed us to systematically investigate the effect of reducing the length of the amino acid-acceptor and TΨ-stems, the effect of TΨ-loop size as well as almost all possible point mutations in the TΨ-loop. The same approach was used earlier to study various RNA-protein interactions and to identify major determinants for tRNA recognition by several aminoacyl-tRNA synthetases (reviewed by Giegé *et al.*, 1993; Martinis & Schimmel, 1995), by the *E. coli* elongation factor Tu (Rudinger *et al.*, 1994, 1996; Nazarenko & Uhlenbeck, 1995), by RNase P (Carrara *et al.*, 1995) and by the *E. coli* tRNA:m^{5}U$_{54}$-methyltransferase (Gu & Santi, 1991; Gu *et al.*, 1996). From the structural point of view, the mechanism of interaction and recognition of RNA minisubstrates by modification enzymes (tRNA:Ψ$_{55}$ synthase and tRNA:m^{5}U$_{54}$-methyltransferase) should be closer to the case of tRNA recognition by the prokaryotic elongation factor Tu than to the model proposed for several aminoacyl-tRNA synthetases. While most aminoacyl-tRNA synthetases interact with large domains of the L-shaped tRNA structure including both amino acid-acceptor and anticodon-stems (Cavarelli & Moras, 1993), the elongation factor Tu (Nissen *et al.*, 1995) and probably the modification enzymes acting on the TΨ-loop approach the tRNA molecule by binding to the domain I (TΨ-stem prolonged by amino acid-acceptor stem). However, in spite of these similarities, the detailed mechanism of interaction appears to be different for *E. coli* or *T. thermophilus* EF-Tu, yeast tRNA:Ψ$_{55}$ synthase and yeast or *E. coli* tRNA:m^{5}U$_{54}$-methyltransferases. While the sequence (and consequently the structure) of the TΨ-loop does not play any important role in tRNA recognition by EF-Tu (Rudinger *et al.*, 1994), the identity determinants located in this loop are crucially important for the recognition by the corresponding modification enzymes.

Enzymatic catalysis is a multistep process which involves substrate binding, chemical reaction(s) within the enzyme active site and the release of the resulting product(s). Complex conformational changes of the enzyme and/or substrate (here a macromolecule) are often implicated. Attempts to define the smallest minisubstrate which can perform accurately the various steps of this complex process allow us to define only the major structural features (identity elements) that are absolutely necessary to the reaction. However, the additional parameters (minor identity determinants, the competition with the other enzymes/substrates in the system) have to be taken into account in order to fully understand the observed efficiency and accuracy of the enzymatic reaction.

Materials and Methods

Reagents

[α-^{32}P]UTP and [γ-^{32}P]ATP (400 and 3000 Ci/mmol, respectively) were from Amersham (UK). Tris, DTT, nucleoside triphosphates, spermidine hydrate, *Penicillium citricum* nuclease P$_1$, *Aspergillus oryzae* RNase T$_2$ and *N*-nitroso-*N*-ethylurea (ENU) were from Sigma (USA), *Aspergillus oryzae* RNase T$_1$ from Pharmacia (Sweden), bacteriophage T7 RNA polymerase, restriction enzymes, calf intestine alkaline phosphatase and IPTG from MBI Fermentas (Vilnius, Lithuania), RNasin from Promega (USA), and T4 DNA ligase, polynucleotide kinase and *S*-adenosyl-L-methionine (SAM) from Boehringer-Mannheim (Germany). T7 RNA polymerase for preparative *in vitro* transcription was purified according to a previously published procedure (Becker *et al.*, 1996). Thin-layer cellulose plates were from Schleicher & Schuell (Germany). All other chemicals were from Merck Biochemicals (Germany).

Deprotected chemically synthesized oligonucleotides were purchased from Genset (France) except for mutants #2, #3, #4, #5, #11, #12, #13, #36 and #42, which were described previously (Rudinger *et al.*, 1994). The plasmids bearing the genes of wild-type yeast tRNA^{Asp} and its variants mut #1 and mut #18 were described elsewhere (Perret *et al.*, 1990; Puglisi *et al.*, 1993).

Synthesis of RNA substrates

In vitro transcription of the cloned tRNA^{Asp} genes (wild-type, mut #1 and mut #18) with radiolabelled [α-^{32}P]UTP was done as described previously (Auxilien *et al.*, 1996). The minihelices and most of the TΨ-stem/loop variants of tRNA^{Asp} were prepared by *in vitro* transcription of single-stranded DNA templates (minus strand). DNA templates were obtained by annealing of a 17-mer oligonucleotide TAATACGACTCACTATA (corresponding to the T7 promoter) to the oligonucleotide containing the complementary sequence of the T7 promoter and of the desired RNA transcript. Equal molar amounts of the two oligonucleotides (25 pmol of each) were mixed in a reaction buffer containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM DTE and 2 mM spermidine. The mixture was incubated for three minutes at 85°C and then cooled slowly over a period of two hours to room temperature. Transcription was performed for three hours at 37°C in the standard reaction buffer containing 1 mM each of CTP, GTP and ATP; 250 μM UTP, 50 μCi [α-^{32}P]UTP, 0.01% (v/v) Triton X-100, 10 mM 5′-GMP, 800 units/ml of RNasin and 1000 units/ml of T7 RNA polymerase. The resulting T7 runoff RNA transcripts were purified to single nucleotide resolution by electrophoresis in 15% (w/v) polyacrylamide gels in the presence of 8 M urea as described earlier (Edqvist *et al.*, 1995).

The RNA transcript of mut #43 (61-mer) with inverted anticodon and TΨ-loops was prepared by T7 transcription using a complete double-stranded DNA template. The template was obtained by stepwise hybridization and subsequent ligation of two sets of five complementary and partially overlapping synthetic oligonucleotides (pre-

viously purified by electrophoresis on a 15% denaturing polyacrylamide gel). Prior to the hybridization step, all synthetic oligonucleotides were phosphorylated at their 5′-ends using ATP and T4 polynucleotide kinase. Ligation reaction was performed overnight at 16°C with T4 DNA ligase as described earlier (Perret *et al.*, 1990; Auxilien *et al.*, 1996). The length of the resulting product (78-mer) was controlled by electrophoresis on a 2% agarose gel.

Large scale *in vitro* transcription of synthetic tRNA genes

Large amounts of wild-type $tRNA^{Asp}$ and $tRNA^{Asp}$ mut #1 transcripts needed for chemical probing were prepared by *in vitro* transcription of the corresponding linearized plasmids using T7 polymerase, by scaling up the same reaction mixture as described above, except that 4 mM of NTP (including unlabelled UTP) was used. For large-scale *in vitro* transcription of $tRNA^{Asp}$ mut #43, the ligation product (described above) was first inserted into the *Sma*I site of pUC118 plasmid. Linearization for transcription was then performed using the *Bam*HI site located downstream from the inserted sequence. The digested plasmid (50 μg) was added to 500 μl reaction mixture containing 40 mM Tris-HCl (pH 8.1), 20 mM $MgCl_2$, 1 mM spermidine, 5 mM DTE, 0.05% Triton X-100, 4 mM each of GTP, UTP, CTP, ATP and T7 RNA polymerase (0.13 mg/ml). After four hours incubation at 37°C, the resulting RNA transcript was purified by electrophoresis under denaturing conditions. tRNA transcripts were dephosphorylated by alkaline phosphatase and 5′-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described elsewhere (Vlassov *et al.*, 1981; Romby *et al.*, 1987).

Enzyme assays

Yeast S100 extract, prepared as described earlier (Auxilien *et al.*, 1996), was used as a source of enzymatic activities for both tRNA:m$^5U_{54}$-methyltransferase and tRNA:Ψ_{55} synthase. Enzymatic formation of Ψ and T in the various RNA transcripts was measured in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$, 2 mM DTT, 0.1 mM EDTA, 100 mM NH_4-acetate, 20 μM of SAM, eight units of RNasin and 50 to 100 fmoles (1 to 2 nM final concentration) of ^{32}P-labelled RNA substrate in a total volume of 50 μl. Reaction was initiated by addition of S100 yeast extract (0.5 to 1 mg protein/ml final concentration). After incubation at 30°C for the indicated time (see Table 1 and Figure legends), samples were treated as described previously (Auxilien *et al.*, 1996). The presence of pseudouridine and ribothymidine in RNA hydrolysates was detected by 2D thin-layer chromatography as described (Auxilien *et al.*, 1996). The relative amount of ribothymidine and/or pseudouridine formed as compared to the total amount of uridine in each RNA transcript was measured by counting the radioactivity in each of the spots corresponding to UMP, ΨMP and/or TMP. Quantification was performed using a PhosphorImager counter (Molecular Dynamics, USA). For substrates corresponding to short RNA transcripts (mut #7 to 10), we observed that significant amounts of RNA duplexes were readily formed in solution at room temperature. Thus, for all RNA substrates tested, such duplexes were systematically dissociated prior the addition of S100 extract and SAM, by heating the reaction mixture for three minutes at 85°C and subsequent chilling on ice.

Alkylation of tRNAs by ethylnitrosourea, splitting of the tRNAs at phosphotriester positions and analysis of the liberated oligonucleotides

Phosphate alkylation in tRNA by ethylnitrosourea (ENU) was performed under both native and denaturing conditions (Vlassov *et al.*, 1981; Romby *et al.*, 1985). Under native conditions, tRNA transcripts (about 10^5 cpm of radioactive tRNA, supplemented with 2 μg RNA of the corresponding unlabelled tRNA transcript), were alkylated for three hours at 30°C in 20 μl of 300 mM sodium cacodylate buffer (pH 8.0) containing 2 mM EDTA, 20 mM $MgCl_2$, 100 mM NaCl and 5 μl of a saturated solution of ethylnitrosourea in ethanol. With denatured tRNA molecules, alkylation was performed at 80°C for two minutes in the same buffer as above except that no $MgCl_2$ and NaCl were present.

In both cases the alkylated tRNA transcripts were recovered by ethanol precipitation. They were cleaved at phosphotriester positions in Tris-HCl buffer (pH 9.0) as described (Vlassov *et al.*, 1981). The cleavage pattern was analysed on a 12% polyacrylamide gel in denaturing conditions. The positions of alkylated and non-alkylated sites in tRNA transcripts were identified using the partial ribonuclease T_1 and alkaline digests as ladders (Donnis-Keller *et al.*, 1977). The extent of phosphate alkylation was quantified by counting the radioactivity on a PhosphorImager.

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