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Pleiotrophic effects of point mutations in yeast tRNA^{Asp} on the base modification pattern

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ABSTRACT

The base-modification pattern has been studied in several synthetic variants of yeast tRNA^{Asp} injected into *Xenopus laevis* oocytes. Certain point mutations in the D-stem and the variable loop of the tRNA led to considerably decreased levels of m^{1}G37, Ψ40 and Q34/manQ34 in the anticodon stem or loop and an increased rate of synthesis for m^{5}C49 in the T-stem. The formation of m^{2}G6 in the aminoacyl-stem was not affected in any of the tRNA-variants. Thus, mutations in one part of the tRNA-molecule can have long-range effects on the interactions between another part of the tRNA and the tRNA modifying enzymes.

INTRODUCTION

The presence of modified nucleosides is one of the most distinctive structural features of tRNA molecules. The formation of base modifications is a post-transcriptional event, and thus a part of the maturation of the precursor tRNAs. More than 75 modified nucleosides have been isolated (1). The structure of most of them is known (2) and their biological functions are now successively being revealed (3).

The interactions between modifying enzymes and tRNAs have not yet been characterized as extensively as those between tRNA and aminoacyl-tRNA-synthetases (4). One reason for this lack of information is that it has been difficult to obtain pure enzymes that are sufficiently active under *in vitro* conditions. Moreover, the enzymatic cofactors that need to be added *in vitro* are in many cases not known and tRNA-substrates that specifically lack the nucleoside modification of interest are scarce (5–8).

One way to circumvent these problems is to microinject synthetic or semi-synthetic tRNAs into *Xenopus laevis* oocytes (9, 10), a system that is inherently optimized for all kinds of tRNA modifications, also those requiring cofactors. Due to its efficiency this *in vivo* test system has been widely used, e.g. to clarify the step-wise maturation of the transcription product from an injected yeast tRNA gene (11). It was also used to show the effects of anticodon point mutations on the potential of the nucleotides at position 34 and 37 in the anticodon loop of yeast tRNAs to be modified. These experiments resulted in that some modification enzymes (12–15) were suggested to have identity elements in close vicinity of the position to be modified, while others (15–17) are less sensitive to the neighbouring nucleotide sequences and recognize identity elements distal from the site of the modification. However, since the anticodon point mutations used in these cases were not affecting the three-dimensional structure of the tRNA-molecule nothing could then be said if the tRNA conformation was important to the recognition by the anticodon modifying enzymes.

The completeness of the oocyte test system led us to utilize this system when we started our studies on the requirements for formation of N^2,N^2-dimethylguanosine in position 26 (m^{2}2G26) in yeast tRNAs. Completely unmodified tRNA^{Asp}, chosen as model tRNA because its 3D-structure is very well characterized (18, 19), was microinjected into *X.laevis* oocytes. By this method we demonstrated that specific sequences neighbouring position 26 and also certain structural parameters in the tRNA were needed for efficient enzymatic formation of m^{2}2G26 (20). The long-range effects of mutations in the variable loop we then found for the synthesis of m^{2}2G26 prompted us to investigate if also the formation of modifications in the anticodon loop were sensitive to point mutations situated far away in the tRNA. Indeed, the data presented here show that the formation of many tRNA-modifications were altered when certain mutations were introduced into the tRNA at a distance from the site of the modification. Mutations in the D-stem and/or the variable loop in tRNA^{Asp} caused drastically decreased levels of m^{1}G37, Q34, manQ34 and Ψ40 in the anticodon loop/stem, while the m^{2}G6 formation was unaffected and the rate of m^{5}C49-formation was enhanced.

MATERIALS AND METHODS

Bacteria used for plasmid propagation, plasmids (pTFMa, pTFMa200, pTFMa204, pTFMa207, pTFMa209, pTFMa210, pTFMa214 and pTFMa215) carrying variants of synthetic yeast tRNA^{Asp}-genes (a, a200, a204, a207, a209, a210, a214, a215) and the preparation of ^{32}P-G labelled tRNA transcripts with T7 RNA polymerase have been previously described (20). In all the tRNA^{Asp} variants used in this study the basepair U1-A72 has been exchanged for G1-C72 as U1 is known to be an unfavourable start for the T7 RNA polymerase (21). This change

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does not influence the cognate aminoacylation (21). Furthermore, all normally occurring modifications in yeast $tRNA^{Asp}$ were found in almost stochiometric amounts after injection of the wt-like $tRNA^{Asp}$a into *X.laevis* oocytes (Table 1). The exchange of U1-A72 for G1-C72 is therefore not considered to disturb the global structure of the tRNA or the interactions between the tRNA and the tRNA modifying enzymes.

The ^{32}P-G labelled $tRNA^{Asp}$ variants were microinjected into the cytoplasm of *X.laevis* oocytes (10, 20). Modification of tRNAs normally occur in the nucleus on pre-tRNAs that are trimmed before passage out to the cytoplasm (11). We injected synthetic, completely unmodified, mature sized tRNA molecules into the cytoplasm, since it has been shown that all the different modifying activities are present also in the cytoplasm (10). Although pre-tRNAs are the biologically normal substrate for modifications in the cell, endtrimmed unmodified tRNAs are known to function equally well and sometimes better than pre-tRNAs as substrates in modification reactions (22). After incubation in the *X.laevis* oocytes for 3–48 hours the radiolabelled tRNA was reisolated, hydrolyzed completely with P1 nuclease or RNase T2 and the presence of modified nucleotides were analysed by 2D-TLC (10, 20).

Complete hydrolysis with P1 nuclease degrades the tRNA into 5′-nucleotides, while RNase T2 degrades it into 3′-nucleotides. Thus, degradation of the ^{32}P-G labelled tRNA with P1 yields information of all G-modifications, and degradation with T2 gives information about the modifications 5′-adjacent to all Gs in yeast $tRNA^{Asp}$ (cf. Fig. 1).

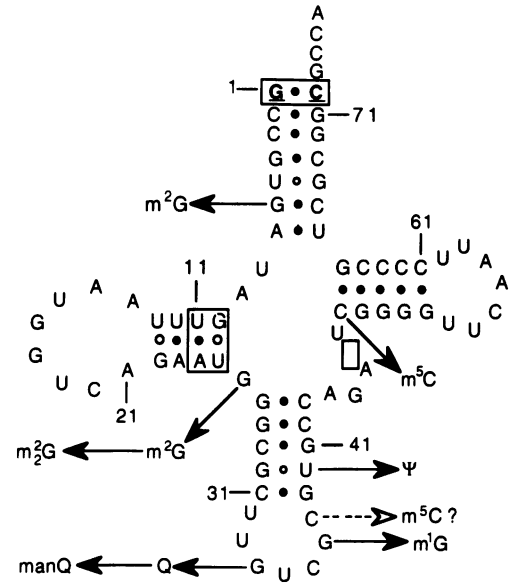

Figure 1. Clover-leaf structure of *in vitro* transcribed yeast $tRNA^{Asp}$ (mutant a). The numbering of bases are according to (23). Modified nucleotides analysed in our experiments are indicated by arrows. In all tested $tRNA^{Asp}$ variants the wt U1-A72 base pair is exchanged for G1-C72 (bold face). □: Mutated positions in $tRNA^{Asp}$ variants.

RESULTS

Mutations in the D-stem and variable loop in $tRNA^{Asp}$ affect the formation of m^1G37 in the anticodon loop

Earlier results demonstrated that the enzymatic formation of m^1G at position 37 in the anticodon loop of yeast tRNA microinjected into *X.laevis* oocytes was rather insensitive to changes in the anticodon sequence (15). The present work show that mutations at other sites but the anticodon loop had more variable effects on the m^1G37 formation (Fig. 2A and Table 1). Conversion of G10-U25 and U11-A24 to G-C basepairs (mutants a200, a207) hardly affected the final level of m^1G37. However, the rate of m^1G-formation was faster in the tRNA with two G-C basepairs in the D-stem (a207), suggesting that strong basepairing in the D-stem would favour m^1G-formation in the anticodon loop. The formation of m^1G37 was completely abolished when an A10-U25 Watson–Crick base pair was created by substituting G10 for A10 (a204). The increase of the variable loop to 5 bases by insertion of a U at position 47 (a210 and a214) drastically lowered the levels of m^1G37 although the modification still could be detected, while no m^1G37 was formed when a C47 was inserted (a209 and a215).

The exchange of G34 for Q34/mannosyl Q34 is also sensitive to changes in the D-stem and the variable loop

In most eubacteria and eukaryotes, except in yeast, queuosine (Q) is present at position 34 of the anticodons in $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$ and $tRNA^{Tyr}$ (23). This tRNA-modification occurs via an enzymatic exchange of guanine for queuine (24). In $tRNA^{Tyr}$ and $tRNA^{Asp}$ from some eukaryotes, including *Xenopus laevis* oocytes, Q34 is further hypermodified to a glycosylated derivative, either galactosylQ (galQ) or mannosylQ (manQ). When yeast $tRNA^{Asp}$ is microinjected into *X.laevis* oocytes manQ34 is formed (12). The sequence U33-G34-U35 has been shown to be one main determinant for Q34-formation (12), while the nucleosides 36, 37 and 38 mainly affect the mannosylation step (13).

Fig. 2B and Table 1 show that the formation of manQ34 via Q34 was very efficient in wild-type like $tRNA^{Asp}$ (mutant a), as well as in mutants a200, a207 and a210. The substitution of G10 to A10 (a204) completely abolished the formation of Q34 and the same was observed for the insertion of C47 in the variable loop (a209 and a215).

U40 is modified into Ψ40 when yeast $tRNA^{Asp}$ is microinjected into *X.laevis* oocytes

In $tRNA^{Asp}$ from yeast and *X.laevis* pseudouridine (Ψ) is formed at positions 13, 32 and 55 (23). These sites are not labelled in the experimental system we have used here. However, when yeast $tRNA^{Asp}$ was microinjected into *X.laevis* oocytes an additional Y occured at position 40 (Fig. 1). Ψ40 was formed at relatively low levels when the wt-like $tRNA^{Asp}$ (mutant a) was used as substrate (Fig. 2C and Table I). Interestingly, base substitutions made to obtain two G-C basepairs at positions 10–25 and 11–24 in the D-stem (a207) increased the yield of Ψ40 with more than 3 times compared to the situation in wt-like $tRNA^{Asp}$ (mutant a). As the introduction of the two G-C base pairs in the D-stem of mutant a207 is required for the synthesis of m^2_2G26 (20), it is tempting to speculate that m^2_2G26-formation might be a prerequisite for efficient formation of Ψ40. This suggestion is supported by the fact that the Ψ40-synthesis in mutant a207 was delayed by about 24 hours (Fig. 2C), a time required for almost complete modification of G26 to m^2_2G26.

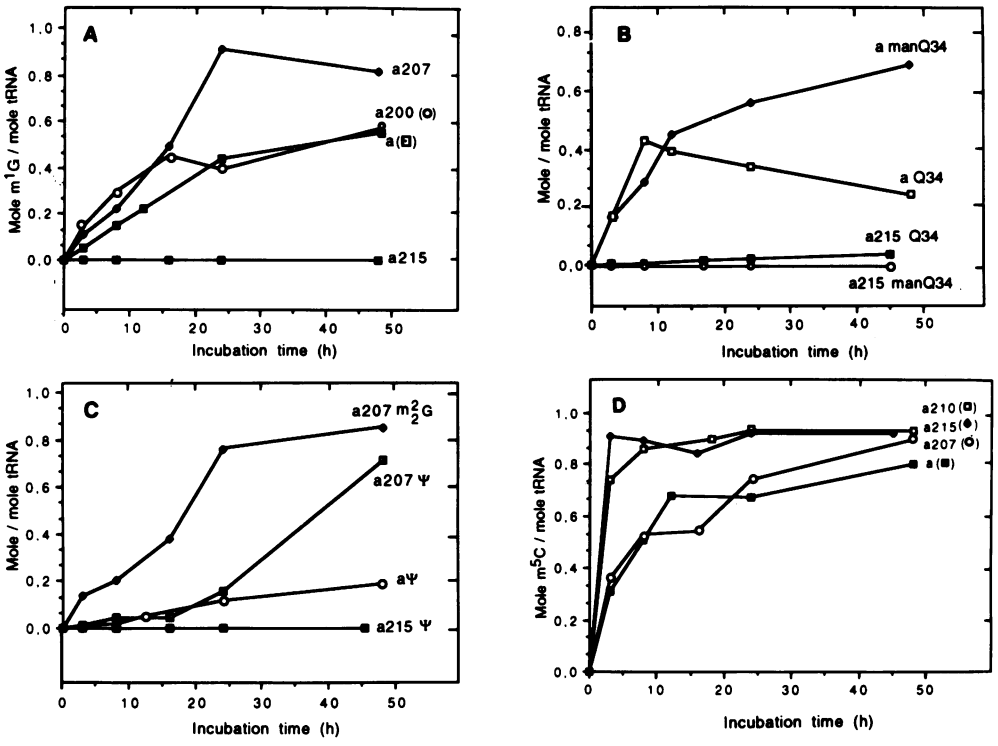

Figure 2. Kinetics of the formation of m^1G37 **(A)**, Q34 and manQ34 **(B)**, Y40 **(C)** and m^5C **(D)** in several $tRNA^{Asp}$-variants when injected into *X.laevis* oocytes. The previously published (20) kinetics of m^2_2G26 formation in $tRNA^{Asp}$ a207 (C) is included for comparison.

Table 1. Levels of modifications[a] in $tRNA^{Asp}$ variants after 48 h of incubation in *X.laevis* oocytes

Yeast $tRNA^{Asp}$	Mutations	m^1G^b (37)[e]	Q^b (34)	$manQ^b$ (34)	Ψ^c (40)	m^5C^c (38+49)	$m^2G^{b,d}$ (6+10)	$m^2G^{c,d}$ (26)	$m^2_2G^{b,d}$ (26)
a	U1 →G1[f] A72→C72[f]	0.6	0.2	0.7	0.2	0.8	1.1	0.9	0.0
a200	U25→C25	0.6	0.2	0.6	0.1	0.3	0.8	0.8	0.1
a204	G10→A10	0.0	0.0	0.0	0.0	0.9	1.0	0.4	0.0
a207	U11→C11 A24→G24 U25→C25	0.8	0.3	0.6	0.7	0.9	1.2	0.2	1.0
a209	+C47	0.0	0.0	0.0	0.0	1.0	1.0	0.1	0.0
a210	U25→C25 +U47	0.1	0.1	0.8	0.2	0.9	0.9	0.5	0.1
a214	U11→C11 A24→G24 U25→C25 +U47	0.1	0.3	0.1	0.1	1.0	1.1	0.4	0.2
a215	U11→C11 A24→G24 U25→C25 +C47	0.0	0.0	0.0	0.0	0.9	0.7	0.2	0.0

a) Modification levels given as mole modified base/mole tRNA. Accuracy ± 0.1
b) Sample degraded with nuclease P1.
c) Sample degraded with RNase T2.
d) Previously published data [20].
e) Possible position of modified base in tRNA are given in ( ).
f) Mutations present in all tRNA-variants.

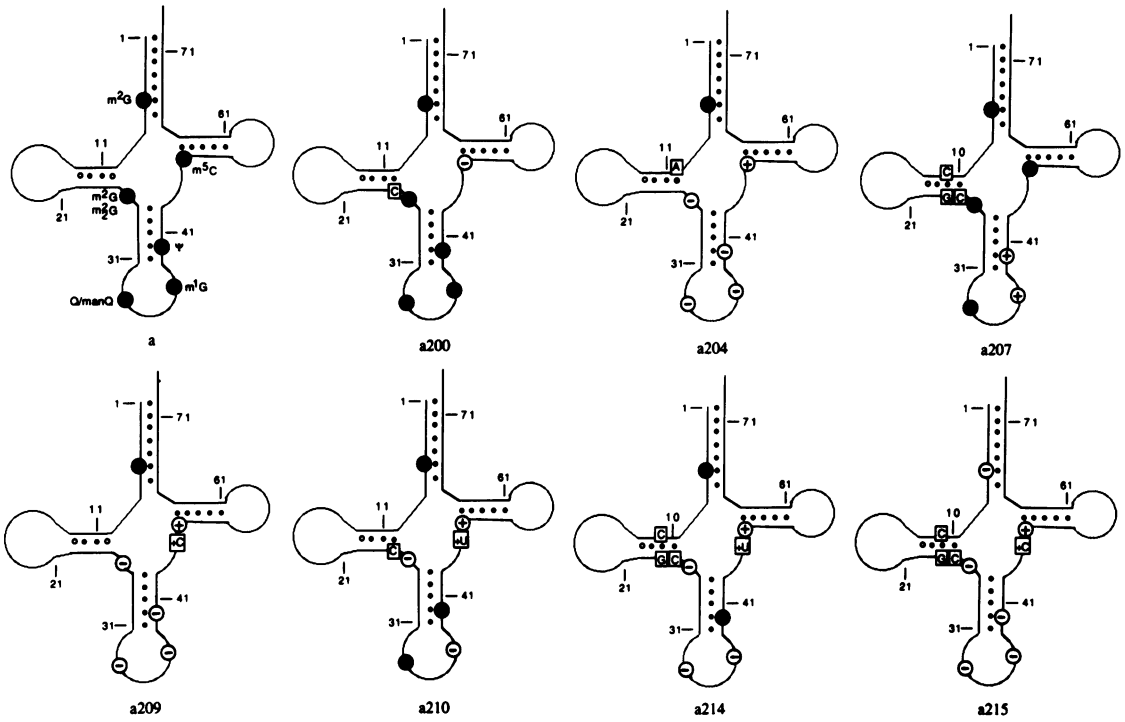

Figure 3. Effects of the mutations in $tRNA^{Asp}$ on the base modification pattern. □: Mutated positions. ●: Modified nucleotide formed to a similar level and with a similar rate as found in wt-like $tRNA^{Asp}$ (mutant a). ⊕: Modified nucleotide formed to an increased level or with an increased rate compared to the situation in wt-like $tRNA^{Asp}$ (mutant a). ⊖: Decreased level or rate of synthesis compared to the situation in wt-like $tRNA^{Asp}$ (mutant a). Position 26 represents the total level of methylation at G26; i.e. the sum of m^2G26 and m_2^2G26.

Synthesis of m^2G6 is insensitive to all tested point mutations in $tRNA^{Asp}$

In eukaryotic tRNAs m^2G is often found in positions 6, 10 and 26, except for in yeast tRNAs where m^2G6 is never formed. In rat liver (25) and yeast (26) different enzymes catalyse the formation of this modification at position 10 and 26. In yeast, $tRNA^{Asp}$ lacks m^2G at all these sites, but when yeast $tRNA^{Asp}$ is injected into *X.laevis* oocytes, G6 and G26 became modified to m^2G. Although G10 was not modified in microinjected wt-like $tRNA^{Asp}$ (mutant a), an m^2G10-site seems to have been created in some of the other tRNA variants (20).

From our results, it is clear that none of the mutations introduced in the different $tRNA^{Asp}$ variants considerably affected the synthesis of m^2G at positions in the aminoacyl-stem and D-stem (Table 1). On the other hand, modifications of G26 (Table 1 and (20)) were drastically reduced when an extra base was inserted in the variable loop (a209, a210, a214, a215) or when G10 was exchanged for A10 (a204). Thus, the tRNA(m^2G6)- and the tRNA(m^2G26)methyltransferases clearly have different structural requirements as shown by the difference in sensitivity to the mutations in $tRNA^{Asp}$.

The rate of m^5C-formation is stimulated in some $tRNA^{Asp}$ variants

The only two putative m^5C sites in yeast $tRNA^{Asp}$ that are followed by a G are positions 38 and 49 (Fig. 1). However, the kinetics of m^5C-formation (Fig. 2D), as well as the fact that only 1 mole of m^5C was formed per mole of tRNA favour that only one of the two putative sites in yeast $tRNA^{Asp}$ becomes modified in *X.laevis* oocytes. In yeast, $tRNA^{Asp}$ contains only m^5C49, while C38 is unmodified. Furthermore, in 79% of the eukaryotic tRNA species having C49 this base is modified to m^5C, while only 27% of the C38 containing tRNAs have m^5C38 (23). Thus, it is reasonable to suggest that C49 was the only m^5C-site which was labelled in our experiments.

All the tested $tRNA^{Asp}$ variants gave relatively high levels of m^5C (Table 1). It is obvious that all point mutations affecting the D-stem or the size of the extra arm did not cause any discriminatory effects on the identity elements for the tRNA(m^5C49)methyltransferase. Furthermore, the rate of formation of this modification was significantly faster (Fig. 2D; a209, a210, a214 and a215) in mutants with five bases in the variable loop.

DISCUSSION

The results presented in this paper on the formation of base modifications in yeast $tRNA^{Asp}$ variants microinjected into *X.laevis* oocytes are summarized in Fig. 3. Clearly point mutations in the D-stem and/or the variable loop have long-range effects on the potential of the tRNA to function as a substrate for various modifying enzymes. The synthesis of modifications in the anticodon stem and loop were shown to be the most sensitive ones. We conclude that the obtained variations in the base modification pattern in the different tRNA variants are due to changes in the normal 3D-structure of $tRNA^{Asp}$ caused by the introduced mutations. Indeed, substitution of G10 to A10 in yeast $tRNA^{Asp}$ (mutant a204) has been demonstrated to change the conformation of the tRNA (27) probably by disturbing the interaction between position 10 in the D-stem and position 45 in the variable loop (19). Other bases in the variable loop of

$tRNA^{Asp}$ are also involved in tertiary interactions, such as A46 hydrogen bonded to G22 in the D-stem and U48 hydrogen bonded to A15 in the D-loop of the tRNA (19). As a consequence, insertion of an extra base between A46 and U48 in $tRNA^{Asp}$ (mutants a209, a210, a214 and a215) affects the structure of the tRNA (28), either by changing the distance between the interacting bases or by forming new interactions.

Our results demonstrate the importance of an intact tRNA-structure for its recognition by the tRNA modifying enzymes tRNA(m^1G37)methyltransferase, Q34insertase, tRNA(Ψ40)-pseudouridylate synthase and tRNA(m^2G26/m^2_2G26)methyl-transferase(s). These data are in line with the recent findings on two other enzymes acting in the anticodon loop, namely tRNA(m^1G37)methyltransferase (5) from *E.coli* and tRNA(Ψ35)pseudouridylate synthase from wheat germ (6) where both of them require that the tRNA-substrates have an intact 3D-structure. The presented data on methylation of G6 and C49 imply that determinants in the tRNA-structure of importance for the methylation of these positions were unaffected or that the corresponding tRNA-modification enzymes, tRNA(m^2G6)- and tRNA(m^5C49)methyltransferases, are less sensitive to changes in the 3D-structure of the tRNA. This last suggestion is in agreement with earlier results demonstrating that cadaverine, a polyamine that destabilizes the tertiary structure of the tRNA in the presence of magnesium ions, also stimulates the enzymatic formation of m^5C49 in *E.coli* $tRNA^{fMet}$ incubated in rat liver extracts (29). Fragments of tRNA, that evidently have lost their intact tertiary tRNA-structure, may also function as substrates for some modifying enzymes, as shown for tRNA(m^5U54)methyltransferase from *E.coli* (7) and tRNA(Gm18)methyltransferase from *Thermus thermophilus* (8). Thus, the different tRNA-modification enzymes vary considerably in their sensitivity to perturbations of the tertiary structure of their tRNA substrates.

Obviously not only the sequence neighbouring the position for a modification but also other determinants in the tRNA influence the interactions between the tRNAs and most tRNA-modifying enzymes. A more systematic investigation on the structural determinants for different tRNA modifying enzymes is now in progress by using several other tRNA variants with mutations causing well characterized changes of the tertiary structures. In this study we have taken advantage of using a complete modification system, the *X.laevis* oocyte, being aware of the fact that yeast tRNA in this heterologous system will get a modification pattern that reflects the properties of the *X.laevis* modification enzymes. The conclusions drawn on the sensitivity of the enzymes towards different structural changes in the tRNA is presumably valid also in the homologous situation and opens the possibility for more specific tests in yeast, for which no complete system yet is available.

The data presented here also imply that before any conclusions are drawn on biological effects obtained through point mutations in tRNAs, it should be investigated if the base-modification pattern was altered and if such alterations might have been the primary cause of the biological observed effect.

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