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Macromolecular interactions in protein synthesis: the interaction of tRNA with its biological partners (aminoacyl-tRNA synthetase, elongation factor EF-Tu, Ribosome)

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ABSTRACT

The accessibility of phosphates towards ethylnitrosourea was studied in tRNA/aminoacyl-tRNA synthetase complexes and in the ternary aminoacyl-tRNA/EF-Tu/GTP complex. When the tRNA is complexed to aminoacyl-tRNA synthetases, several phosphates are protected against the alkylation agent, the protection patterns varying according to the tRNA/aminoacyl-tRNA synthetase system. However in all cases phosphates of the anticodon stem are protected. In the ternary aminoacyl-tRNA/EF-Tu/GTP complex, no protection of phosphates is observed. On the contrary, the accessibility of several phosphates is increased, suggesting a structural rearrangement of the tRNA induced by complex formation.

To identify ribosomal components involved in the peptidyl-tRNA binding site two approaches have been investigated : (i) $tRNA^{Phe}$ molecules have been prepared in which cytidine has been randomly converted into 4-thiouridine by pressurized hydrogen sulfide. Direct irradiation at 335 nm of N-Ac-Phe-(S^4U)tRNA(Phe)/PolyU/70S ribosome complex induced covalent crosslinking of the Phe-tRNA(Phe) molecules exclusively to protein S10 in the 30S subunits. (ii) Photochemical crosslinking of ribosomal P site bound Ac-Val-tRNA(Val) to a region near the 3'-end of small subunit RNAs from prokaryotes and eukaryotes occurs, via cyclobutane dimer formation, between the 5'-anticodon base cmo^5U34 or mo^5U34 of tRNA and C1400 of **E.coli** 16S rRNA or C1626 of yeast 18S rRNA. The crosslinking site is in the centre of a sequence which is evolutionarily conserved, has a single stranded structure and is located at the ribosomal subunit interface.

1. INTRODUCTION

Among the various functions of transfer ribonucleic acids (tRNAs) in living cells, their best understood role is their participation in ribosome-mediated protein biosynthesis (1,2). These functions lead tRNAs to many interactions with different proteins and nucleic acids. With aminoacyl-tRNA synthetases, enzymes which attach the correct amino acid to the 3'-end of their cognate tRNAs, the molecular recognition must be highly specific. With elongation factor which carries the aminoacylated tRNAs to the ribosome and also with the ribosomal components which are involved in peptide bond synthesis it is expected to find other recognition mechanisms allowing interactions of various tRNAs with common macromolecular partners. In this study we will illustrate these three different situations and discuss which structural features in tRNA are important for the interactions with aminoacyl-tRNA synthetases, elongation factor and ribosome. Components of the ribosomes involved in complex formation with tRNA will also be described.

2. INTERACTIONS BETWEEN tRNA AND AMINOACYL-tRNA SYNTHETASE

Many methods have been used to determine the regions of tRNAs in contact with aminoacyl-tRNA synthetases (3,4). Among them, ultraviolet light-induced crosslinking was the first successfull experimental approach to define directly the contact points (3,5). We applied this approach on the valine system from yeast (6) and found, roughly in agreement with the model of Rich and Schimmel (7) deduced from $tRNA^{Phe}$ data, that the cross-linked T1 RNase oligonucleotides are located along and around the diagonal side of the structure which contains the acceptor-, D- and anticodon-stems. Other informations came from enzymatic mapping experiments. Digestions of complexed tRNAs by the double-strand specific RNase from cobra venom showed in all systems so far studied contacts of the anticodon-stem with the synthetases (8-11). According to the system under investigation, protections occur at the 5'-side, at the 3'-side or at both sides of the anticodon-stem.

Another way to determine the parts of tRNA interacting with aminoacyl-tRNA synthetase is to compare its accessibility to chemical probes in the free and complexed states. This can easily be done since many specific chemical reagents are available which can attack functional groups on the individual nucleotides. The regions interacting with the probe in the free tRNA but not in the complexed molecule can be considered to be in close contact with the interacting aminoacyl-tRNA synthetase and thus protected by it.

Here we present results obtained with ethylnitrosourea (ENU), an alkylating reagent which essentially ethylates the phosphate residues of nucleic acids (12,13). The principle of the method derives from the chemical sequencing methodologies of nucleic acids and relies on statistical and low yield modification at each phosphate, in such a way that each tRNA undergoes less than one modification. The tRNA molecules labelled at their 3'- or 5'-end with radioactive ATP are specifically split at the modified position and the resulting end-labelled oligonucleotides are analyzed by high voltage electrophoresis on sequencing gels followed by autoradiography. Assignment of bands is done by comparing their migrations to ladders obtained after limited T1 RNase digestion and alkaline hydrolysis. In such a way it is possible to probe the entire tRNA molecule in one experiment. In the presence of aminoacyl-tRNA synthetases, experimental conditions must allow both chemical reactivity and good complex formation. Therefore samples are incubated for 3 hours at pH 8.0 and at 20°C in the presence of magnesium at a rather low ionic strength and with concentrations of tRNA (1.5 µM) and enzyme (5 µM) in the range where the complex formation is guaranteed. Detailed experimental procedures were published for experiments with $tRNA^{Phe}$ and $tRNA^{Val}$ (14).

A typical alkylation experiment of 5'-labelled $tRNA^{Asp}$ is shown on Figure 1. This experiment allows to probe the phosphate groups located between positions 6 to 55. Similar assays with 3'-labelled tRNA probing the phosphates from the 3'- to the 5'-side of the molecule were also carried on.

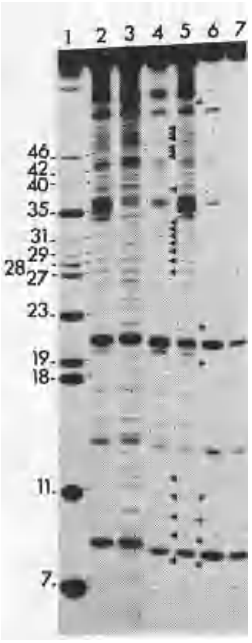

Fig. 1

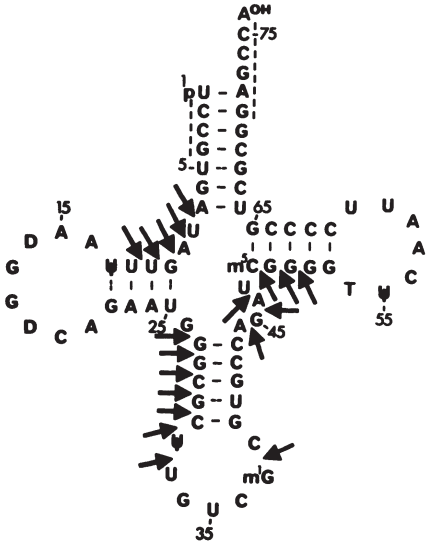

Fig. 2

Fig. 1 : Autoradiogram of 15% acrylamide gel of a phosphate alkylation experiment with ethylnitrosourea of 5'-end labelled tRNA(Asp) from yeast in the presence of yeast aspartyl-tRNA synthetase. Lane 1 : partial T1 RNase digest ; lane 2 and 5 : alkylation at 20°C in the presence of aspartyl-tRNA synthetase ; lane 6 and 7 : control incubations in the absence of reagent and in the presence of enzyme (6) and in the absence of reagent (7). The phosphates protected against alkylation in the native tRNA (lanes 2 and 5) and in the tRNA complexed with its synthetase (lane 4) are indicated by diamonds and arrows respectively.

Fig. 2 : Cloverleaf structure of yeast tRNA(Asp) (15) with positions of the phosphates strongly protected by yeast aspartyl-tRNA synthetase against ethylnitrosourea. Full arrows show the phosphates protected by the aspartyl-tRNA synthetase ; regions not tested for technical reasons (14) are indicated by dashed lines.

In the presence of aspartyl-tRNA synthetase the splitting at some phosphate positions is nearly suppressed or is strongly reduced (for instance P27 to P33), suggesting that these groups are protected from alkylation by the enzyme and thus most likely are in close contact with the protein. The results are summarized on Figure 2 in which the protected phosphate groups are indicated by arrows on the cloverleaf structure of yeast $tRNA^{Asp}$. Among the parts of the molecule not tested it is clear that the CCA-end or at least the terminal adenosine is also in contact with the enzyme for catalytic necessity.

If one compares the protected phosphates in $tRNA^{Asp}$ with results obtained by the same approach in the phenylalanine and valine systems the striking feature which emerges is the large difference between contact areas. In the three systems, the only common contact areas, beside the terminal adenosine, are the variable loop and the neighbourhood of P9 (Figure 2). Not surprisingly these regions are very close in space. Biochemical experiments have also emphasized the involvement of U8 in a contact interaction of tRNAs with their cognate synthetase (16).

For $tRNA^{Asp}$ one side of the L-shaped molecule is clearly involved in the protein-nucleic acid association. This face includes the variable-loop, the 5'-end of the anticodon-stem and part of the 3'-end of the amino acid-arm. The surface involved is quite important and this observation is consistent with neutron diffraction results which imply the existence of large contact areas between the protein and the nucleic acid (17). This interaction differs significantly from the one proposed for $tRNA^{Phe}$ derived from crosslinking experiments (7). If one assumes a similar folding for $tRNA^{Asp}$, another type of interaction between the enzyme and the tRNA must be postulated.

These observations underline the differences which are likely to exist in the recognition between tRNAs and their cognate synthetases. It is worthwhile noticing that the oligomeric structure of the aminoacyl-tRNA synthetases in the aspartic acid, phenylalanine and valine systems are quite different : aspartyl-tRNA synthetase is a dimer (α_2) of MW ≃ 125,000 which can bind two tRNA molecules, whereas phenylalanyl-tRNA synthetase is an $\alpha_2\beta_2$ tetramer (MW ≃ 270,000) which also binds two tRNA molecules and valyl-tRNA synthetase is a large monomer (MW ≃ 130,000) which binds one tRNA molecule.

The former results essentially gave a static pictures, of tRNA/synthetase complexes. In fact complex formation is a dynamic and multistep adaptation process involving conformational changes of both macromolecules. The acylation activity of synthetases towards simplified substrates like free adenosine or the CCA_{OH} oligonucleotide illustrates this concept (18,19). Indeed the acylation of isolated adenosine or CCA_{OH} becomes only possible after activation of the catalytic centre of the synthetases upon addition of tRNA lacking 1, 2 or 3 terminal nucleotides (18,19).

The basic understanding, at the molecular level, of the recognition mechanism between tRNA and aminoacyl-tRNA synthetases, however, will require the knowledge of the three-dimensional structure of the different components in their free and complexed states. This aim might be approached in the aspartic acid system from yeast for which both free $tRNA^{Asp}$ (20) and aspartyl-tRNA synthetase (11), as well as the complex between tRNA and synthetase (22,23) could be crystallized under rather similar conditions in the presence of ammonium sulphate. New data on the three-dimensional structure of $tRNA^{Asp}$ and the current progress on the crystallographic analysis on the tRNA/synthetase complex are reported in this issue (24,25).

3. INTERACTION BETWEEN AMINOACYL-tRNA AND ELONGATION FACTOR Tu

Elongation factor Tu promotes the A-site specific binding of aminoacyl-tRNAs (aa-tRNA) to the ribosome via a ternary aa-tRNA/EF-Tu/GTP complex. In the presence of GTP, the factor binds preferentially charged non-initiator tRNAs, but it discriminates against initiator tRNA, uncharged tRNAs and aminoacyl-tRNAs modified in their 3'-end (e.g. 26,27). Mapping

experiments with nucleases demonstrate that EF-Tu as expected binds to the amino acid 3'-end of elongation tRNAs but also along the combined amino acid and T-stem ; no interaction was found in the anticodon-loop (e.g. 28,29).

Here we report the effect of EF-Tu on the conformation of aminoacylated $tRNA^{Phe}$ upon ternary complex formation. For this we investigated the chemical stability of tRNA and the accessibility of phosphates towards ENU, in complexed Phe-$tRNA^{Phe}$ and in free $tRNA^{Phe}$ using end-labelling and rapid sequencing gel methodologies (30). Unexpectedly ENU experiments did not allow to define contact points between EF-Tu and the phosphate groups of the aminoacylated tRNA. This result, however, is not contradictory to the nuclease mapping experiments. Because of the small size of ENU, phosphate residues could be readily more accessible to this reagent, even when they are covered by the Tu factor, than to nucleases which are much more bulky. In addition, phosphates at positions 9, 10, 11, 19, 49, and especially 58-60, are already protected by the tertiary structure of the tRNA (13) (phosphates located near the 3'- and 5'-termini could not be tested because of technical limitations).

This study brings evidence about 6 discrete conformational changes in tRNA upon ternary complex formation. One of them, occurring at position 53, takes place in the T-stem, a region which has been shown to be covered by EF-Tu (28,29). This change could be visualized due to the small size of the

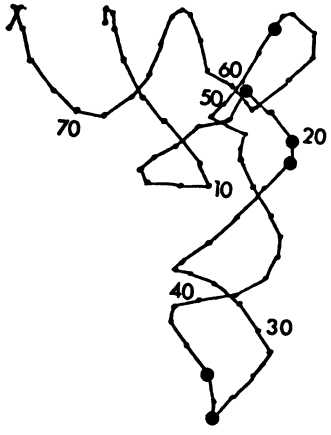

Fig. 3 : Effect of elongation factor Tu on the conformation of Phe-$tRNA^{Phe}$. The figure represents a ORTEP drawing of yeast tRNA(Phe) three-dimensional structure (taken from ref. 14). The heavy circles represent positions which undergo conformational changes upon ternary complex formation.

chemical probe which is not hindered by the factor in reaching this phosphate. The 5 other structural alterations occur in the D- and anticodon-loops at positions 18, 20, 21 and 34, 36, respectively (Fig. 3). In that case the appearance or disappearance of cuts in complexed tRNA reflects a changed flexibility in its ribose-phosphate backbone. These results clearly indicate that the T-region, the D- and anticodon-loops undergo a conformational rearrangement upon ternary complex formation. In the particular case of the D-loop, the occurrence of conformational change would explain the

contradictory results obtained in T1 RNase accessibility studies and in kethoxal modification experiments (31,32). Concerning the anticodon-loop, these results agree well with spectroscopic (33,34) and accessibility mapping experiments (28,29).

4. INTERACTION BETWEEN tRNA AND RIBOSOME

4.1 Objectives

One of the main events in protein biosynthesis is the specific codon-anticodon interaction on the ribosome. Nevertheless this interaction is not sufficient to explain the high stability of the tRNA/ribosome complex and the high fidelity with which it is formed. Additional interactions between both molecules are necessary and it will be of great importance to localize which sites of the tRNA and which components of the ribosome are involved in the formation of this complex.

To identify ribosomal components involved in the peptidyl-tRNA binding site, we developed two different approaches. In the first approach a photoactive derivative of $tRNA^{Phe}$ was prepared in which cytidine residues were randomly converted into 4-thiouridines. Thiouridine is photoactivable at 335 nm and is able to react covalently with nearby nucleophilic groups. Direct irradiation of the N-Ac-Phe-(S^4U)$tRNA^{Phe}$/PolyU/70S ribosome complex induced specific crosslinking of the tRNA to the ribosome. The second approach is based on the photochemical crosslinking of the anticodon of N-Ac-Val-$tRNA^{Val}$ to the ribosomal P site by photoactivation at 310 nm of the wobble base cmo^5U34 in **E.coli** $tRNA^{Val}$ or mo^5U34 in **B. subtilis** $tRNA^{Val}$.

4.2 Crosslinking of N-Ac-Phe-(S^4U)$tRNA^{Phe}$ to the ribosomal P site

4.2.1 Chemical conversion of cytidine residues into 4-thiouridines

Treatment of RNA molecules with pressurized H_2S leads to a highly specific conversion of cytidines into 4-thiouridine residues (S^4U) (35). The extent of RNA thiolation can easily be determined since the the S^4U moiety has a maximum of absorption at 335 nm. The conditions adopted here for the thiolation procedure are described in a previous paper (36). Kinetics of thiolation showed that a seven hours-treatment at 37°C led to an average conversion of one cytidine residue per molecule. A maximum of about 10 S^4U residues per tRNA was obtained after 24 hours. We used here conditions in which a single cytidine was randomly converted. The integrity of the thiolated tRNA was verified by electrophoresis on a polyacrylamide urea containing gel (36).

In order to characterize the cytidine residues able to be converted into S^4U, the thiolated tRNA was subjected to limited formamide hydrolysis leading to one cut per molecule (37). The oligonucleotide mixture was then submitted to 5'-end labelling and fractionated. Each oligonucleotides was totally digested by P1 nuclease and the 5' terminal nucleotide was identified.

As described by Riehl et al. (38), nine residues can be converted into S^4U. These residues are spread along the tRNA molecule (Fig. 4) : C2, C74 and C75 in the amino acid acceptor-stem ; C56, C60, C61, C63 in the T-loop and stem ; C27 and Cm32 in the anticodon -stem and -loop. Thiolation of the entire tRNA dramatically affects its aminoacylation capacity, whereas the activity of the modified tRNA containing an intact 3'-CCA_{OH} is not affected. This suggests that the single stranded cytidines in the 3'-terminal -CCA_{OH} sequence is essential for the aminoacylation reaction of $tRNA^{Phe}$.

According to the secondary and tertiary structure models of $tRNA^{Phe}$ (40), only four modified cytidines (Cm32, C60, C74 and C75) are located in single stranded regions. Studies of the ultraviolet and infrared spectra of S^4U showed that the neutral form is a 2-keto-4-thione (C=S) structure (41). In the light of this structural feature, G-S^4U base pairing would contain

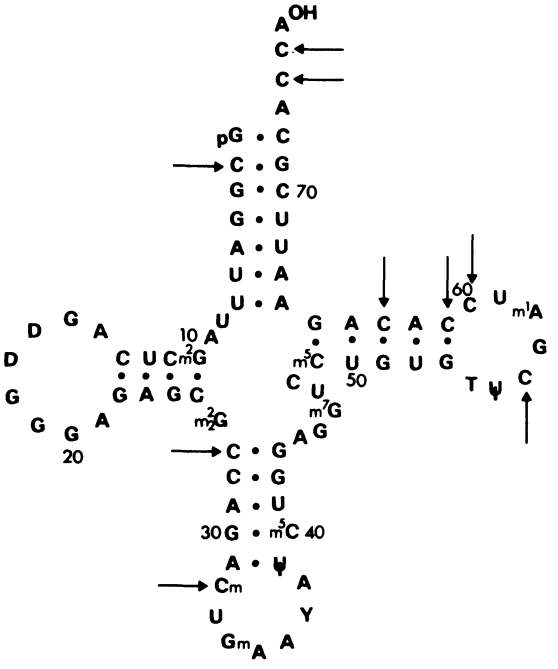

Fig. 4 : Cloverleaf representation of yeast tRNA(Phe) (39). Cytidines which can be modified by thiolation are designed by an arrow.

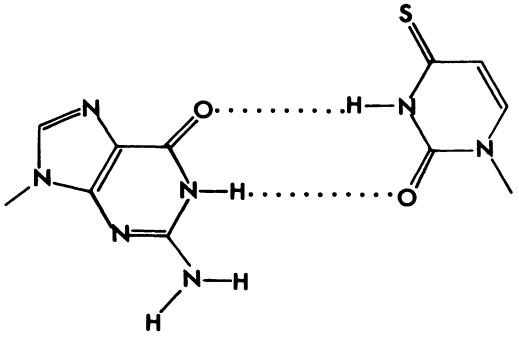

Fig. 5 : Hydrogen bonding in guanosine-4-thiouridine base pair.

only two hydrogen bonds (Fig. 5). This base pair would resemble a G-U interaction, as it is found between G4 and U69 in $tRNA^{Phe}$.

The substitution of the classical G-C base pair involving three hydrogen bonds by the G-S^4U one would lead to a minor structural distortion (40). Such changes of base pairing in the tRNA do not affect its biological activity. This is supported by crystallographic studies performed on yeast $tRNA^{Asp}$ which contains two G-U base pairs in the D-stem. Their presence does not fundamentally perturbe the helical conformation of this stem (42), suggesting that the overall biological active conformation is maintained.

4.2.2 Determination of the ribosomal components involved in the peptidyl-tRNA binding site on the ribosome

tRNA molecules lacking 3'-terminal-CCA_{OH} were thiolated in conditions allowing a randomly conversion of one cytidine per molecule. After the thiolation procedure, modified tRNA was renatured before reincorporation of an intact -CCA_{OH} terminus, and aminoacylated. Phe-(S^4U)$tRNA^{Phe}$ was acetylated and bound at the ribosomal P site. The non covalent N-Ac-Phe-(S^4U)$tRNA^{Phe}$/PolyU/70S ribosome complex was directly irradiated at 335 nm to photoactivate the S^4U residues. Complex measuring after irradiation showed that 10% of the tRNA molecules located at the ribosomal P site could be crosslinked to the ribosome. Specificity of P site binding was verified by puromycin reactivity ; specificity of the crosslinking reaction was tested in the absence of mRNA or in the presence of a non-specific mRNA.

Analysis of the covalent complex revealed that the tRNA was exclusively crosslinked to 30S subunits. Within the 30S subunits, a single covalent bond occurred with protein S10. Initially this was an unexpected result ; numerous experiments have shown crosslinking of tRNA in the P site to 50S subunits ; however these experiments were performed on aminoacyl-tRNAs derivatized in their amino acid moiety (43). Moreover if the S^4U residues were randomly spread along the tRNA molecule, one would have expected crosslinking to more than one ribosomal component.

The fact that protein S10 was found to be crosslinked to this derivative of peptidyl-tRNA is of particular interest in the light of results obtained with the second approach described here.

Our results, together with those of others, thus indicate that protein S10 is located near the site of tRNA binding, although it cannot be explicitly localized to either A or P site. This may reflect a partial overlapping of both sites or a close proximity of protein S10 to both of them.

Nevertheless experiments performed in our laboratory (N. Riehl and B. Ehresmann, unpublished results) showed that Phe-(S^4U)$tRNA^{Phe}$ could not be covalently bound to the ribosome when located at the A site, but a covalent tRNA/EF-Tu complex could be isolated. These results suggest a conformational change of the tRNA molecule when located at the A site on the ribosome. Experiments are in progress to determine the crosslinking point on the (S^4U)tRNA and on the elongation factor.

4.3 Crosslinking of the anticodon of E.coli and B.subtilis N-Ac-valyl-tRNA(Val) to the ribosomal P site

Several tRNAs, containing cmo^5U_{34} or mo^5U_{34} at the 5'-anticodon position, can be crosslinked to 70S or 80S ribosomes after irradiation between 310 and 330 nm. Covalent attachment only occurs to 16S or 18S RNA when tRNA is bound to P site and is codon specific. The crosslink which is formed is a pyrimidine-pyrimidine cyclobutanodimer between the 5'-anticodon

base and a pyrimidine of 16S-18S RNA. In collaboration with Ofengand, we have determined the site of crosslinking in **E.coli** $tRNA^{Val}$, which contains cmo^5U_{34} and we have used **B.subtilis** $tRNA^{Val}$ which has mo^5U at position 34 in order to see if the crosslinked residue in rRNA remains unchanged when the carboxyl group is absent from the tRNA anticodon. In addition we have determined the site of crosslink in yeast 18S RNA in order to see if the same crosslinking site is involved both in prokaryotes and eukaryotes (48,49).

The crosslink was formed by irradiation of **E.coli** or **B.subtilis** Ac-Val-$tRNA^{Val}$ bound to P site of **E.coli** ribosome or by irradiation of **E.coli** Ac-Val-$tRNA^{Val}$ to yeast ribosomes in the presence of pGUU or poly (U_2, G) as codon. In all cases, the crosslinked tRNA-rRNA was isolated by SDS-sucrose-gradient centrifugation. In each case, both photolyzed and covalent heterodimers were subjected to total T1 RNase hydrolysis followed by 5'- or 3'-labelling. Comparison of electrophoresis patterns showed the presence of covalent duplexes as additional bands, present only in the non irradiated complexes. Two-dimensional electrophoresis including photolysis before the second dimension allowed to isolate the photolyzed oligomers as off-diagonal products. The pairs of released oligomers were isolated and identified. One of the oligomers was shown to be UACACACCG, a unique rRNA monomer present in an evolutionarily conserved region. This oligomer was found in all three heterodimers. This finding is in agreement with that of Taylor et al. (50) obtained by a different approach in a **E.coli** tRNA-rRNA complex. The other oligomer of the dimer had the sequence expected for the T1 RNase product encompassing the anticodon of the tRNA.

The precise site of crosslinking was determined by two novel methods. Bisulfite modification of the oligonucleotide dimer converted all C residues to U, except for any crosslinked C which would be resistant by being part of a cyclobutane dimer. Sequencing gel analysis of the UACACACCG oligomer showed that the C residue protected was the 3'-penultimate C residue, C1400 in **E.coli** rRNA or C1626 in yeast rRNA. This position in **E.coli** rRNA was also identified by Prince et al. (51), who used a totally different methodology. We developed a new technology based on random hydrolysis of the covalent oligonucleotide duplex followed by two-dimensional gel electrophoresis, which yields the crosslinking site in both oligomers in one single step. This method fully confirms the crosslinking site in rRNA and excludes the possibility of small amounts of crosslinking at other positions. In particular, crosslinking of C1399 or C1625 was totally excluded. This method also directly proved that the site of crosslinking in tRNA was the wobble base, cmo^5U34 in **E.coli**, or mo^5U34 in **B.subtilis**. Since both mo^5U34 and cmo^5U34 crosslink to the same C1400, the -COOH group of cmo^5U34 cannot be involved in either crosslink formation or nucleotide selection.

It is striking that the crosslinking site is restricted to a single site in rRNA. Since the reaction is pyrimidine specific by its chemistry, the adjacent C residue should have been equally reactive. The failure to detect

crosslinking to C1399 or its equivalent C1621 in yeast implies that a very specific three-dimensional structure is formed between this region of the rRNA and the tRNA anticodon. The conservation of the sequence surrounding the crosslinking site, its location in a region which is highly exposed at the surface of the subunit and its crosslinking ability indicates that this RNA region is essential to both procaryotic and eucaryotic protein synthesis.

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