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tRNA Stabilization by Modified Nucleotides†

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ABSTRACT: Post-transcriptional ribonucleotide modification is a phenomenon best studied in tRNA, where it occurs most frequently and in great chemical diversity. This paper reviews the intrinsic network of modifications in the structural core of the tRNA, which governs structural flexibility and rigidity to fine-tune the molecule to peak performance and to regulate its steady-state level. Structural effects of RNA modifications range from nanometer-scale rearrangements to subtle restrictions of conformational space on the angstrom scale. Structural stabilization resulting from nucleotide modification results in increased thermal stability and translates into protection against unspecific degradation by bases and nucleases. Several mechanisms of specific degradation of hypomodified tRNA, which were only recently discovered, provide a link between structural and metabolic stability.

With more than 100 distinct chemical structures, the high frequency and chemical diversity of ribonucleotide modifications occurring in natural RNA present a complicated puzzle; clearly, modifications must be of high importance or else this complexity would not have evolved. A pronounced hot spot for sophisticated nucleotide modification is found in the anticodon loop of tRNA, in particular at positions 34 and 37. These are known to optimize the mRNA decoding function by directly affecting codon−anticodon interactions (*1*). The sophisticated involvement of modifications at the latter positions in codon recognition and related issues has been expertly reviewed elsewhere (*2*, *3*). Many such modifications were shown in early work to be essential, which can be easily rationalized with their important function in the crucial process of accurate translation.

In contrast, the functions of most modifications in the second cluster of modifications, situated in the structural core of tRNA, are ill understood. These modifications are typically unsophisticated with respect to their chemistry: in addition to pseudouridine (Ψ), they consist mostly of methylated nucleotides. Some examples discussed in this review are shown in Figure 1. Although a distinction between structure and function can never be complete, we will treat these chemically simple core modifications as "structural" modifications for the purpose of this work. In tRNAs, which are the most heavily modified and also most investigated RNAs, efforts to clearly define the function of single modifications suffer from the apparent collective nature of the ensemble of modifications. Indeed, the absence of only a single modification remains very frequently without obvious consequence (*4*), and effects become apparent only when additional modifications or elements of tRNA structure are also missing (*5*, *6*). The common perception in the field (*7*−*10*) pictures nucleotide modifications in the tRNA core as modulators of its structural flexibility, which can rigidify certain areas and render others flexible (*11*). A central topic of this review is the structural impact of core modifications, and the difficulties in studying such an impact. Because tRNAs exhibit a very conserved and well-defined base pairing pattern, significant structural alterations as a consequence of nucleotide modification are unexpected. Rather, it is typically expected that primary transcripts already form the classical cloverleaf structure and the three-dimensional L shape known from crystallography (*12*). As has been shown in hundreds of cases with unmodified in vitro transcripts, this is indeed the case; most unmodified tRNAs are functional, e.g., in aminoacylation in vitro (*13*−*15*) or even in in vitro translation (*16*). There are, however, several reported cases in which transcripts fail in vitro (*17*−*19*), and these are clearly of great interest with respect to post-transcriptional modifications. Here again, it is helpful to distinguish modifications that directly interact with partners of the translation machinery from those that shape and fine-tune the tRNA core structure for peak performance. Recent evidence indicates that tRNAs do not function in vivo without post-transcriptional modifications that reinforce the structural tRNA core (*5*, *6*, *20*).

As the laws of thermodynamics literally dictate that there must be more than one structure for any given RNA (*10*), it is advisible to think of one's favorite structure not as the "correct" structure but rather as the "predominant", most populated conformation in a given equilibrium. As a direct consequence of modification, we will repeatedly discuss cases of structural equilibrium and alternative structures. Indeed, tRNA participates in a multitude of interactions during its involvement in protein biosynthesis: depending on its binding partner, it has been found in a multitude of structural variations of the L shape (*12*, *21*−*24*) that require structural flexibility in addition to stability.

In what follows, we will review our knowledge of the stabilizing impact of nucleotide modifications, first on the level of large structural alterations that involve significant rearrangements of Watson−Crick interactions, i.e., nanoscale structural changes, and then touching on the more subtle structural effects that can

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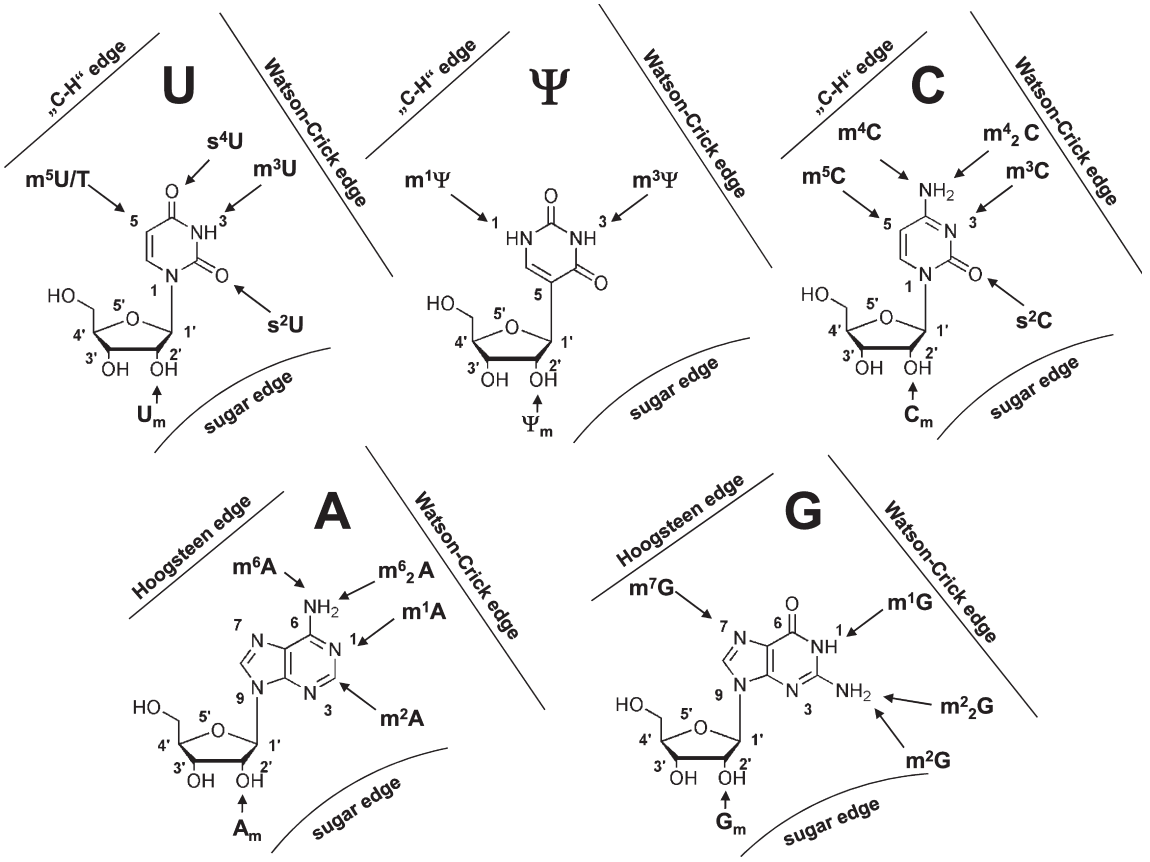

FIGURE 1: Sites of chemically simple modifications on the Watson−Crick edges and the Hoogsteen edges (purines) or C−H edges (pyrimidines) (*162*, *163*). Single methylation of endocyclic nitrogens of the Watson−Crick edge or double methylation of exocyclic amines ablates the possibility of standard Watson−Crick pairing. Methylations of the 2′-OH group, which may modulate the hydrogen bond pattern of the sugar edge, are also indicated. Modified nucleotides are abbreviated according to common nomenclature (*164*).

be measured on the angstrom scale. The stabilization of RNA will then be discussed from a thermodynamic point of view, i.e., thermal stability. Finally, we turn to biological consequences for tRNAs lacking structural stabilization, in particular, degradation pathways of unstructured tRNA.

NANOSCALE REARRANGEMENTS OF tRNA AS AN EFFECT OF NUCLEOTIDE MODIFICATION

Large structural effects in RNA, measured on the nanometer scale, are likely to include the dissolution and re-formation of helical structures. The latter implies alteration on the level of hybridization, and thus of numerous Watson−Crick interactions. Modifications are suitable for altering hydrogen bond interactions on the Watson−Crick edge, the Hoogsteen edge, or the sugar edge of the target nucleotide. Methylations are the most simple and most widespread modifications known to block formation of hydrogen bonds between nucleotides. Accordingly, the few known examples of modification-dependent Watson−Crick rearrangements involve nucleotide methylations. Among the best understood cases is human mitochondrial $tRNA^{Lys}$ which adopts a noncanonical extended hairpin structure when unmodified (*19*, *25*). As was shown by single-molecule fluorescence energy transfer studies, this structure is the predominant one in a dynamic equilibrium with the originally expected cloverleaf structure (*26*). Despite the presence of this correctly folded subpopulation, the unmodified form is not a substrate for aminoacylation by the cognate lysyl-tRNA synthetase (*27*). Significantly, the introduction of m^1A at position 9 shifts the dynamic equilibrium in favor of the cloverleaf structure (*25*, *26*, *28*) (Figure 2A). Biochemical investigations suggest that m^1A formation is an early maturation event (*28*), which may occur directly after tRNA 5′ maturation by RNase P (*29*). Studies of DNA indicate a penalty of ~6.5 kJ/mol for the introduction of m^1A into a helical structure, where m^1A is reported to form a T(*anti*)·m^1A(*syn*) Hoogsteen base pair with the thymidine residue in the opposing strand (*30*). In $tRNA^{Lys}$, the methyl group afforded a stabilization of 3−4 kJ/mol of the cloverleaf relative to the extended hairpin structure depending on the magnesium ion concentration (*31*). A mutational analysis of various transcripts is consistent with the major contribution of this effect coming from the interdiction of a Watson−Crick interaction by the methyl group. Mutations that imitate this effect lead to a predominance of the cloverleaf conformation and to good substrates for aminoacylation (*27*). Initial studies, conducted by structural probing, had already yielded strong indications of high structural plasticity and of alternative structures in this tRNA. Similar studies revealed structural plasticity in transcripts of several other human mitochondrial tRNAs, including $tRNA^{Leu(UUR)}$ and $tRNA^{Asp}$ (*32*−*34*). For the latter, a noncloverleaf conformation with a strong resemblance to the aforementioned extended hairpin of $tRNA^{Lys}$ has been proposed (*35*)(Figure 2B). Aminoacylation studies in vitro show that transcripts of animal mitochondrial tRNAs are altogether worse substrates than transcripts of canonical tRNAs in the cytosol (*33*, *34*, *36*−*40*). These findings imply that such plasticity is a common characteristic of animal mitochondrial tRNAs and that despite a general conservation of the base pairing in stem regions (*41*, *42*), the minimal structural characteristics of the cloverleaf may frequently be reached only after certain modification steps.

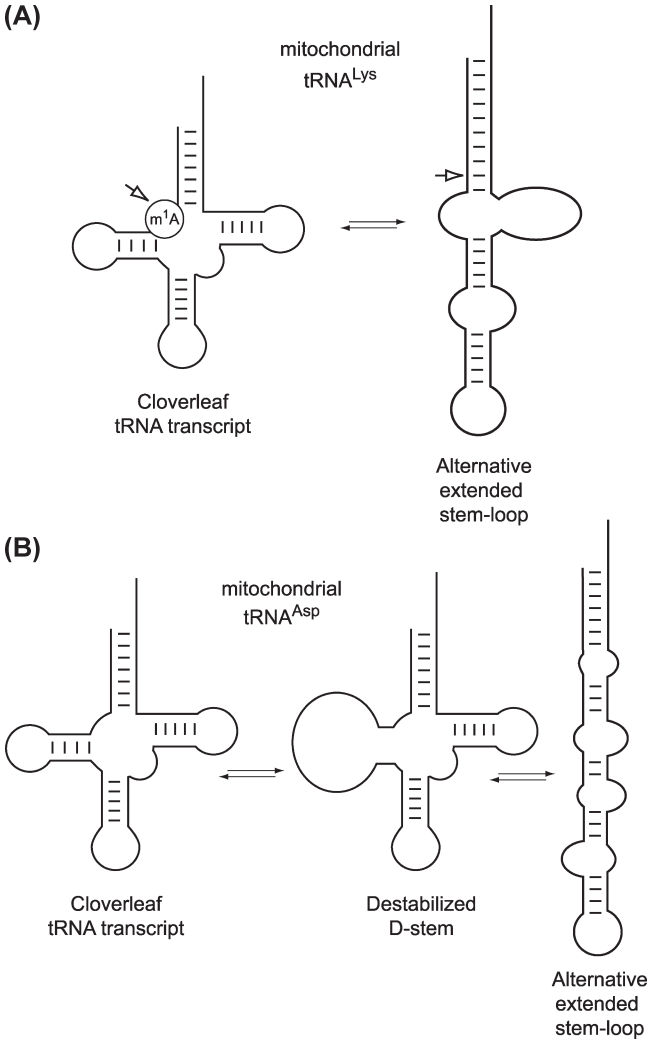

FIGURE 2: Alternative secondary structures related to nucleotide modification of mitochondrial tRNAs. (A) Structural equilibrium of human mitochondrial $tRNA^{Lys}$. The equilibrium is displaced toward the structure on the right by a single methylation on N9 of adenosine 9 as evidenced by structural probing (*19*, *25*) and single-molecule FRET experiments (*26*). (B) Alternative structures proposed for $tRNA^{Asp}$ based on structural probing (*35*).

There has been speculation (*10*) that the apparent folding problem of mitochondrial tRNAs may in part be related to the nucleotide bias in the mitochondrial genome and in mitochondrial tRNAs (*42*) . In brief, the "RNA folding problem" is related to the fact that Watson−Crick pairs are quite strong and govern RNA folding in such a predominant way that their low diversity of elemental units (only four different base pairs) makes it difficult for a given sequence to specify a truly unique secondary structure (*10*). The resulting problem of various alternative structures with similar energy is obviously compounded in sequences with a nucleotide bias, such as RNAs transcribed from mitochondrial genomes, which are rich in A-T pairs. As we have seen in the case of $tRNA^{Lys}$, the introduction of post-transcriptional nucleotide modifications may partially remedy a problem of alternative structures by interdiction of certain Watson−Crick interactions.

Consequently, one might expect similar problems and solutions for many tRNAs that are rich in G and C nucleotides. Indeed, although there is no direct experimental proof so far, circumstantial evidence that suggests that m^2_2G might have a role in tRNA from thermophiles that is similar to that of m^1A in mitochondrial $tRNA^{Lys}$ is accumulating. Interestingly, those hydrogen bond donors and acceptors that are involved in the formation of the G-U wobble base pair are left unaltered upon modification of G to m^2_2G. While its ability to form a Watson−Crick pair with cytosine is ablated, m^2_2G may still undergo noncanonical interactions involving the N1 and O2 positions and thus form an m^2_2G-U wobble interaction. Scenarios in which this property might favor the functional cloverleaf structure have been proposed for m^2_2G at positions 10 (*43*) and 26 (*44*).

Large RNA structural alterations on the nanometer scale, which do not involve significant changes in base pairing, may be achieved by changes in the relative reorientation of structural domains. Modifications that may trigger or contribute to such effects are situated in the structural core of the tRNA, where they are thought to stabilize a number of tertiary interactions, which, in turn, govern the interstem angle between the anticodon and acceptor domains among other things. A compilation of biochemical and biophysical data for modified and unmodified tRNA (*10*, *12*, *14*, *15*, *45*−*52*) indicates strong interplay between magnesium ion coordination and post-transcriptional modification. Apparently, tRNA reaches its functional optimum at physiological concentrations of magnesium ions when fully modified, but the absence of modification can be partially compensated by increasing the magnesium ion concentration. In general, small RNAs have been observed to undergo compaction at increased concentrations of magnesium ions, likely as a general consequence of the increased level of shielding of the phosphates (*31*, *53*). There is evidence that structural core modifications (*54*), including, e.g., m^5C40 (*51*, *55*, *56*), improve the binding of magnesium ions. As the interstem angle of unmodified tRNA is known to strongly decrease at elevated concentrations of magnesium ions (*57*−*59*), nucleotide modifications are expected to promote a decrease in the angle at significantly lower, namely, physiological, concentrations of Mg^{2+} ions.

SUBTLE EFFECTS ON THE ANGSTROM SCALE

Only a few of the plethora of nucleotide modifications exert their influence via blocking of Watson−Crick interactions. Rather, most influence parameters of the local RNA structure and lead to stabilizing effects by restricting conformational flexibility on the angstrom scale. Viewed on that scale, e.g., by nuclear magnetic resonance (NMR), RNA appears as a string of nucleotides whose moieties are constantly and very rapidly changing their relative orientations. This fact is often less obvious in the commonplace presentation of crystal structures, but readily obvious in studies of RNA dynamics by NMR (reviewed in ref *60*); it also forms the basis of molecular dynamics (MD) simulations (*61*, *62*).

The structural characterization of a nucleotide unit in an RNA chain includes the sugar pucker and the relative conformation of the sugar and base as central parameters that are readily investigated by NMR (*60*). In unstructured RNAs, there is an equilibrium between the two predominant sugar conformations, 2′-endo and 3′-endo (Figure 3), which is shifted in favor of the 3′-endo conformation by hybridization to A-form helices. Nucleotide modifications favoring the 3′-endo conformation, such as methylation of the sugar 2′-OH group, pseudouridinylation, or thiolation of the pyrimidine O2 atom (Figure 3), will thus stabilize helical conformations (for references, see, e.g., refs (*63*−*65*); reviews in refs *60* and *66*).

Because NMR experiments are subject to restrictions of RNA size, only a limited number of studies on full-length tRNAs have

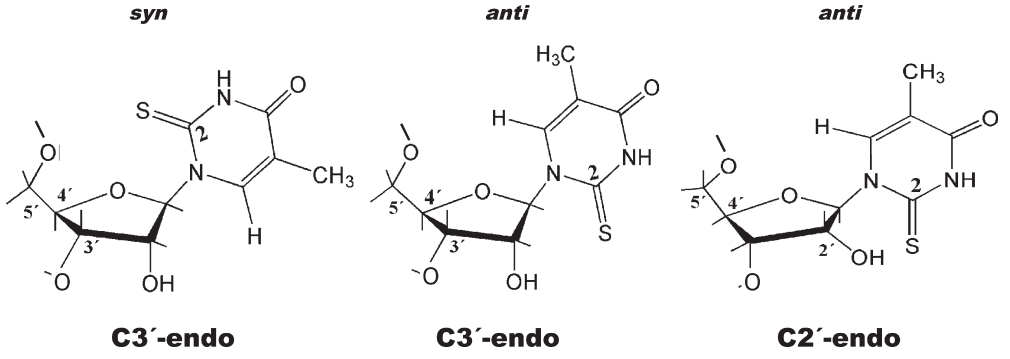

FIGURE 3: Local structural impact of chemically simple modifications. Nucleotide conformations are illustrated with s^2T, a modification typically occurring at position 54 in thermophiles growing above 80 °C: (left) 3′-endo conformation of the ribose and *syn* conformation of the base, (middle) 3′-endo conformation of the ribose and *anti* conformation of the base (most favored in s^2T), and (right) 2′-endo conformation of the ribose and *anti* conformation of the base.

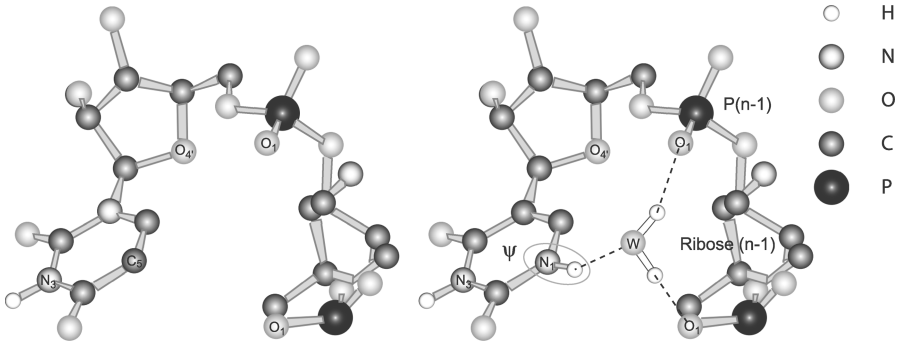

FIGURE 4: Coordination of water molecules to N1 of Ψ as modeled onto the X-ray structure of *E. coli* $tRNA^{Gln}$ based on an electron density difference map of modified vs unmodified $tRNA^{Gln}$ in a complex with its cognate amino acyl-tRNA synthetase (*90*). On the right side, the N1H group in Ψ is encircled, and the coordinated water molecule as well as their presumed hydrogen bonds to the neighboring phosphates is indicated.

been conducted (*47*, *50*, *67*, *68*). Truncated T-stem loops (*69*) and truncated anticodon stem loops (ASLs) of ~17 nucleotides are typical model systems, in which subtle to strong effects of various modified nucleotides, including Cm, Gm, m^1G, m^5C, Ψ, m^6A, i^6A, ms^2i^6A, t^6A, ms^2t^6A, mnm^5s^2U, mcm^5s^2U, and cmo^5U, have been studied. Although it has been suggested on the basis of tRNA crystal structures (*12*, *70*) that potential long-range effects of the tRNA core structure on the anticodon loop conformation may have been lost, ASLs are, in principle, functional in ribosome binding assays (*71*). This underscores the biological significance of studies including modified nucleotides in $tRNA^{Phe}$ from *Escherichia coli* (*72*−*75*) and yeast (*76*−*78*), $tRNA^{Lys}$ from *E. coli* and humans (*79*−*85*), and $tRNA^{Val}_3$ from *E. coli* (*86*). Of particular interest are studies on the shaping of the *E. coli* $tRNA^{Phe}$ ASL by modifications, because in the absence of modifications the characteristic anticodon loop structure is collapsed into a three-nucleotide loop at the expense of an elongated helix featuring U32-A38 and U33-A37 pairs as extra base pairs (*72*−*75*). Recent studies revealed an interesting effect of Ψ residues at positions 32 and 39, which contribute to the native loop structure and disrupt the abnormal U33-A37 base pair. Again, the interplay among magnesium ion binding, modification, and RNA structure appears to be an important feature (*12*, *72*).

Pseudouridine, once named the fifth ribonucleotide (*87*) because of its abundance, is an isomer of uridine. Formation of Ψ is an isomerization reaction catalyzed by pseudouridine synthases (Pus), which proceeds by cleavage of the N-glycosidic bond, base rotation, and reconnection to the sugar at C5 in a C−C bond (*88*). The structural impact of Ψ has been studied not only in a large number of RNA sequences, including various duplexes (*88*, *89*) and somewhat more complex anticodon stem−loop structures (ASLs), but also in structurally more complex systems. Ψ is present in numerous crystal structures of native RNAs, of which one is particularly instructive. Crystal structures of glutaminyl-tRNA synthetase have been obtained in complex with the native $tRNA^{Gln}$, as well as with the unmodified in vitro transcript. An electron density difference map revealed additional electron density near the "signature" endocyclic N1 of every Ψ present in the structure, which was interpreted as a water molecule whose order stems from coordination to the extra hydrogen bond site (*90*) (Figure 4). Depending on the exact structural context, this water molecule is thought to undergo additional interactions, in particular with the phosphate on the 5′ side of the Ψ (Figure 4). A coordinated water molecule as a mediator of a stabilizing influence of Ψ on RNA structure has also been predicted by MD simulations (*91*, *92*), and its presence is furthermore supported by results from advanced NMR techniques. In addition, in modified RNAs containing Ψ studied to date, the 3-endo sugar pucker conformation predominates (*93*−*95*). Both effects are consistent with numerous studies using CD and NMR spectroscopy and UV melting, which consistently reveal a structural stabilization of helices by Ψ residues, typically identified as an increased melting temperature (*88*, *89*, *93*−*97*).

THERMOSTABILITY

The structural impact of nucleotide modifications is often evaluated via UV melting experiments, in which certain structural transitions, in particular, melting of helices, are detected as an increase in UV light absorption along a temperature gradient.

Other types of spectroscopy, including NMR, have been used to the same end (*64*, *66*, *98*, *99*). An increase in the characteristic midpoint temperature of a transition, the so-called melting temperature, is indicative of a structural stabilization. This interpretation is well-grounded in thermodynamics, but interpretation is straightforward only for the melting of simple RNA duplexes (*100*). In biological interpretations of melting temperatures, it is often tacitly assumed that an increase in thermal stability and the associated structural stabilization are equivalent to increased resistance against unspecific catalytic degradation by general bases or nucleases (*101*), and thus associated with increased biochemical and metabolic stability. Support for this notion comes from the fact that hydrolysis of the phosphodiester backbone requires a linear "in-line" geometry of the phosphate and the neighboring 2′-OH moiety, which is the attacking nucleophile (*102*). This geometry is most easily achieved in largely unstructured, single-stranded regions, which can be distinguished from double-stranded and otherwise stable structures by detection of spontaneous hydrolysis in so-called in-line probing experiments (*102*). Because the 3-endo sugar pucker of helical regions prevents the in-line geometry, it protects the structure from spontaneous cleavage, as do nucleotide modifications that favor the 3-endo conformation or which lend thermal stability to RNA structures in general.

An interesting case in which thermal stabilization of tRNA structure by modifications is equivalent to physiological stabilization is given in thermophilic organisms. Their RNA must obviously retain a functional structure at temperatures as high as that of boiling water. In addition to the denaturation of secondary and tertiary structure, RNA is ordinarily subject to rapid spontaneous in-line-type hydrolysis at these temperatures. Thermophiles have developed several strategies for increasing the stability of tRNA structure (reviewed in ref *103*), including the synthesis of polyamines (*104*, *105*) and a high content of G·C base pairs, which is linearly correlated with an increase in melting temperature (*66*, *106*). Interestingly, certain nucleotide modifications, including G_m, $m^2{}_2G_m$, ac^4C_m, s^2T, and m^1A, are induced at high temperatures (*107*−*110*). Biosynthesis of these modifications conferred significant thermal stabilization to thus modified tRNAs (*109*−*111*), and null mutants deficient in the synthesis of certain modifications exhibit impeded growth at elevated temperatures (*112*, *113*). Among the best understood modifications from thermophiles with respect to biosynthesis and structural stabilization are G_m18, s^2T54, and m^1A58 (*66*, *114*). These modifications are clustered near or directly at those nucleotides forming the "classical" G18−Ψ55 and G19−C56 interactions between loop D and loop T, implying that they may (together with Ψ55) serve to stabilize this important tertiary interaction. Intriguingly, investigations of G_m18, which is also known in mesophiles, did not produce convincing evidence of significant tRNA thermal stabilization by this modification (*108*), and the same has been reported for m^1A58 (*66*, *108*). However, the presence of m^1A58 is required for the thiolation of T54 to s^2T54 in vitro and in vivo (*113*), and thus, the fact that growth of knockout strains of m^1A58 was impeded at elevated temperatures (*112*) was interpreted as being due to the lack of s^2T54 (*113*).

The thermophile-specific hypermodification s^2T54, however, is very effective in increasing the thermal stability of tRNA (*66*, *114*). In a groundbreaking early study, comparison of the melting profiles of purified *Thermus thermophilus* $tRNA^{Ile}_1$ containing T or s^2T at position 54 revealed an increase of 3 °C in thermal stability due to the presence of sulfur in s^2T54 (*64*, *111*, *115*). Studies of the structure and dynamics of s^2T as a nucleoside and in the context of the tRNA scaffold have revealed a strong stabilization for the 3′-endo/*anti* conformation (*66*, *114*) (Figure 3). Although residue 54 is not formally part of the T-stem helix as depicted in the cloverleaf structure (e.g., Figure.2), the three-dimensional loop T structure features an extension of the helix, also including T54, which undergoes an additional reverse Hoogsteen interaction with A58/m^1A58.

BIOLOGY OF tRNA MODIFICATIONS

Although synthesis and maturation of tRNA vary significantly among organelles, organisms, and kingdoms (*116*), many common features have been retained during evolution. With only one known exception (*117*), primary tRNA transcripts, which are frequently polycistronic, are processed at the 5′ end by RNase P. This enzyme removes leader sequences and leaves a 5′-phosphate on the tRNA's first nucleotide. Various mechanisms for removal of 3′ trailer sequences are known, and the universal 3′ end may be added or reconstituted by the CCA-adding enzyme (*118*−*120*). Early experiments in eukaryotes (*121*) suggest that certain modifications already appear at the level of the primary transcript, while some others take place only at the level of almost mature tRNA . It has been proposed that this may reflect the capability of these enzymes to recognize simple RNA structural elements present in the primary transcript (*122*), although differential expression and catalytic efficiencies of the various modification enzymes may arguably be an important factor. The tRNA maturation process is further complicated by elimination of introns inserted into the anticodon loops of many eukaryotic and archaeal tRNAs (*123*), and in eukaryotes, yet another layer of complexity is added by the export of partially mature tRNA species to the cytoplasm and possible later retrograde movement to the nucleus (*120*). The stepwise character of tRNA maturation and modification was clearly demonstrated in eukaryotes (*121*). Figure 5 (top panel) gives a brief visual outline of this complex maturation process and the implications of tRNA modifications in it. Recent findings suggest that tRNA modification does not passively accompany tRNA maturation but rather actively shapes the unstructured tRNA precursor into the fully functional tRNA having a complex three-dimensional structure. The interdependence of certain tRNA modifications (*124*−*126*) strongly indicates a step-by-step nature of at least parts of the whole tRNA maturation process. Indeed, speculation about a "molecular assembly line", in which the maturing tRNA is handed over from one modification enzyme to the next, has been supported by a certain amount of experimental evidence through the characterization of multienzyme complexes (*29*, *127*, *128*), and synthetic lethality mutants between the La protein implicated in tRNA processing and several tRNA modification enzymes, including tRNA:$m^2{}_2G26$-methyltransferase Trm1 or tRNA:pseudouridine synthase Pus4 (*129*).

Fully trimmed but incompletely modified tRNA can already partake in protein synthesis. This is obvious from studies on multiple deficiencies of tRNA modification enzymes, which lead to slow growth and translational defects both in bacteria [*E. coli trmH trmA truB* mutants (lacking Gm18, m^5U54, and Ψ55) (*130*)] and in yeast (lacking Ψ26−28, Ψ34, Ψ36, Ψ65, and Ψ67 from Pus1 and Ψ55 from Pus4) (*131*); further examples will be mentioned below. In yeast, this happens despite a certain extent of proofreading before export from the nucleus (*132*). It follows that systems for further surveillance and degradation act on a population of tRNAs, which are active in the translation

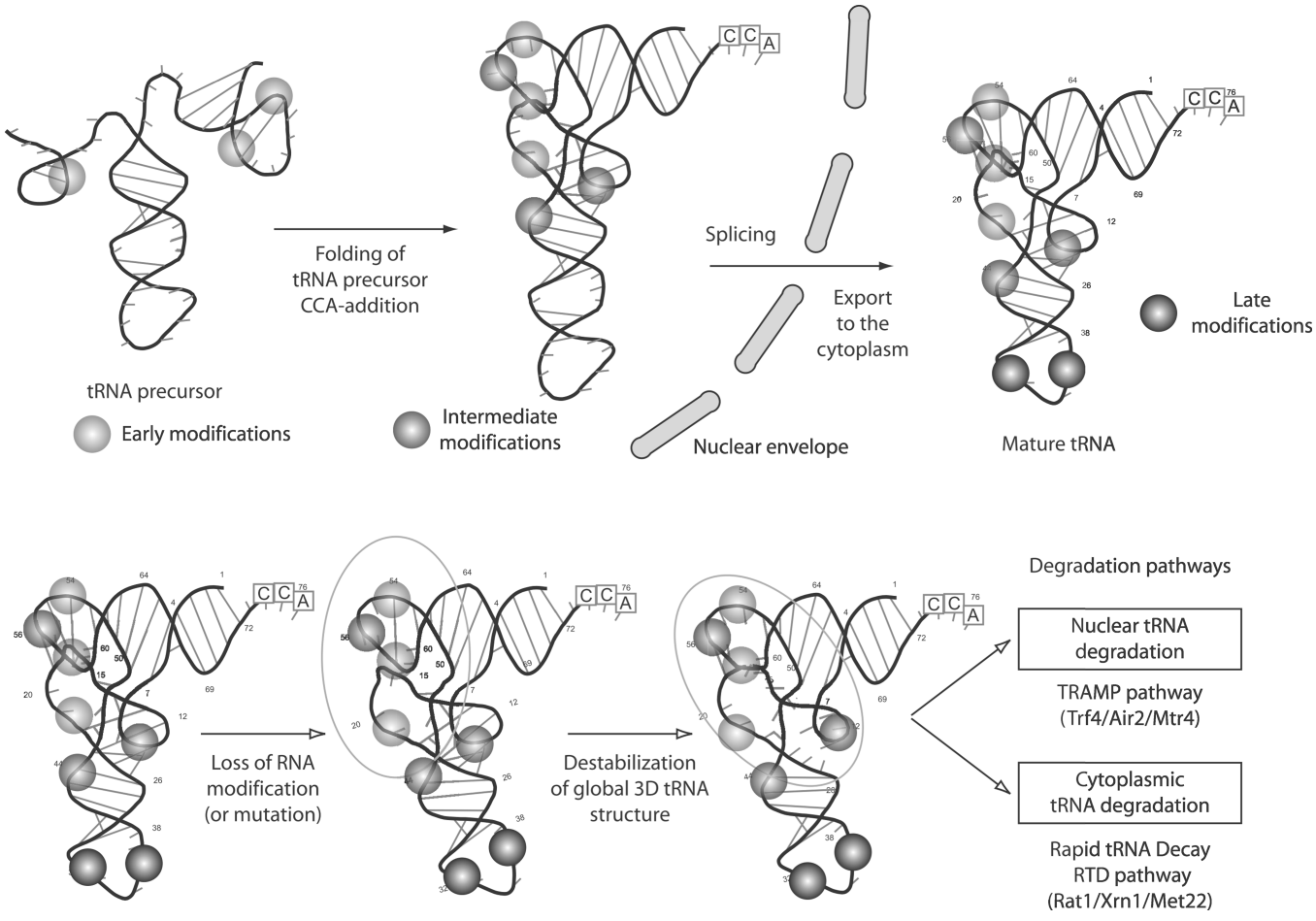

FIGURE 5: Biogenesis and degradation of tRNA. The top panel is a sketch of modification-induced structure acquisition during maturation. Early modification events are thought to be effected by enzymes recognizing partial tRNA features (*122*). The precursor is processed at the 3′ and 5′ ends on the basis of its intermediate tRNA structure, and further modifications are added (*121*), which lend additional stabilization to the tertiary structure. Certain modification enzymes are known to act on the unspliced precursor tRNA (*123*). In eukaryotes, splicing is effected after partial modification, but before transport from the nucleus to the cytosol. The bottom panel shows a sketch of targeted degradation of hypomodified tRNAs. It is thought that degradation proceeds as a consequence of destabilized three-dimensional interaction between loops D and TΨ of tRNA, followed by increased accessibility of the three-dimensional tRNA core and extremities to nucleases and poly(A) polymerase.

machinery and were traditionally considered to be shielded from degradation (*133*).

Impact of Modifications on tRNA Processing, Stability, and Degradation. Tight relationships between the integrity of global tRNA structure and stability have become especially apparent in mitochondrial tRNA, possibly because these are less well structured and less extensively modified than their cytosolic counterparts. Certain tRNA-dependent mitochondrial dysfunctions have been described as a consequence of the relative instability of aberrant tRNA species. Nucleotide substitutions observed in pathogenic tRNAs may affect various aspects of tRNA metabolism, like processing (*134*), modification, or aminoacylation (*135*, *136*). Certain point mutations touch the tRNA's structural scaffold itself (*39*), while a mutation in the pus1 gene causes MLASA[1] (myopathy with lactic acidosis and sideroblastic anemia) as a consequence of absent structural Ψ residues, e.g., at positions 27 and 28 of human mitochondrial tRNA (*137*, *138*). In certain cases, the consequence of mitochondrial tRNA mutations clearly was an intrinsic instability of tRNA species in vivo. For example, mutation in human mitochondrial $tRNA^{Ile}$ reduces the stability of this tRNA in vivo and, as a consequence, reduces the level of amino acyl-tRNA. The half-life of mutant tRNA is considerably reduced both in vivo and in vitro, and the melting temperature was also found to be reduced (*139*). Similar observations were also made for other point mutants of mitochondrial tRNAs (*135*, *136*).

Recent studies of tRNA degradation pathways in yeasts clearly demonstrated that tRNA stability in vivo depends not only on the tRNA sequence itself but also on its modification profile. Several yeast deletion mutants with multiple deficiencies in tRNA modification exhibit a strong growth phenotype, indicating the importance of these modified residues in vivo.

Pathways for Degradation of Hypomodified tRNA in Vivo. Accelerated degradation of hypomodified tRNA species was observed in both bacteria and eukaryotes. Careful characterization of the Δ*TrmB* mutant of the extreme thermophilic eubacterium *T. thermophilus* demonstrated the absence of m^7G46 in tRNAs in vivo. Unexpectedly, the lack of m^7G46 also led to hypomodification of Gm18 and m^1G37. Even if aminoacylation properties of hypomodified tRNA species seem not to be affected, the melting temperature and stability of certain tRNAs in vivo were found to be considerably reduced. As a consequence, the protein synthesis is strongly depressed and the ΔTrmB mutant exhibits a strong growth phenotype at 80 °C (*140*).

Degradation pathways of hypomodified tRNA species are far better studied in lower eukaryotes. Yeast strains deficient in

[1]Abbreviations: MLASA, myopathy with lactic acidosis and sideroblastic anemia; RTD, rapid tRNA decay; tRFs, tRNA fragments or tRNA halves.

Trm6 (or Trm61) tRNA:m^1A58-methyltransferases display a reduced level of initiator $tRNA^{Met}_i$ at elevated temperatures (*20*, *141*). The selective degradation of $tRNA^{Met}_i$ lacking m^1A58 can be attributed to a weak tertiary interaction between loops D and T that is characteristic of $tRNA^{Met}_i$ (*142*). Similar data were also obtained for $tRNA^{Val(AAC)}$ in *trm8*-Δ *trm4*-Δ mutants, which lack m^7G46 and m^5C34, -40, -48, and -49 in the respective tRNAs (*6*). Reduced in vivo levels of $tRNA^{Ser(CGA)}$ were observed in yeast strains accumulating both a $tRNA^{Ser}$ mutation and deficiencies in Trm2 or Pus4 activities (*143*). The same observation was also made for yeast in strains lacking Um44 and ac^4C12 due to deletion of *TRM44* and *TAN1* genes (*144*). In all studied cases, hypomodified tRNAs are rapidly degraded, but their degradation seems to proceed by two different independent pathways, depending on the cellular compartment.

The first degradation pathway for elimination of destabilized tRNA species in the nucleus is mediated by the TRAMP complex which contains the poly(A) polymerase Trf4, the RNA-binding protein Air2, and the DExH RNA helicase Mtr4p (*20*, *145*). Following polyadenylation, these molecules are degraded by exonuclease Rrp6 and nuclear exosome.

An alternative pathway of tRNA degradation in the cytoplasm ("rapid tRNA decay", abbreviated RTD) is independent of TRF4/RRP6. The existence of an Trf4-independent pathway was initially suggested by analysis of La protein synthetic mutants, and its mechanism was determined by analysis of $tRNA^{Val(AAC)}$ degradation in the *trm8*-Δ *trm4*-Δ strain lacking m^7G46 and m^5C34, -40, -48, and -49 (*5*, *6*). This RTD pathway includes the 5′−3′ exonucleases Rat1 and Xrn1 and an auxiliary protein Met22. Met22 is probably not directly involved in RNA degradation but rather in cleavage of the pAp intermediate resulting from Rat1 and Xrn1 activity. However, the role of the RTD pathway seems to be more complicated since the aminoacylation level of $tRNA^{Val(AAC)}$ is affected by mutations of its components.

Taken together, these observations provide a link between specific pathways of tRNA degradation and general surveillance mechanisms ensuring RNA quality control in the cell.

CONCLUSIONS AND OUTLOOK

Lessons learned from studying various species of modified RNA and its structural rearrangements (*93*−*95*, *146*−*150*) supplement the principal conclusions drawn from tRNA-based investigations. Numerous RNA modifications are present not only in tRNAs but also in most other cellular RNAs, like rRNA, snRNA, snoRNA, and mRNA (*7*−*9*). Particularly interesting RNA structural data were obtained for a fragment of 28S rRNA bearing five highly conserved pseudouridine residues (*150*) and for a short RNA duplex imitating the interaction between the mRNA branch point and U2 snRNA (*93*−*95*, *146*). The presence of modified residues was also revealed in recent studies of regulatory RNAs (*151*).

Coming full circle, modifications of the "structural" type would be present in the small fragments of cleaved tRNAs (tRFs or tRNA halves), which were recently discovered in eukaryotic cells (*152*, *153*). These are thought to play a role in regulation of various functions (*154*, *155*) and, possibly, malfunctions such as cancer. Indeed, the differential occurrence in cancer cells of modifications such as G_m and C_m, albeit in the anticodon, has been observed in the early going (*156*−*159*), and urinary modified nucleosides are still being considered as cancer markers (*160*, *161*). Little is known to date about the functional and structural importance of these modifications, but undoubtedly, the knowledge accumulated from tRNA studies will have a direct impact on our ability to detect and interpret modifications in newly discovered RNA species.

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