RNA Biology 7:6, 634-641; November/December 2010; © 2010 Landes Bioscience

RNA folding in living cells

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Key words: RNA folding, in vivo chemical probing, ribozyme, RNA-protein interactions

Abbreviations: POL, RNA polymerase; POLs, RNA polymerases; RNAP, RNAse P RNA; C-domain, catalytic domain of RNAse P RNA; S-domain, specificity domain of RNAse P RNA; DMS, dimethyl sulphate; RBS, ribosome binding site; NAIM, nucleotide analog interference mapping; 5'SS, 5' splice-site; asRNAs, antisense RNAs; sRNA, small non-coding RNA; ncRNA, non-coding RNA; 5'UTR, 5' untranslated region

RNA folding is the most essential process underlying RNA function. While significant progress has been made in understanding the forces driving RNA folding in vitro, exploring the rules governing intracellular RNA structure formation is still in its infancy. The cellular environment hosts a great diversity of factors that potentially influence RNA folding in vivo. For example, the nature of transcription and translation is known to shape the folding landscape of RNA molecules. Trans-acting factors such as proteins, RNAs and metabolites, among others, are also able to modulate the structure and thus the fate of an RNA. Here we summarize the ongoing efforts to uncover how RNA folds in living cells.

Introduction

RNA molecules play a central role in virtually all cellular processes. To exert their function RNAs have to fold into specific three-dimensional structures. The process of folding describes how an RNA molecule undergoes the transition from the unfolded, disordered state to the native, functional conformation. In vitro RNA folding has been intensely studied, mostly using catalytic RNAs as model systems. Measuring formation of the native structure as a function of catalysis provides an immense advantage for investigating ribozyme folding. Hitherto several folding paradigms have been discovered.[1-10] In principle, RNA encounters two major folding problems:[11] (i) RNA molecules are prone to misfold, thereby becoming trapped in inactive, often long-lived conformations, the escape from which becomes rate-limiting during the folding process; (ii) the native, functional RNA conformation might not be thermodynamically favored over other intermediate structures, thus requiring the assistance of a specific RNA-binding protein (or high salt) for stabilization of the tertiary structure.

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Submitted: 07/30/10; Revised: 09/02/10; Accepted: 09/05/10
Previously published online
www.landesbioscience.com/journals/rnabiology/article/13554
DOI: 10.4161/rna.7.6.13554

Studying RNA folding in vitro typically starts with a random coil of in vitro transcript, which is first denatured at high temperature and subsequently refolded in the presence of metal ions. In most cases, this involves non-physiological conditions with respect to the ionic concentration and folding temperature.[1-10] As the cellular environment differs significantly from the in vitro refolding scenario, the question of how well in vitro and in vivo folding pathways correlate remains to be addressed. In the past decade there has been considerable effort to investigate RNA folding in vivo, providing the first profound insights into the forces governing intracellular RNA structure formation.[12] In the cell RNA folding is likely to be influenced by the speed and directionality of transcription and, in prokaryotes, of translation. In addition, bimolecular interactions with other RNA molecules, proteins, metabolites and ligands (i.e., polyamines) as well as ion homeostasis play a key role in successful folding of a functional transcript. Aside from the nature of such trans-acting factors, their charge, specificity, concentration and localization are crucial aspects inasmuch they might shape intracellular RNA folding. Due to the complexity of the cellular environment and the restricted availability of suitable methodologies to address this issue, our knowledge on RNA folding in vivo is still limited. Therefore, complementary in vitro approaches, in which the intracellular situation is mimicked, remain an invaluable resource. Such in vitro folding studies allow dissecting the contribution of individual cellular aspects independent from one another (e.g., co-transcriptional folding in absence of RNA processing and RNA-binding proteins). The novel insights gained from the in vivo and near-physiological in vitro studies will be discussed in this review.

Co-transcriptional RNA Folding

In vitro the full-length transcript is denatured and then refolded at optimal ionic strength and temperature. While small RNAs fold on the μs to ms timescale in vitro, large RNA molecules may require minutes to hours to reach the functional state.[1-10] In the cell, however, most nascent transcripts are likely to fold more rapidly during transcription. The first evidence for co-transcriptional folding came from the observation that autocatalytic splicing of the Tetrahymena group I intron occurs on the same timescale as synthesis by the RNA polymerase (POL).[13] Considering that the

elongation speed ranges from 10–20 nt·s^{-1} for human POL II, to 20–80 nts^{-1} for bacterial POLs and to 200 nt·s^{-1} for phage POLs, most nascent transcripts are likely to fold during transcription.[14] The order in which RNA structural elements are synthesized and the elongation speed significantly influence the folding rate and the type of folding intermediates that are formed, supporting the idea of RNA folding being co-transcriptional.[15-19] Interestingly, while folding of the Tetrahymena intron is affected by the different transcription rate of POLs from *E. coli* and T7-phage,[17] the RNAse P RNA (RNAP) did not respond to this difference in transcription speed.[20,21]

The pioneering work of Sosnick, Pan and coworkers revealed that in addition to the elongation rate, transcriptional pausing, which is a property of bacterial POLs, and its duration affect RNA folding as well.[20,23] They demonstrated that the protein NusA drastically increased transcriptional pausing at a specific site in the RNAP C-domain, thereby preventing the formation of a non-native interaction between the C- and S-domains of RNAP.[20] In turn the folding rate of the RNAP S-domain was significantly accelerated. However, co-transcriptional folding can also be slower than Mg^{2+}-initiated re-folding, as evidenced by the RNAP C-domain, which folds at timescales of 0.2 s^{-1},[14] and 6 s^{-1},[22] respectively. Furthermore, folding of three conserved *E. coli* ncRNAs, RNAP, signal-recognition particle RNA and tmRNA is facilitated by pausing-induced non-native interactions.[23] The cognate POL pauses between the upstream and downstream portions of native long-range helices and these sites are also conserved among γ-proteobacteria.[23]

Transcriptional pausing was also found to play a role in folding of the FMN riboswitch, in which two major pause sites are found between the riboswitch structure and an intrinsic terminator stem, whose formation is induced by RNA-FMN complex.[19] As the bimolecular interaction of the riboswitch and the metabolite FMN is coupled to transcription, pausing could provide additional time to allow formation of the riboswitch structure, thereby sensing and responding to the intracellular FMN concentration and in turn proceeding or terminating transcription. Notably, there is also evidence that transcriptional pausing occurs in vivo as well, as a nascent hairpin of a reporter transcript restricts the lateral POL movements.[24]

Even though folding of nascent transcripts has almost exclusively been studied in vitro, recent reports provided important insights into other events that take place co-transcriptionally in vivo. Neugebauer and coworkers demonstrated that the spliceosome assembles in a stepwise manner on the nascent pre-mRNA in yeast, suggesting that splicing occurs also co-transcriptionally.[25] Importantly, the Cap-binding complex appears to mediate coupling of pre-mRNA splicing and transcription. Aside from intron splicing, most RNAs have to undergo additional processing events. For example, there has been considerable evidence for co-transcriptional assembly, modification and processing of pre-rRNA,[26,27] whereby the assembly factors are recruited by POL I.[28] Only recently Kos and Tollervey were able to show that the 35S primary rRNA transcript is synthesized in ~170 s in yeast and indeed almost ¾ of the nascent transcripts were cleaved at the early processing sites. In addition, the nascent 20S precursor transcript is predominantly methylated.[29] These events are critical for proper rRNA folding and in turn for ribosome subunit assembly.

Numerous functional and physical links have been described between transcription, splicing, polyadenylation, decay, processing and export.[30,31] This could be mediated by the specific subset of RNA-binding proteins which are delivered by the RNA polymerase to the nascent transcript. These "hitchhiking" proteins could potentially modulate RNA folding. A first insight comes from the finding that the Tetrahymena intron partitions into active and misfolded, non-functional pools in vivo, whereby the pool size differs depending on the transcribing POL.[32]

The Contribution of Kinetics vs. Thermodynamics to Intracellular RNA Folding

Folding kinetics and thermodynamic stability have been found to make distinct contribution to folding of several RNAs in vitro.[1-10] Among these RNAs is the hairpin ribozyme, which is a small RNA catalyzing a well-characterized cleavage and ligation reaction in vitro.[33,34] The equilibrium of these two competing reactions depends on secondary and tertiary structure stability and reaction conditions. Due to its simple reaction mechanism and the detailed description of its in vitro kinetic framework[33,34] the hairpin ribozyme is an ideal candidate to explore forces driving intracellular RNA folding. During the past years, Fedor and coworkers perfomed elegant experiments,[35-38] showing first that the self-cleavage rate of the minimal hairpin ribozyme is 5-fold slower in yeast (0.06 min^{-1}) than in vitro (0.3 min^{-1}).[35] In contrast, the increased stability of the natural ribozyme, which consists of a 4-way junction, results in a rapid cleavage reaction in yeast with comparable kinetic parameters measured in vitro under near-physiological conditions.[38] Also, mutations that slow down cleavage rates in vitro, display a comparable effect in yeast,[35] indicating that the ribozyme uses the same cleavage mechanism in yeast and that folding is not rate-limiting neither in vitro nor in vivo.

To assess whether kinetics and thermodynamics make the same contribution in vitro and in yeast, co-transcriptional folding of hairpin ribozyme variants containing complementary insertions upstream or downstream of the ribozyme was analyzed.[36] In vitro the upstream insertions inhibited ribozyme assembly more than downstream ones in line with a sequential folding mechanism, in which structures that assemble first dominate folding. In contrast, when expressed in yeast, both upstream and downstream inserts blocked the ribozyme activity, providing evidence that the intracellular fold reflects the relative stability of alternative structures.[36] Mahen et al. recently extended their previous analysis[36] and reported that a narrow thermodynamic threshold determines whether kinetics or thermodynamics guide intracellular RNA folding.[37] Thus, the hairpin ribozyme follows a sequential folding mechanism in yeast and in vitro; the exchange between adjacent structures is however faster in vivo than in vitro.[36,37] This fast exchange could potentially be mediated by proteins, such as specific RNA-binding proteins or RNA chaperones, among others.

There is also considerable in vivo evidence that the Tetrahymena intron folds co-transcriptionally, as the probability of correct folding both in *E. coli* and in yeast depends on the RNA polymerase by which the pre-rRNA is transcribed.[32,39] Despite the apparent difference in size and complexity between these two ribozymes, co-transcriptional RNA folding seems to be an important driving force in vivo.

Exploring RNA Folding Intermediates In Vivo

The pathway to the functional structure is hierarchical interspersed by intermediate folding states.[1-10] Most of these folding intermediates are on-path presenting an increased amount of native contacts. Thus, identifying folding intermediates and describing their structural content are among the most intriguing questions about an RNA folding pathway. Hitherto, significant progress has been made in characterizing intermediate folding states in vitro, in part even at atomic resolution.[1-10,40-42] However, little is known about the folding energy landscape that RNAs traverse in vivo.[12] In other words, how well do in vitro and in vivo folding pathways correlate? Given the disparate environments during in vitro refolding and intracellular RNA structure formation, a comprehensive comparison of RNA folding in vitro under non-physiological conditions and in vivo is of tremendous interest. To our knowledge there is only a single study in which this fascinating aspect had been addressed. Group I introns consist of two major structural domains, the P4-P6 and P3-P9 domains, which assemble to form a cleft for binding stem P1, which contains the 5'splice-site (5'SS). During the past decades folding of group I introns, in particular of the Tetrahymena ribozyme, has been carefully dissected in vitro.[4,6-10] In short, the Tetrahymena ribozyme traverses a rough free-energy folding landscape, but in the direct pathway the P4-P6 domain folds rapidly followed by the slow assembly of the P3-P9 domain.[43] These main characteristics are shared by other group I introns.[4,9,10] To assess group I intron folding in vivo, the structure of *td* wild-type and mutant introns was monitored using DMS chemical probing in *E. coli*. Intron mutations have a distinct impact on the tertiary structure of the *td* ribozyme, suggesting that they interfere with folding at different stages: destabilization of stem P6 caused structural perturbations in both major domains, while weakening of stem P7 only interferes with folding of the P3-P9 domain. Monitoring the intracellular structure of these folding intermediates allowed describing a putative order of events in a hierarchical in vivo folding pathway, which is reminiscent of the main folding steps observed in vitro.[43]

Misfolded RNA and RNA Chaperones

RNA is composed of four main building blocks; however, despite this apparent simplicity RNAs can fold into more than one stable conformation of which only one represents the functional state.[1-12] The formation of non-native interactions results in alternative secondary or tertiary structures. As these non-native elements can be as stable as native contacts, the misfolded structures are commonly long-lived (min to hours) trapping the RNA in an off-pathway intermediate.[1,3,4,6-10] Thus, the escape from such a kinetic trap is often the rate-limiting folding event. In vitro such dominant folding barriers have been extensively studied for the Tetrahymena intron and RNAP among others. Notably, only few RNAs have been found to follow a two-state pathway or to traverse a smooth landscape.[2,5,44]

In case of the Tetrahymena intron the RNA can be trapped in misfolded conformations through both native and non-native interactions.[45,46] The former is caused by a tertiary interaction that is formed too early along the pathway[46] and the latter by mispairing in the catalytic core.[45] Thus, there exist multiple, parallel folding pathways for Tetrahymena ribozyme in vitro.[47,48] However, are such aberrant conformations in vitro artifacts or does RNA misfold in vivo? For a long time it had been known that RNA folding is facilitated in vivo.[12,49,50] Nevertheless, there is accumulating evidence that RNA misfolding also occurs in cells.[32,51] Recently, Woodson and coworkers described another important parallel between in vitro and intracellular folding, when they found that the Tetrahymena intron indeed partitions into native and misfolded populations in yeast.[32] Notably, the amount of native vs. misfolded intron molecules is strongly influenced by flanking sequence context and by the transcribing POL. In other words, most active intron was formed upon integrating the ribozyme into a natural-like rDNA context, which is transcribed by POL I, compared to inserting the intron into a POL II pre-mRNA transcript. Most strikingly, however, the misfolded intron species was sequestered and degraded without undergoing RNA chaperone-guided refolding.[32]

One of the first examples of in vivo misfolding was observed for the *td* group I intron. Schroeder and coworkers revealed that translation is required for efficient splicing of the T4-phage derived *td* intron in *E. coli*.[51] A folding trap consisting of the 3' end of the intron and a complementary sequence in the upstream exon forming a stable hairpin is located close to the 5'SS. By introducing a stop-codon in the upstream exon the ribosome is prevented from reaching the 5'SS and in turn from ironing out the trap, resulting in a strong decrease in splicing.[51] Alternatively, in the absence of translation a diverse group of proteins, the so-called RNA chaperones, are able to resolve the misfolded *td* intron structure and in turn to promote efficient splicing in vivo.[52] The best-studied RNA chaperone is the *E. coli* transcriptional regulator StpA. To obtain the very first insights into the mechanism of RNA chaperones, the impact of StpA on the structure of the *td* intron was assessed in vivo.[53] StpA was found to open the misfolded intron structure by resolving tertiary contacts and in turn reverting the intron conformation to an earlier folding intermediate, giving the RNA another chance to reach the native, splicing-competent state. This destabilizing activity of StpA rendered splicing of *td* intron mutants with reduced structural stability sensitive to StpA.[53,54]

Meanwhile a large number of proteins have been described as RNA chaperones.[3,55] This heterogeneous set of proteins does not share any sequence conservation or common motifs. Instead they have distinct primary cellular functions in transcription, translation, RNP assembly and stabilization as well as virus replication, among others. In addition to their main task, these proteins with

RNA chaperone activity are capable of resolving kinetic traps by destabilizing RNA structural elements.[3,55] The precise mechanism of action has not been deciphered yet. It is possible that there are different modes underlying RNA chaperone activity. So far some general principles have been revealed: many RNA chaperones bind RNA non-specifically and with low affinity, implying that the interaction is transient and in some cases of mere electrostatic nature.[3,55] On the other hand, there are also RNA chaperones, like ribosomal proteins, that bind RNA with rather high affinity.[3,55] Once the RNA is properly folded, the protein does not have to remain associated with the RNA to maintain its native conformation. The most intriguing hallmark of RNA chaperones is the fact that their activity does not require ATP binding and/or hydrolysis.[3,11,55,56] One attractive model of how RNA chaperones might facilitate refolding of non-native conformers is the entropy transfer model,[57] wherein RNA chaperones containing intrinsically unstructured regions become structured upon unfolding a misfolded RNA molecule.

RNA and its Protein Collaborators

Many RNAs including large, multi-domain ribozymes are able to fold into their functional conformation without assistance of ligands other than metal ions in vitro. Thus, in vitro metal ions are sufficient to neutralize the repulsion of the negatively charged RNA backbone during compaction and to stabilize RNA folding intermediates and/or the native state.[58,59] The ionic conditions required for native state formation in vitro are, however, typically non-physiological. For example, group II introns, like *Sc.* ai5γ, depend on 0.5 M monovalent ions, 100 mM Mg^{2+} and 42°C as optimal folding and splicing conditions.[60] While ion homeostasis is known to play a critical role for RNA splicing in yeast,[61] the intracellular ion concentration is well below that necessary for in vitro folding. On the other hand numerous trans-acting factors, such as specific RNA-binding proteins, are present in vivo and potentially facilitate RNA structure formation. The interaction of some of these cofactor proteins with their target RNA(s) have been extensively studied in vitro.[62-73] These proteins were found to recognize and bind a distinct sequence or structure, thereby guiding folding and stabilizing the structure of their target RNA. Notably, stabilizing RNA cofactors, like Cyt18, LtrA, Cbp2, CRS1 were observed to lower the Mg^{2+} requirements for folding of their target RNAs in vitro.[56,67,74,75,142] For example, the *Neurospora crassa* aminoacyl-tRNA synthase Cyt18 also functions as group I intron splicing factor both for the endogenous introns ND1 and mtLSU and can also stabilize the catalytically active structure of heterologous introns (e.g., the T4 phage td, yeast mitochondrial bI5 or Tetrahymena introns).[76-78] By binding to a folding intermediate, Cyt18 guides RNA folding and stabilizes the intron structure.[53,64,77,79] Importantly, Cyt18 uses completely different binding interfaces to recognize and interact with these two distinct classes of RNA, tRNAs and self-splicing group I introns.[68,69] Consistent with its role as stabilizing cofactor, Cyt18 is capable of rescuing splicing of *td* intron mutants with a reduced structural stability.[53,77,80] Indeed, the structural perturbation of these intron mutants was monitored in vivo and found alleviated in the presence of co-expressed Cyt18, in that the splicing factor contributes to the overall compactness of the intron structure and strengthens tertiary contacts in vivo, thereby rescuing splicing.[53,79] This is also consistent with the fact that mutating Cyt18 impairs group I intron splicing in *N. crassa*,[66,81] suggesting that the native intron structure is not thermodynamically the most favored one in vivo and thus a specific protein is required to stabilize the tertiary intron structure.

Another stabilizing RNA cofactor is the yeast protein Cbp2, which promotes splicing of the yeast mitochondrial bI5 intron in vitro and in vivo.[73,75,82,83] At near-physiological conditions Cbp2 captures the collapsed folding intermediate of the bI5 intron in vitro and promotes the native state without inducing large scale rearrangements.[72,73,78,84] In vitro Cbp2 is also capable of binding the extended bI5 intron, but this complex represents a kinetic trap.[72] Thus, the fast collapse of the bI5 intron to a compact state represents a self-chaperoning mechanism.[72] Interestingly, in vivo the bI5 intron requires an additional cofactor, the DEAD-box helicase Mss116p, in order to splice efficiently.[85] So far, the role of Mss116p in bI5 splicing remains enigmatic.

RNA Helicases—Unwinding and Annealing of RNA

RNA helicases are an ubiquitous protein family involved in virtually all cellular processes by promoting RNA structural rearrangements and RNP remodeling in an ATP-dependent manner.[86-90] While a few helicases (e.g., NS3A, NphII) have been described as highly processive enzymes, members of the DEAD-box helicase subfamily are non-processive. Interestingly, some of these proteins (e.g., Ded1p, Mss116p) can unwind and anneal RNA strands, whereby the latter activity is ATP-independent.[91-93] While most mechanistic insights were gained from studying helicases acting on model RNA substrates, the function of the yeast mitochondrial DEAD-box protein Mss116p has recently been examined for one of its natural target RNAs.

Mss116p is essential for efficient splicing of all yeast mitochondrial introns (group I and II introns).[85,94] Like for stabilizing RNA cofactors, Mss116p was observed to reduce the [Mg^{2+}] necessary for intron folding in vitro[91,92] albeit displaying unwinding and annealing in vitro.[91,92,95] While Mss116p's ATPase activity is required to promote intron splicing both in vitro and in vivo,[85,92] it has been a matter of debate whether its unwinding activity is also essential for this process.[96] As a consequence two distinct models for how Mss116p facilitates RNA folding have been put forward: it might function as a splicing factor by acting in an RNA chaperone-like fashion[91,96] or by providing stability to the intron RNA.[92] While the early information on Mss116p's mechanism was entirely inferred from splicing kinetics, Pyle and coworkers recently directly monitored DEAD-box protein-facilitated folding of the ai5γ intron.[97] Mss116p was observed to directly stimulate ai5γ folding by accelerating the collapse to the near-native state in an ATP-independent manner through stabilization of an early folding intermediate.[97] ATP on the other hand is required for the protein turnover. Importantly, Mss116p is not required

to stabilize the native state of the ai5γ intron, but stabilization comes from binding of flanking exon sequences. Along this line, we have probed the structure of the ai5γ intron in different yeast genetic backgrounds. In the absence of Mss116p the intron is largely unfolded, suggesting that this protein is required for the formation of an early folding intermediate in vivo as well.[143] In addition, Mss116p influences the folding mechanism in another way: long exon sequences interfere with ai5γ splicing in vitro.[98] Abolishing the unwinding activity of Mss116p strongly affected splicing of pre-RNA with long exons but not with short exons, indicating that unwinding is essential for exon unfolding, but not for intron folding.[98] Thus, a protein known for its unwinding activity functions as a stabilizing cofactor as well. Aside from the conserved helicase motifs located on the N-terminal domain of Mss116p, this DEAD-box protein contains an arginine-rich, positively charged C-terminal domain, which is shared by only a few other DEAD-box proteins (e.g., Cyt19 and Ded1p).[99,100] The very same helicases were also shown to functionally substitute for Mss116p in vitro and in vivo.[85,92,101]

At a first glance the mechanism of action of helicases appears related to that of RNA chaperones. However, in contrast to RNA chaperones, DEAD-box proteins require an external energy source to unwind RNA and many of them depend on a loading platform.[3,55,87,88,90]

Modulation of RNA Structure Mediated by ncRNA

In the past decade a vastly growing number of small non-coding RNAs (sRNAs) have been identified in numerous organisms (i.e., archaea, bacteria and eukaryotes). These sRNAs are ribo-regulators affecting all steps of gene expression through modulating RNA structure, thereby allowing the cell to adapt to environmental cues and to control bacterial pathogenesis.[102-105] In general, one has to distinguish between sRNAs acting in trans and those in cis.

Of sRNAs the antisense RNAs (asRNAs) are a large class that target mRNAs through base-pairing.[102-105] The asRNA regulation typically results in the inhibition of transcription or translation or it induces degradation of target RNAs. These asRNAs can be transcribed from the opposite strand of genes which they regulate. These cis-asRNAs usually display extensive base-pairing with a single target mRNA (e.g., the *hok-sok* pair).[102-105] Commonly such asRNAs are associated with plasmids, phages and transposons or act as antitoxins. Alternatively, asRNAs are encoded from an autonomous locus in the genome. These trans-asRNAs only share short stretches of complementarity with their multiple target mRNAs.[102-105] The majority of trans-asRNAs depend on an accessory protein for exerting their activity. The best-studied example is the *E. coli* Sm-like protein Hfq, which, for instance, stabilizes the sRNA DsrA and facilitates its interaction with the *rpoS* mRNA, thereby altering its structure and in turn activating translation.[106-110] Similarly, in *S. aureus* the quorum sensing RNAIII interacts with the transcriptional regulator *rot*, among others.[111,112] Upon interaction the RBS of the *rot* mRNA becomes inaccessible, thereby repressing its translation and subsequently the asRNA-mRNA complex is degraded by the ds-specific RNAse III. In general, the most common type of regulatory mechanism by trans-asRNAs is translation repression, but a few notable examples for an activating mode of action have been described.[102-105]

In addition, mRNAs, specifically their 5'UTR, can function as a direct sensor for the physical and metabolic state of the cell. One class of cis-regulatory elements, the riboswitches, responds to trans-acting ligands such as the intracellular concentration of metabolites (i.e., nucleobases, coenzymes, sugars, amino acids) and metal ions.[113,114] In an allosteric manner, binding of the ligand induces a structural change in the riboswitch, thereby affecting the expression of the downstream element by controlling either transcription termination, translation initiation or mRNA processing (i.e., alternative splicing).[113,114] Many of the regulated genes encode proteins involved in the transport or biosynthesis of the effector molecule. Thus, if the intracellular concentration of the effector molecule is high, it binds to the regulatory element and induces a conformational change that results in a shut down of transcription/translation of the respective mRNA. Another type of cis-acting elements are the thermosensors, which sense temperature shifts.[115,116] Consistent with their function, these elements are found in mRNAs encoding heat- or cold-shock proteins among others. At low temperature the RBS is part of a helical structure preventing the formation of the translation initiation complex. Upon raising temperature the RBS-containing helix melts allowing translation of the mRNA. In case of the *cspA* mRNA, encoding a cold-shock protein, the cold activates its translation and stabilizes the transcript (see also review by Phadtare and Severinov in this issue). In vivo this cold-shock response depends on an alternative mRNA structure, which forms co-transcriptionally.[117] Thus, the transcription velocity, RNA folding kinetics, ribosome recognition may all contribute to the activity of thermosensors. In brief, we are only beginning to understand the scope of ncRNA function, which these molecules exert through modulating their target RNA's fold.

Current Methods Employed to Study RNA Structure in vivo

A diversity of experimental methods including physical and chemical approaches is applied to probe RNA/RNP structures in vitro. The cellular complexity limits however their applicability in analyzing the RNA structure and function within living cells. To date, several chemical reagents, which are sensitive to secondary and/or tertiary structure, have been used for probing RNA structure in vivo (reviewed in ref. 118–122). DMS is the most successfully used chemical to probe RNA structure in a variety of organisms, ranging from bacteria to eukaryotes.[120,123-131] A major convenience of DMS is that it rapidly penetrates all compartments of cells without prior cell permeabilization. After uptake, DMS methylates N7 of guanines, N1 of adenines and N3 of cytosines, if they are not involved in H-bonding or protected by proteins. Similarly, lead-(II)-acetate was used to probe RNA structure in bacteria.[121,132,133] This ion easily enters bacterial cells and primarily induces specific cleavages at positions of

tight metal ion binding. Lastly, hydroxyl radical footprinting can be applied to probe RNA tertiary structure and intermolecular interfaces.[118] In brief, X-rays from a high flux synchrotron generates hydroxyl radicals directly inside cells, which in turn can abstract a hydrogen atom from the ribose and initiate cleavage of the RNA backbone.[118] By this, hydroxyl radical cleavage correlates with the solvent accessibility of the backbone, providing information at nucleotide resolution. Notably, the modification or cleavage sites are then mapped by primer extension of total RNA extracts, revealing information about RNA structure and RNA-protein interaction in vivo.

UV-crosslinking is another powerful tool for identifying novel RNA-protein interaction partners or for characterizing RNA-protein interactions in vivo.[134-139] This method provides important spatial constraints and thus information on the organization of RNP complexes. Recently, the chemogenetic approach NAIM has been used for the first time to investigate RNA-protein interactions at atomic level in *Xenopus laevis* oocytes.[140,141] Random modifications of base or backbone moieties are incorporated into the transcript as nucleotide analog phosphorothioates and then microinjected in oocytes followed by identifying functional groups essential for RNP assembly in vivo. Both UV-crosslinking and NAIM could be modified to study intra- or intermolecular RNA interactions.

Perspectives

Despite the significant progress in understanding RNA folding in vivo, many aspects of intracellular RNA structure formation remain to be explored. For example, it will be of great interest to understand how intracellular proteins associated with various transcription or RNA processing complexes influence RNA folding. Along this line, the effect of temperature in intracellular RNA folding is also largely unexplored. However, any progress in understanding the forces driving RNA folding in living cells depends on developing novel methodologies to monitor RNA structure formation in vivo. In particular, techniques like NMR that would allow studying folding dynamics in vivo are of significant interest. Progress in experimental techniques might also enable to study inhomogeneous RNA population in vivo, which cannot be structurally characterized by conventional ensemble methods. Although the complex cellular environment poses many challenges, this topical research field holds many fascinating facets to discover.

Acknowledgements

We thank Renée Schroeder, Olga Fedorova, Katharina Semrad, Nora Sachsenmaier, Michael Wildauer and Andreas Liebeg for comments on the manuscript. C.W. acknowledges the financial support from the Austrian Science Foundation FWF (grants P21017 and Y401).

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