Published in final edited form as:

Curr Opin Struct Biol. 2015 February ; 30: 134–146. doi:10.1016/j.sbi.2015.02.011.

Characterizing excited conformational states of RNA by NMR spectroscopy

Bo Zhao^{1,2} and Qi Zhang¹

¹Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

²Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

Abstract

Conformational dynamics is a hallmark of diverse non-coding RNA functions. During these functional processes, RNA molecules almost ubiquitously undergo conformational transitions that are tuned to meet distinct structural and kinetic requirements for proper function. A complete mechanistic understanding of RNA function requires comprehensive structural and dynamic knowledge of these complex transitions, which often involve alternative higher-energy conformational states that pose a major challenge for high-resolution structural study by conventional methods. In this review, we describe recent progress in RNA NMR that has started to unveil detailed structural, thermodynamic and kinetic insights into some of these excited conformational states of RNA and their functional roles in biology.

Introduction

The last few decades have witnessed explosive discoveries of diverse non-coding RNA (ncRNA) functions, ranging from enzymatic catalysis by ribozymes to gene regulation by riboswitches [1–4]. These ever-growing discoveries have not only placed these simple biomolecules — composed of only four chemically similar building blocks — center stage in biology, but have also opened new avenues for developing RNA-targeted therapeutics and for engineering RNA-based devices to probe, interfere, and manipulate biological processes. A remarkable feature common to many of these newly discovered ncRNAs is that they almost ubiquitously undergo conformational transitions, which can take place in multiple steps, each triggered by distinct cellular inputs, leading to distinct structures that serve unique functions on their multi-step functional pathways [5–8]. Examples include ribozymes [9–12], which undergo conformational changes to achieve distinct structures essential for substrate binding, catalysis, and product release; and riboswitches [13–15], which undergo ligand-dependent structural changes to regulate gene expression at either the transcriptional or translational level. While our understanding of the chemical basis of these fascinating

Corresponding author: Zhang, Qi (zhangqi@unc.edu).

Conflicts of interest

The authors declare no conflicts of interest.

RNA functions has been significantly advanced in the past two decades, benefiting from the tremendous progress in determining high-resolution RNA structures by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, it has been increasingly recognized that RNA molecules are highly dynamic entities. Therefore, a deep mechanistic understanding of these RNA functions requires complementary knowledge of intrinsic conformational dynamics that underlie these structural transitions.

Such a synergistic view of an RNA molecule, including its structures, dynamics, thermodynamics, and kinetics, can be described by its free-energy landscape, a concept that was originally introduced by Frauenfelder and co-workers to describe protein dynamics and folding [16]. Dissecting the free-energy landscape of an RNA molecule, delineating its sequence dependence, and elucidating its perturbations due to specific cellular cues have been of major interest in the fields of RNA structural biology and biophysics. The energy landscape can be depicted as a series of valleys and hills defining individual states that RNA can transit. Located at the global minimum on the free-energy landscape is the lowest-energy state, the most thermodynamically stable and populated state. This state is often regarded as the native state and is also referred to as the ground conformational state of an RNA molecule. Due to thermal energy, the native state is not static but rather fluctuates around its average conformation, which leads to occasional transition to alternative, higher-energy states, which are located at various local minima on the free-energy landscape. Therefore, a comprehensive description of the free-energy landscape requires structural and dynamic characterizations of these interconverting states as well as their exchange kinetics. While the ground states as well as various higher-energy states that can be kinetically trapped and hence long-lived, are amenable to and have been the focus of most high-resolution studies, it still remains a major challenge for structural biology to characterize a large number of higher-energy RNA states that are dynamically sampled by the ground states at thermodynamic equilibrium. A fundamental experimental obstacle is that many of these states exist in insufficient abundance and for too little time to be studied and sometimes even detected by conventional high-resolution approaches. Here, we specifically refer to these higher-energy states with fractional populations (i.e. less than 10%) and/or short lifetimes (i.e. less than a second) as excited conformational states of RNA, which are the focus of the review.

In the past decade, development of sophisticated biophysical techniques with significantly improved sensitivity, including single molecule [17–19], time-resolved hydroxyl radical footprinting [20], and fluorescence techniques [21], have allowed for discovery and characterization of novel excited conformational states involved in both RNA folding and recognition of proteins and ligands at single-molecule and single-nucleotide resolution. However, accurate assignment of a specific molecular configuration to an excited state remains a challenge for these low-resolution experimental approaches. NMR spectroscopy has been well established as a powerful ensemble-based tool for high-resolution structural and dynamic studies of biomolecules. In the past few years, developments in solution-state NMR [22], in particular relaxation dispersion (RD) techniques based on NMR chemical exchange phenomena [23,24*,25–31,32**], have greatly extended the limits of NMR in characterizing kinetically distinct conformational states of RNA at the atomic level, where the population of an excited state can be as little as ~0.5% and the lifetime as short as tens-

of-microseconds [31,32**]. In this review, we focus on recent progress in NMR that has provided high-resolution structural and dynamic insights into excited conformational states of RNA, which have facilitated developing a deep mechanistic understanding of various ncRNA functions.

A hierarchical free-energy landscape of RNA conformational transitions

Excited states of RNA differ dramatically between each other in their intrinsic structural, thermodynamic, and kinetic properties. This is a result of their underlying complex conformational transitions, which can occur over a broad range of timescales from picoseconds to seconds and longer, as well as across a large scale of amplitudes, from local base reorientation to global secondary structure reorganization. In this review, we classify these excited states of RNA based on the nature of the dynamic processes in which they are involved. To simplify the description of complex RNA dynamics, Al-Hashimi and co-workers have recently introduced a general framework that is based on the highly hierarchical and rugged RNA free-energy landscape [33]. In this framework, RNA dynamics are classified as distinct motional modes that represent transitions between different states located within three hierarchical free-energy tiers — Tiers 0, 1 and 2 (Figure 1a). At Tier 0, the lowest tier on the free-energy landscape, conformational transitions occur between states with different secondary structures, such as a transition between a stable hairpin and a less-stable pseudoknot. Since energy barriers separating different secondary structures are usually orders of magnitude higher than $k_B T$ (where k_B is the Boltzmann constant and T is the temperature in Kelvin) ($\gtrsim 17$ kcal/mol), Tier-0 conformational transitions are the slowest transitions and occur at the timescales of tens-of-milliseconds to seconds and longer. Residing within individual Tier-0 basins are Tier-1 conformational states, which share similar global secondary structures but differ in local base-pairing configurations and/or tertiary interactions. Four distinct dynamic processes are formulated to describe these Tier-1 transitions: tertiary-structure formation, base pair reshuffling, base pair melting, and base pair isomerization. Shown in Figure 1a is an example of base pair reshuffling between a stable hairpin and a less-stable hairpin. Since energy barriers between Tier-1 states (~ 9 – 16 kcal/mol) are smaller than those between Tier-0 states, Tier-1 transitions occur at faster timescales of microseconds to tens-of-milliseconds. Tier 2 is the subsequent tier on the hierarchy, where a given Tier-1 state samples additional fine-tuned conformations via localized dynamic processes, such as base flipping, sugar re-puckering, and interhelical reorientations. At this tier, energy barriers separating different conformations are only a few $k_B T$ (~ 2 – 9 kcal/mol), and dynamic interconversion between Tier-2 states can be ascribed to two fast motional modes, base-stacking motions at the nanosecond-to-microsecond timescale and jittering motions at the picosecond-to-nanosecond timescale.

On the basis of this framework, we classify excited states of RNA into three tiers — Tiers 0, 1, and 2 — the same tiers used in defining RNA dynamics (Figure 1a). It is worth noting that thermodynamic and kinetic properties of an excited state are strictly tied to a specific free-energy landscape, which in turn highly depends on various experimental conditions, such as temperature, pH, and salt concentrations. Manipulating these conditions is commonly used to optimize population and lifetime of an excited state for experimental characterization, and, in some cases, an excited state can even be converted to become the

new ground state. Furthermore, an excited state, due to having higher free energy, is always thermodynamically well defined as a lower populated state relative to its corresponding ground state. On the contrary, since conformational transitions at different tiers have distinct kinetics, the lifetime of an excited state can be dramatically different from tier to tier; for an example, a Tier-0 excited state may have a longer lifetime than a Tier-1 ground state. Finally, we want to point out that experimental identification of an excited state is based on the ability of the experimental technique to distinguish different conformational states. NMR spectroscopy has been a powerful tool for quantifying conformational interconversion and obtaining structural and kinetic insights of excited states in nucleic acids (Figure 1b). These NMR methodologies have been extensively reviewed recently [22,34,35], hence will not be covered in detail here. Briefly, ZZ-exchange spectroscopy can allow thermodynamic (i.e. population) and kinetic (i.e. rate of exchange) characterizations of the equilibrium conformational transition occurring at subsecond-to-second timescales, provided that the state of interest is sufficiently populated (i.e. $\gtrsim 10\%$) [36,37*]. We would like to also point out that, due to this requirement of a relatively high population, a high-energy conformational state that can be characterized by ZZ-exchange spectroscopy is often referred to as a minor state instead of an excited state. As the rate of exchange increases and the population of an excited state decreases, which can result in complete resonance disappearance of an excited state. In these cases, a set of powerful NMR techniques based on NMR chemical exchange phenomena become suited, which not only allow accurate quantification of both thermodynamics and kinetics of the equilibrium conformational transition, but also allow obtaining detailed structural insights into such an ‘invisible’ excited state from extracted NMR chemical shifts. These techniques include Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion spectroscopy [38,39], which can be applied to characterize transitions occurring at the rate of exchange ($k_{\text{ex}} = k_{\text{GE}} + k_{\text{EG}}$) between ~ 200 and $\sim 2000 \text{ s}^{-1}$ when site-specific labeling schemes are employed to reduce extensive carbon–carbon scalar couplings in RNA [28–30]; chemical exchange saturation transfer (CEST) spectroscopy [37*,40–42], which can be applied on uniformly $^{13}\text{C}/^{15}\text{N}$ labeled samples to characterize transitions occurring at the rate of exchange between ~ 20 and $\sim 5000 \text{ s}^{-1}$; and rotating-frame $R_{1\rho}$ RD spectroscopy [23,24*,43], particularly low spin-lock field $R_{1\rho}$ RD [25–27,31,32**,37*], which can also be applied on uniformly $^{13}\text{C}/^{15}\text{N}$ labeled samples but can characterize transitions occurring at a broad range of exchange rates between ~ 60 and $\sim 40,000 \text{ s}^{-1}$. Hence, these diverse NMR techniques provide ample tools and methods to directly or indirectly characterize kinetically distinct Tier-0 and Tier-1 excited states and Tier-2 excited states that involve in base-stacking motions. Tier-2 states that participate in picosecond-to-nanosecond jittering motions are often described by a statistical distribution of dynamic ensembles rather than individual excited states and will not be discussed in this review.

Tier-0 excited conformational states of RNA

Tier-0 excited states participate in Tier-0 conformational transitions, which are the basis for many ncRNA regulatory functions ranging from regulating gene transcription and translation to mRNA splicing and viral replication [8,33]. During these dynamic processes, key regulatory RNA elements are either exposed or sequestered via global secondary

structural transitions in response to specific cellular cues. Due to relatively large energy barriers separating different secondary structures, the presence of Tier-0 excited states can often be directly detected by observing various low-intensity NMR signals that coincide with high-intensity resonances from the ground state. Optimizing these weak signals by varying temperatures or changing salt/divalent cation concentrations are often employed to facilitate direct structural and dynamic studies of Tier-0 excited states by NMR, providing mechanistic insights into specific pathways through which the default-functional and the signal-activated states interconvert to carry out their regulatory functions.

A recent NMR study of the *add* adenine riboswitch in human Gram-negative pathogenic bacterium *Vibrio vulnificus* provides one such example (Figure 2a) [44**]. Riboswitches, usually consisting of a ligand-sensing aptamer domain and an expression platform, are an important class of ncRNAs that regulate gene expression at either the transcriptional or translational level [13–15]. The *add* adenine riboswitch is a translational riboswitch that regulates adenine biogenesis by controlling protein expression of adenosine deaminase. Upon specific binding to adenine by the aptamer domain, the *add* adenine riboswitch activates translation of the downstream adenosine deaminase gene by exposing the Shine–Dalgarno sequence within its expression platform, which is otherwise sequestered in the absence of adenine. While the mechanism of riboswitch function is often simplified to a two-state exchange between mutually exclusive ligand-free and ligand-bound states, the *add* adenine riboswitch was discovered to employ a sophisticated three-state mechanism to maintain robust gene regulation over a broad range of temperatures (Figure 2a). In the absence of adenine, the full-length *add* adenine riboswitch adopts two conformational states — a major state with a population of ~69% and a minor state with a population of ~31%, which dynamically interconvert at a rate of exchange $k_{ex} \sim 1.3 \text{ s}^{-1}$ at 25 °C as measured by ^{15}N ZZ-exchange NMR spectroscopy. The relatively high populations allowed determination of the secondary structures of both states using conventional NOESY and HSQC based approaches together with fragment constructs derived from the full-length RNA. The major state is a binding-inactive translational-off state, as NMR characterizations revealed the aptamer domain with a secondary structure that is ligand-binding incompetent and the expression platform with the Shine–Dalgarno sequence being sequestered. In contrast, the minor state is a binding-active translational-off state, where the aptamer domain is ligand-binding competent and the expression platform sequesters the Shine–Dalgarno sequence. The minor state, upon recognition of adenine, is switched to the translational-on state. The equilibrium between these two ligand-free states is dynamically regulated by temperature, with the population of the minor state ranging from ~40% at 30 °C to only ~10% at 10 °C, which can be regarded as an excited conformational state based on our definition. Remarkably, it was shown that the decreased population of the minor state at lower temperatures is counterbalanced by the increased binding affinity to adenine, which results in a stable switching efficiency between 83% at 30 °C and 67% at 5 °C. It was further shown that, once the dynamic equilibrium between the two ligand-free states was completely shifted toward the minor/excited state via mutations, the switching efficiency of the riboswitch becomes sensitive to temperature, dropping significantly from 85% at 30 °C to only 14% at 5 °C. Hence, this minor state, together with its unique thermodynamic and ligand binding properties, serves as a critical intermediate to bridge the default translational-

off state and the adenine-activated, translational-on state for proper and robust functioning of the *V. vulnificus* add adenine riboswitch, whose working environment spans a broad range of temperatures.

Direct observation of resonances from an excited state provides a spectroscopic basis for NMR studies. However, assignment of a specific configuration to a Tier-0 excited state can still be challenging, in particular when a complex structure is involved. In an NMR study of the *Fusobacterium nucleatum* preQ1 riboswitch, a site-specific labeling strategy, coupled with solid-phase RNA synthesis, was employed to facilitate secondary structure confirmation of an excited state of the ligand-free aptamer domain (Figure 2b) [45*]. The ground state of this ligand-free aptamer consists of a P1 hairpin and an unfolded 3'-end tail. The presence of additional low-intensity peaks in 1D imino ^1H NMR spectra indicated structural heterogeneity in the ligand-free aptamer. Comparisons between imino proton resonances of the ligand-bound aptamer and the ligand-free aptamer at lower temperature lead to a proposed excited state in the ligand-free RNA that resembles the pseudoknot conformation of the ligand-bound state [46–48]. To probe the structural nature of potential excited states, a 5-fluorine (5-F) label was introduced to residue U32, located at the 3'-end tail. The observation of two distinct fluorine signals in 1D ^{19}F NMR spectra, ~90% and ~10% at 25 °C, respectively, immediately suggests one alternative conformation in addition to the ground state. These two states dynamically interconvert, as tuning temperatures can re-distribute their relative populations. The major ^{19}F signal has a chemical shift typical for an unpaired 5-F uridine, agreeing well with an unfolded 3'-end tail in the ground state. The minor ^{19}F signal, composed of two overlapping peaks, has a chemical shift typical for Watson–Crick (WC) base-paired 5-F uridines, which agrees well with a pseudoknot-like excited state where the 3'-end tail is sequestered into the P2 stem. To provide further support for this configuration, NMR J_{NN} -COSY experiments were carried out using a site-specific labeled construct, where two potential A-U WC base pairs, one at the P1 stem (A3-U20) and the other at the P2 stem (A10-U32), were labeled with $^{15}\text{N}(1)$ -modified adenines and $^{15}\text{N}(3)$ -modified uridines. Simultaneous formation of both A-U WC base pairs was detected either at 10 °C or in the presence of 2 mM Mg^{2+} , providing strong evidence that the RNA folds into a pseudoknot upon transiting to the excited state. Given the ligand-bound preQ1 aptamer adopts a pseudoknot fold, as revealed by various crystal structures [46–48], it was further proposed, but yet to be validated, that this excited, pseudoknot state may represent the state being selected by preQ1 during adaptive recognition.

Although Tier-0 excited states can often be directly observed by NMR, as in the previous two examples, some Tier-0 excited states do not give observable NMR signals and their existence is often inferred from various biophysical and biochemical measurements [49,50]. These states are 'invisible' to NMR, which can be due to either a low population and/or a short lifetime. Characterizing these NMR 'invisible' states requires applications of new NMR techniques to complement conventional NMR methods. CEST NMR spectroscopy [40] is a powerful technique for characterizing sparsely populated states that undergo slow conformational exchange at the millisecond-to-second timescale [41,42], the timescale for many Tier-0 conformational transitions. A nucleic-acid-optimized 2D ^{13}C CEST experiment was recently developed and applied on the aptamer domain of the *Bacillus cereus* fluoride

riboswitch (Figure 2c) [37]. Only one conformation consisting of P1 and P2 stems was observed for the ligand-free aptamer using conventional 1D and 2D NMR experiments. However, the presence of an alternative conformation was directly visualized on CEST profiles measured for base (C8) and sugar (C1') carbons of two P1 loop residues, G8 and G10, using a $^{13}\text{C}/^{15}\text{N}$ G-labeled NMR sample. Global fitting of these CEST profiles to a single two-state exchange model allowed simultaneous structural, thermodynamic, and kinetic characterization of this NMR 'invisible' excited state. On the basis of the extracted exchange parameters, the excited state has a population of $p_E = 10\%$ and inter-converts with the ground state at a rate of $k_{\text{ex}} = 112 \text{ s}^{-1}$ at 30°C . The lifetime of this excited state is calculated to be only 9.9 ms, which is ~ 100 times shorter than the lifetime of the excited state observed in the ligand-free *add* adenine riboswitch discussed above. In addition, the extracted chemical shifts of the excited state provide key atomic fingerprints to the structure of the excited state. While base (C8) and sugar (C1') carbons of both G8 and G10 in the ground state have chemical shifts similar to those of free GTP, consistent with an unfolded P1 loop, their corresponding chemical shifts in the excited state are significantly shifted toward those of base-paired G residues. Given that the crystal structure of a ligand-bound fluoride riboswitch aptamer revealed a compacted pseudoknot [51], these chemical shift fingerprints lead to a proposed formation of a pseudoknot-like ligand-free excited state where both G8 and G10 form base pairs upon folding of the P3 stem. Hence, detection and identification of an NMR 'invisible', potential pseudo-knot-like excited state by ^{13}C CEST experiments provide a structural basis for proposing conformational selection as a potential mechanism for the adaptive recognition of fluoride, which otherwise would be attributed to a ligand-induced mechanism for pseudoknot formation based on conventional NMR characterizations. However, it needs to be pointed out that these chemical shift fingerprints alone only suggest and would agree with the proposed pseudoknot-like excited state. Further experiments, such as an application of the recently developed 'mutate-and-chemical-shift-fingerprint' (MCSF) strategy, as discussed below, should be performed to validate this model.

Tier-1 excited conformational states of RNA

With overall similar secondary structures, Tier-1 excited states differ from their ground state counterparts in local base-pairing configurations via tertiary-structure formation, base pair reshuffling, base pair melting, and base pair isomerization [8,33]. These conformational transitions occur at the timescales of microseconds to tens-of-milliseconds, which are faster than the Tier-0 transitions. Thus, these alternative conformations can be employed to carry out functions complementary to the ground states in a more efficient manner [33]. While glimpses into these Tier-1 excited states were obtained by NMR a decade ago [23], their structures and functional roles remain elusive. In several recent NMR studies, various Tier-1 excited states have been identified and thoroughly characterized, providing structural insights into their distinct functional roles.

One example of a functional Tier-1 excited state that forms a new tertiary structure is the *murine leukemia virus* (MLV) recoding signal [52^{**}]. With specific interactions between *cis*-regulatory RNA elements and the ribosome during translation, ribosomal frameshifting and read-through provide a mechanism for alternative protein expression from a single mRNA

template [53]. The translation of retroviral reverse transcriptase requires such a recoding signal on the mRNA to allow the ribosome to bypass the Gag stop codon and produce Gag-Pol fusion protein [54]. Notably, the ratio of Gag to Gag-Pol, an important factor in virus infectivity, is tightly regulated in retroviruses, with a readthrough rate of ~5%. One mechanism for this regulation was unveiled by a recent NMR study of the MLV recoding signal, which has an overall pseudoknot secondary structure (Figure 3a) [52**]. This MLV pseudoknot was found by NMR to interconvert between two distinct tertiary conformations in a pH dependent manner. The ground state, whose solution structure was solved by NMR at physiological pH, revealed a loose pseudoknot conformation with coaxially stacked helices S1 and S2, looped-out residues A17 and A29, and a flexible loop L2. While NMR cannot directly detect the excited state at physiological pH, its formation was identified with the observation of additional NMR resonances at lower pH (pH ~ 6.5). Chemical shift and NOE analysis revealed that residue A17 is protonated in the excited state, which allows the formation of a base triple with residues C23 and G53. This new tertiary contact between A17 and S2 would introduce an inter-helical bend between S1 and S2, which can subsequently release the steric constraints that prevent S1-L2 tertiary interactions in the ground state. A remarkable feature of the protonation-dependent dynamic equilibrium is that the population of the active excited state is determined to be ~6% at physiological pH, tracking with the read-through percentage in native MLV translation. This observation suggests that this protonation-dependent tertiary structure transition to an excited state with a compact pseudoknot conformation is the functional switch allowing the ribosome to bypass the *gag* stop codon. The functional role of this excited state in ribosome read-through was further supported by structure-guided mutagenesis. It was shown that residue-specific mutations, which stabilize this excited conformation, such as U38A and A29C, cause significant increases in the in vivo readthrough levels, ~80% and ~20%, respectively. Thus, the structural and thermodynamic characterizations of this compact pseudoknot excited state provide a framework for developing a mechanistic understanding of the functional role of a frameshifting signal in translational recoding and the important role of transient tertiary interactions in RNA function.

The other types of Tier-1 conformational transitions, which can occur at much faster timescales compared to tertiary structure formation, present a significant challenge for NMR studies. Not only are these Tier-1 excited states often 'invisible' in NMR spectra, even initial testable configurations of these states require de novo determination rather than being inferred from various presumed models. Recently, a novel strategy was developed, which provides a versatile approach that opens new routes for characterizing structures of these sparsely populated and transiently lived excited conformational states in RNA [32**]. This strategy synergistically combines NMR $R_{1\rho}$ relaxation dispersion spectroscopy, particularly the low spin-lock $R_{1\rho}$ RD, mutagenesis, and computational secondary structure prediction by MC-Fold [55]. Applications of this strategy have started to reveal the remarkably rich structural diversity that can be encoded in a single RNA molecule [32**,56**]. The HIV-1 transactivation response element (TAR) RNA is a classic example (Figure 3b). HIV-1 TAR is an essential element for the activation of the viral promoter. While only one conformation can be identified in the NMR spectra of TAR, NMR $R_{1\rho}$ relaxation dispersion measurements on the apical loop residues revealed two conformational states that interconvert at a rate of

$k_{\text{ex}} = 25.4 \times 10^3 \text{ s}^{-1}$. The apical loop is a functionally critical region of TAR, containing the recognition site for viral transactivation factor protein Tat and human cyclin T1 [57]. In the ground state, the apical loop adopts a conformation where residues C30 and G34 form a WC base pair, residue G33 stacks on top of G34, and residues U31, G32 and A35 are flexible as evidenced by their sugar puckers existing in equilibrium between 2'-endo and 3'-endo configurations. In contrast, the excited state of the apical loop, which has a population of 13%, exhibits significant differences in local conformation. These structural insights were obtained by analyzing the extracted excited-state chemical shifts from NMR $R_{1\rho}$ relaxation dispersion measurements. The downfield shifted sugar (C1') carbon chemical shifts of residues C30, U31, and A35 in the excited state suggest their adoption of a 3'-endo sugar pucker configuration, which is characteristic of a helical conformation; the base (C8) carbon resonance of G34 in the excited state is also downfield shifted and has a chemical shift typical for a G-U wobble base pair. These chemical shift signatures, together with kinetic measurement as well as secondary structure predictions by the MC-Fold program, have provided a basis for de novo proposing a unique secondary structure of the apical loop in the excited state, which has a tetraloop conformation with C30–A35⁺ and U31–G34 wobble base pairs, a stacked G33 residue, and a flexible G32 residue. To validate this proposed structure, another elegant strategy — so-called 'MCSF' — was also developed, where a mutation or a chemical modification is introduced to a specific residue to manipulate the dynamic equilibrium of the exchange. As a result, the proposed excited state can either be enriched or depleted and the corresponding chemical shift finger-print can be compared with those of the excited state to validate the proposed structure. With this MCSF strategy, a series of mutants that either stabilize (C30U and A35G) or destabilize (A35-DMA) the proposed excited-state structure were generated. Comparing the carbon chemical shifts of the excited state to those observed in C30U and A35G mutants revealed similar directions and magnitudes, providing strong evidence that the excited state involves the formation of a C30-A35⁺ wobble base pair. On the other hand, dimethylation of the amine of residue A35, which sterically prevents it from base pairing with residue C30, does not greatly perturb the observed chemical shifts compared to those of the ground state but abolishes detection of the observed exchange phenomena, providing further confirmation for the proposed excited-state structure. While the hexanucleotide loop in ground state allows key residues C30, U31, G34, and A35 to interact with Tat and Cyclin T1, the unique tetraloop conformation of the excited state sequesters all of these residues into base pairs and forms an autoinhibited state that prevents specific recognition by Tat and Cyclin T1. Hence, detection and structural characterization of this excited conformational state provides a structural basis for potential mechanisms in down-regulating transactivation of the HIV-1 genome or releasing Tat and Cyclin T1, which can be further applied in developing TAR-targeted anti-HIV therapeutics by stabilizing this auto-inhibited excited state.

While the initial $R_{1\rho}$ relaxation dispersion study on the HIV-1 TAR revealed an autoinhibited excited state in the functionally critical apical loop (ES1), the base pair reshuffling process in this RNA turned out to be much more complex [56*]. Despite the microsecond-timescale dynamic exchange within the apical loop, the initial study also revealed a separate, slower, millisecond-timescale exchange process in two apical loop residues, G33 and A35. Following up on this original discovery, a recent investigation that

expanded $R_{1\rho}$ relaxation dispersion measurements to the entire HIV-1 TAR element has identified a second exchange process that occurs at a much slower rate of $k_{\text{ex}} = 474 \text{ s}^{-1}$ [56*]. Multiple residues across the upper helix and the three-nucleotide bulge were found to undergo simultaneous dynamic transitions to an excited conformation (ES2) that is populated at only 0.4%. On the basis of carbon chemical shifts of this excited state, secondary structure prediction by MC-Fold, and the MCSF strategy, the secondary structure of this excited state was revealed to be a structure where the upper two bulge residues pair up with cross-strand residues in the upper helix, leading to a global register shift and formation of four non-canonical base pairs. This recent study provides a structural basis for potential mechanisms that allow efficient long-range communication to facilitate RNA folding and ribonucleoprotein assembly. Together, these comprehensive NMR characterizations of HIV-1 TAR have unveiled the intrinsic, complex structural plasticity encoded in a single RNA sequence, which in turn forms the molecular foundation for diverse functions.

This synergistic NMR/mutagenesis/MC-Fold approach has also been employed to unveil functionally critical excited conformational states in the ribosomal A-site internal loop (Figure 3c) and the HIV-1 stem loop 1 (SL1) (Figure 3d) [32**]. The ribosomal A-site internal loop has an essential role in ribosomal decoding, where loop residues A1492 (A92) and A1493 (A93) stabilize the codon–anticodon pairs between the cognate tRNA and mRNA for translational fidelity (Figure 3c). $R_{1\rho}$ relaxation dispersion measurements on an A-site RNA construct revealed the existence of an exchange process that occurs at a rate of exchange $k_{\text{ex}} = 4.1 \times 10^3 \text{ s}^{-1}$ with an excited state populated at ~2.5%. The extracted chemical shifts provided initial structural insights into the excited state. For example, the upfield-shifted chemical shift of base carbon C6 of U95 suggested a flipped-out conformation of this residue in ES, whereas the downfield-shifted chemical shifts of base carbons of A92, A93, G94, and C96 suggested increased stacking interactions for these residues. Together with predicted secondary structures by MC-Fold, these structural fingerprints indicate a base reshuffling process that loops out U95 and forms three non-canonical base pairs. Utilizing the MCSF strategy, a series of mutants that aim to stabilize either GS or ES were made in order to validate the identity of the ES. For example, an U06G/U95C mutant, which aims to stabilize the GS by replacing the U–U mismatch with a stable G–C base pair, has a GS-like NMR spectrum and also lacks detectable exchange behavior. As an alternative test, a U95 mutant was also made to stabilize the ES by removing the looped out residue in the ES, which indeed has ES-like chemical shifts. Even more remarkably, when a methyl group was introduced to the N3 position at residue U95, which is designed to disrupt the non-canonical U–U interaction, two equally populated sets of chemical shifts were observed, corresponding to GS and ES, respectively. Together, these comprehensive results unveiled a global exchange process that breaks the C07–G94 base pair and loops out U95, resulting in an ES formation with three non-canonical base pairs. This excited A-site state sequesters both loop residues (A92 and A93) into a helix, which not only impairs the decoding ability but also disrupts the formation of B2a intersubunit bridge. Identification of this functionally inactive excited state provides some structural basis for various defect mutants, which can be attributed to a more stabilized ES. Finally, this inactive excited state may further provide a novel target for developing antibiotics.

The HIV-1 SL1 forms an initial kissing dimer during HIV-1 viral maturation, which leads to the final formation of a stable duplex dimer via unfolding and re-folding of the entire SL1 hairpin (Figure 3d). Previous NMR characterizations have revealed complex NMR chemical exchange processes occurring around the HIV-1 SL1 bulge region. $R_{1\rho}$ relaxation dispersion measurements on a monomeric SL1 construct, where the kissing loop residues were replaced with a stable GAGA tetraloop, revealed two distinct exchange processes, which are centered on seven base pairs around the two-nucleotide bulge of SL1 [32**]. The ground state undergoes two conformational transitions toward a 9% populated ES1 and a 2% populated ES2, occurring at rates of exchange $k_{\text{ex}} = 9.2 \times 10^3 \text{ s}^{-1}$ and $5.1 \times 10^3 \text{ s}^{-1}$, respectively. On the basis of secondary structure prediction by MC-Fold, carbon chemical shift fingerprints of both excited states, and MCSF analysis, structural models for both ES1 and ES2 were identified. While the internal bulge shifts upward by three base pairs from GS to ES1 via the first base pair reshuffling process, the second reshuffling process migrates the internal bulge downward by two base pairs from GS to ES2. Interestingly, these two excited states are mutually exclusive, as mutations stabilizing residues above the bulge simultaneously stabilize residues below the bulge. This branched three-state reshuffling process generates a ‘moving zipper’ — as defined by the authors — that provides a potential mechanism for ATP-independent HIV-1 SL1 isomerization, wherein the two-nucleotide bulge, upon formation of the kissing dimer, forms base-pairs with the adjacent helix, reducing the activation energy necessary for melting of the stem loop and re-annealing of the dimer duplex. It was further shown that mutations that either stabilize ES1 or prevent ES2 significantly reduced spontaneous isomerization rate, providing strong support for the proposed functional role of both excited states in HIV-1 SL1.

Tier-2 excited conformational states of RNA

Tier-2 excited states have the shortest lifetimes among all excited states in the three tiers of the free-energy landscape [33]. The kinetically distinct Tier-2 excited states, which are often involved in base-stacking motions at the nanosecond-to-microsecond timescale, occur at single-stranded regions, such as apical loops and bulges, to optimize local conformations for functions such as adaptive recognition of ligands. The existences of Tier-2 excited states in RNA have been indicated from early NMR dynamic studies based on conventional high spin-lock $R_{1\rho}$ relaxation dispersion experiment [23,24*,58], which detects chemical exchange process occurring at the very fast microsecond timescale. Among these, the GNRA tetraloop is a RNA motif that has been extensively characterized by both conventional $R_{1\rho}$ and CPMG relaxation dispersion experiments [23,28]. On the basis of these relaxation dispersion studies on both base and sugar carbons, it was found that sugar repuckering in the loop is coupled with base stacking motions, which leads to conformational exchange between the ground state to an excited state with alternative stacking at an exchange rate of $k_{\text{ex}} \sim 2 \times 10^4 \text{ s}^{-1}$. Another recent example of a Tier-2 excited state involved in the stacking motion came from the NMR study of the loop dynamics in the ligand-bound *B. subtilis* preQ1 riboswitch aptamer [59]. By applying on-resonance $R_{1\rho}$ relaxation dispersion experiments, three well-stacked loop residues were found to undergo a concerted motion at an exchange rate of $k_{\text{ex}} = 9.4 \times 10^3 \text{ s}^{-1}$. These loop residues are located right above the binding pocket, and the stacking motions may open the cap to an unstacked

excited state to facilitate ligand binding. Compared with NMR structural and kinetic characterizations of Tier-0 and Tier-1 excited states, most of these NMR studies on Tier-2 excited states are relatively limited at the level of detecting the existence rather than delineating the structural properties of a Tier-2 excited state. However, in a study of the U6 spliceosomal RNA intramolecular stem-loop (ISL), a Tier-2 excited state was identified and confirmed by collaborative analysis of NMR $R_{1\rho}$ RD measurement and existing structural knowledge of the pH-dependent conformational equilibrium of U6 ISL [24*]. It is also worth noting that this study is one of the very first examples to have unveiled structural properties of a transiently lived excited state of RNA.

The spliceosome is a large ribonucleoprotein complex responsible for splicing eukaryotic pre-mRNA [60]. The U6 ISL is a critical component of the spliceosome that binds an essential metal ion around the bulge region for pre-mRNA splicing [61]. Early NMR structural studies revealed a pH-dependent conformational equilibrium of the U6 ISL [62,63]. At high pH (pH 7.0), loop residue U80 stacks between two helices and creates an interhelical bend. At low pH (pH 5.8), not only is residue U80 looped out, residue A79 is also protonated and forms a non-canonical base pair with residue C67, resulting in a 25° change in the interhelical bend. With a looped-out U80, the conformational transition at low pH has also been shown to be antagonistic to magnesium binding at the U80 pro-S oxygen, a process required for splicing. By applying conventional high spin-lock $R_{1\rho}$ relaxation dispersion measurements at pH 7.0, pH 6.0, and various magnesium concentrations, a global conformational transition was unveiled for the U6 ISL that occurs at a rate of exchange $k_{\text{ex}} = \sim 1.2 \times 10^4 \text{ s}^{-1}$. Since the applied $R_{1\rho}$ RD measurements on fast exchange processes can only allow quantification of the overall exchange parameter (the product of populations and chemical-shift difference between ground and excited states) rather than the individual components, it is a major challenge to delineate the structural properties of the excited state. Since the U6 ISL undergoes an intrinsic pH-dependent conformational equilibrium, an elegant approach was taken to examine whether the observed conformational transition occurs between the two pH-dependent states. Here, the population of each state was calculated based on the protonation state of residue A79 N1 nitrogen, chemical shift differences between ground and excited states were estimated based on direct chemical shift measurement at pH 6.0 and at pH 7.0, and the overall exchange parameter was calculated based on this simple model for every residue. Remarkably, these calculated exchange parameters agree very well with their experimental counterpart, providing strong support that the conformational exchange does occur between the two pH-dependent conformers, where the low pH state is the excited state that is dynamically sampled at a higher pH. It was further proposed that this dynamic transition involving looping out residue U80 provides a pH-sensitive switch for controlling Mg^{2+} binding at the critical location of U6 ISL RNA.

Outlook

It has become increasingly clear that RNA molecules are highly dynamic entities that can fold into diverse conformations with distinct functional roles [8]. By providing quantitative insights into structures, thermodynamics, and kinetics of alternative higher-energy states in various ncRNAs, the recent progress in RNA NMR, which includes applications of conventional methods on complex RNA molecules as well as developments and applications

of novel NMR spectroscopy in characterizing sparsely populated transient RNA states, has not only expanded our basic knowledge of the intrinsic conformational plasticity of RNA but also greatly contributed toward our mechanistic understanding of ncRNA functions. However, a major challenge still remains in the field, which is the de novo three-dimensional structural determination of these excited RNA conformational states with atomic resolution. Developments of novel NMR methodologies, such as residual dipolar coupling (RDC) RD [64,65] and paramagnetic relaxation enhancement (PRE) techniques [66], have recently opened new avenues for structural determination of excited conformational states in proteins and protein–DNA complexes. We anticipate nucleic-acid-dedicated RDC RD and PRE methods will facilitate de novo structural determination of excited states in RNA. Together with exciting developments in NMR chemical shift guided computational prediction of 3D RNA structures in recent years [67,68], we are optimistic that high-resolution 3D structures of RNA excited states will become available in the near future. With these ongoing developments of novel techniques and their subsequent applications, we believe that NMR spectroscopy will continue to play an important role in facilitating mechanistic delineation of even more complex ncRNA functions as well as in developing a deep physicochemical understanding of RNA that provides a basis for rational design of novel RNA-based nanodevices.

Acknowledgments

QZ acknowledges start-up fund from the University of North Carolina at Chapel Hill and a grant from the March of Dimes Foundation.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of *Tetrahymena*. *Cell*. 1982; 31:147–157. [PubMed: 6297745]
2. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell*. 1983; 35:849–857. [PubMed: 6197186]
3. Mattick JS. RNA regulation: a new genetics? *Nat Rev Genet*. 2004; 5:316–323. [PubMed: 15131654]
4. Sharp PA. The centrality of RNA. *Cell*. 2009; 136:577–580. [PubMed: 19239877]
5. Williamson JR. Induced fit in RNA–protein recognition. *Nat Struct Biol*. 2000; 7:834–837. [PubMed: 11017187]
6. Leulliot N, Varani G. Current topics in RNA–protein recognition: control of specificity and biological function through induced fit and conformational capture. *Biochemistry*. 2001; 40:7947–7956. [PubMed: 11434763]
7. Cruz JA, Westhof E. The dynamic landscapes of RNA architecture. *Cell*. 2009; 136:604–609. [PubMed: 19239882]
8. Dethoff EA, Chugh J, Mustoe AM, Al-Hashimi HM. Functional complexity and regulation through RNA dynamics. *Nature*. 2012; 482:322–330. [PubMed: 22337051]

9. Hertel KJ, Uhlenbeck OC. The internal equilibrium of the hammerhead ribozyme reaction. *Biochemistry*. 1995; 34:1744–1749. [PubMed: 7849034]
10. Zhuang X, Kim H, Pereira MJ, Babcock HP, Walter NG, Chu S. Correlating structural dynamics and function in single ribozyme molecules. *Science*. 2002; 296:1473–1476. [PubMed: 12029135]
11. Liu S, Bokinsky G, Walter NG, Zhuang X. Dissecting the multistep reaction pathway of an RNA enzyme by single-molecule kinetic fingerprinting. *Proc Natl Acad Sci USA*. 2007; 104:12634–12639. [PubMed: 17496145]
12. Ferre-D'Amare AR, Scott WG. Small self-cleaving ribozymes. *Cold Spring Harb Perspect Biol*. 2010; 2:a003574. [PubMed: 20843979]
13. Garst AD, Edwards AL, Batey RT. Riboswitches: structures and mechanisms. *Cold Spring Harb Perspect Biol*. 2011:3.
14. Breaker RR. Riboswitches and the RNA world. *Cold Spring Harb Perspect Biol*. 2012:4.
15. Serganov A, Patel DJ. Metabolite recognition principles and molecular mechanisms underlying riboswitch function. *Annu Rev Biophys*. 2012; 41:343–370. [PubMed: 22577823]
16. Frauenfelder H, Sligar SG, Wolynes PG. The energy landscapes and motions of proteins. *Science*. 1991; 254:1598–1603. [PubMed: 1749933]
17. Bokinsky G, Zhuang X. Single-molecule RNA folding. *Acc Chem Res*. 2005; 38:566–573. [PubMed: 16028891]
18. Li PT, Viereggs J, Tinoco I Jr. How RNA unfolds and refolds. *Annu Rev Biochem*. 2008; 77:77–100. [PubMed: 18518818]
19. Savinov A, Perez CF, Block SM. Single-molecule studies of riboswitch folding. *Biochim Biophys Acta*. 2014; 1839:1030–1045. [PubMed: 24727093]
20. Sclavi B, Sullivan M, Chance MR, Brenowitz M, Woodson SA. RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. *Science*. 1998; 279:1940–1943. [PubMed: 9506944]
21. St-Pierre P, McCluskey K, Shaw E, Penedo JC, Lafontaine DA. Fluorescence tools to investigate riboswitch structural dynamics. *Biochim Biophys Acta*. 2014; 1839:1005–1019. [PubMed: 24863161]
22. Bothe JR, Nikolova EN, Eichhorn CD, Chugh J, Hansen AL, Al-Hashimi HM. Characterizing RNA dynamics at atomic resolution using solution-state NMR spectroscopy. *Nat Methods*. 2011; 8:919–931. [PubMed: 22036746]
23. Hoogstraten CG, Wank JR, Pardi A. Active site dynamics in the lead-dependent ribozyme. *Biochemistry*. 2000; 39:9951–9958. [PubMed: 10933815]
24. Blad H, Reiter NJ, Abildgaard F, Markley JL, Butcher SE. Dynamics and metal ion binding in the U6 RNA intramolecular stem-loop as analyzed by NMR. *J Mol Biol*. 2005; 353:540–555. This study presents one of the very first examples to unveil structural property of a transiently lived excited state of RNA by collaborative analysis of NMR $R_{1\rho}$ RD measurement and existing structural and biochemical knowledge. [PubMed: 16181635]
25. Massi F, Johnson E, Wang C, Rance M, Palmer AG 3rd. NMR R_1 rho rotating-frame relaxation with weak radio frequency fields. *J Am Chem Soc*. 2004; 126:2247–2256. [PubMed: 14971961]
26. Korzhnev DM, Orekhov VY, Kay LE. Off-resonance $R(1\rho)$ NMR studies of exchange dynamics in proteins with low spin-lock fields: an application to a Fyn SH3 domain. *J Am Chem Soc*. 2005; 127:713–721. [PubMed: 15643897]
27. Hansen AL, Nikolova EN, Casiano-Negroni A, Al-Hashimi HM. Extending the range of microsecond-to-millisecond chemical exchange detected in labeled and unlabeled nucleic acids by selective carbon $R(1\rho)$ NMR spectroscopy. *J Am Chem Soc*. 2009; 131:3818–3819. [PubMed: 19243182]
28. Johnson JE Jr, Hoogstraten CG. Extensive backbone dynamics in the GCAA RNA tetraloop analyzed using ^{13}C NMR spin relaxation and specific isotope labeling. *J Am Chem Soc*. 2008; 130:16757–16769. [PubMed: 19049467]
29. Klotz K, Spitzer R, Tollinger M, Konrat R, Kreutz C. Probing RNA dynamics via longitudinal exchange and CPMG relaxation dispersion NMR spectroscopy using a sensitive ^{13}C -methyl label. *Nucleic Acids Res*. 2011; 39:4340–4351. [PubMed: 21252295]

30. Wunderlich CH, Spitzer R, Santner T, Fauster K, Tollinger M, Kreutz C. Synthesis of (6-(13)C)pyrimidine nucleotides as spin-labels for RNA dynamics. *J Am Chem Soc.* 2012; 134:7558–7569. [PubMed: 22489874]
31. Nikolova EN, Kim E, Wise AA, O'Brien PJ, Andricioaei I, Al-Hashimi HM. Transient Hoogsteen base pairs in canonical duplex DNA. *Nature.* 2011; 470:498–502. [PubMed: 21270796]
- 32••. Dethoff EA, Petzold K, Chugh J, Casiano-Negroni A, Al-Hashimi HM. Visualizing transient low-populated structures of RNA. *Nature.* 2012; 491:724–728. This study presents a novel strategy that opens new routes for characterizing excited-state structures. This strategy has been applied in three distinct RNAs, which have allowed for the first time detailed structural, thermodynamic, and kinetic characterizations of sparsely populated and transiently lived excited conformational states in RNA. [PubMed: 23041928]
33. Mustoe AM, Brooks CL, Al-Hashimi HM. Hierarchy of RNA functional dynamics. *Annu Rev Biochem.* 2014; 83:441–466. [PubMed: 24606137]
34. Sekhar A, Kay LE. NMR paves the way for atomic level descriptions of sparsely populated, transiently formed biomolecular conformers. *Proc Natl Acad Sci USA.* 2013; 110:12867–12874. [PubMed: 23868852]
35. Palmer AG 3rd. Chemical exchange in biomacromolecules: past, present, and future. *J Magn Reson.* 2014; 241:3–17. [PubMed: 24656076]
36. Farrow NA, Zhang O, Forman-Kay JD, Kay LE. A heteronuclear correlation experiment for simultaneous determination of 15N longitudinal decay and chemical exchange rates of systems in slow equilibrium. *J Biomol NMR.* 1994; 4:727–734. [PubMed: 7919956]
- 37•. Zhao B, Hansen AL, Zhang Q. Characterizing slow chemical exchange in nucleic acids by carbon CEST and low spin-lock field R1rho NMR spectroscopy. *J Am Chem Soc.* 2014; 136:20–23. This study presents the development of 13C CEST NMR technique, which allows structural, thermodynamic and kinetic characterizations of an excited state of the ligand-free fluoride aptamer domain that is 'invisible' to conventional NMR methods. [PubMed: 24299272]
38. Palmer AG 3rd, Kroenke CD, Loria JP. Nuclear magnetic resonance methods for quantifying microsecond-to-millisecond motions in biological macromolecules. *Methods Enzymol.* 2001; 339:204–238. [PubMed: 11462813]
39. Korzhnev DM, Kay LE. Probing invisible, low-populated States of protein molecules by relaxation dispersion NMR spectroscopy: an application to protein folding. *Acc Chem Res.* 2008; 41:442–451. [PubMed: 18275162]
40. Forsen S, Hoffman RA. Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonance. *J Chem Phys.* 1963; 39:2892.
41. Fawzi NL, Ying J, Ghirlando R, Torchia DA, Clore GM. Atomic-resolution dynamics on the surface of amyloid-beta protofibrils probed by solution NMR. *Nature.* 2011; 480:268–272. [PubMed: 22037310]
42. Vallurupalli P, Bouvignies G, Kay LE. Studying invisible excited protein states in slow exchange with a major state conformation. *J Am Chem Soc.* 2012; 134:8148–8161. [PubMed: 22554188]
43. Palmer AG 3rd, Massi F. Characterization of the dynamics of biomacromolecules using rotating-frame spin relaxation NMR spectroscopy. *Chem Rev.* 2006; 106:1700–1719. [PubMed: 16683750]
- 44••. Reining A, Nozinovic S, Schlepckow K, Buhr F, Furtig B, Schwalbe H. Three-state mechanism couples ligand and temperature sensing in riboswitches. *Nature.* 2013; 499:355–359. This study presents the discovery of a novel three-state mechanism that underlies the gene regulation by the *add* adenine riboswitch. The excited state in the ligand-free riboswitch serves as a critical intermediate state to ensure robust gene regulation across a broad range of temperatures. [PubMed: 23842498]
- 45•. Santner T, Rieder U, Kreutz C, Micura R. Pseudoknot preorganization of the preQ1 class I riboswitch. *J Am Chem Soc.* 2012; 134:11928–11931. This study presents a site-specific labeling strategy that allows unambiguous secondary structure determination of an excited state of the ligand-free preQ1 aptamer domain. [PubMed: 22775200]
46. Klein DJ, Edwards TE, Ferre-D'Amare AR. Cocrystal structure of a class I preQ1 riboswitch reveals a pseudoknot recognizing an essential hypermodified nucleobase. *Nat Struct Mol Biol.* 2009; 16:343–344. [PubMed: 19234468]

47. Spitale RC, Torelli AT, Krucinska J, Bandarian V, Wedekind JE. The structural basis for recognition of the PreQ0 metabolite by an unusually small riboswitch aptamer domain. *J Biol Chem*. 2009; 284:11012–11016. [PubMed: 19261617]
48. Kang M, Peterson R, Feigon J. Structural insights into riboswitch control of the biosynthesis of queuosine, a modified nucleotide found in the anticodon of tRNA. *Mol Cell*. 2009; 33:784–790. [PubMed: 19285444]
49. Wilson RC, Smith AM, Fuchs RT, Kleckner IR, Henkin TM, Foster MP. Tuning riboswitch regulation through conformational selection. *J Mol Biol*. 2011; 405:926–938. [PubMed: 21075119]
50. Chen B, Zuo X, Wang YX, Dayie TK. Multiple conformations of SAM-II riboswitch detected with SAXS and NMR spectroscopy. *Nucleic Acids Res*. 2012; 40:3117–3130. [PubMed: 22139931]
51. Ren A, Rajashankar KR, Patel DJ. Fluoride ion encapsulation by Mg^{2+} ions and phosphates in a fluoride riboswitch. *Nature*. 2012; 486:85–89. [PubMed: 22678284]
52. Houck-Loomis B, Durney MA, Salguero C, Shankar N, Nagle JM, Goff SP, D'Souza VM. An equilibrium-dependent retroviral mRNA switch regulates translational recoding. *Nature*. 2011; 480:561–564. This study presents the discovery of a novel equilibrium-based mechanism that underlies the regulation of readthrough efficiency by the MLV recoding signal. The excited state has a compact pseudoknot conformation that interacts with the ribosome to ensure proper bypass to produce Gag-Pol fusion protein. [PubMed: 22121021]
53. Baranov PV, Gesteland RF, Atkins JF. Recoding: translational bifurcations in gene expression. *Gene*. 2002; 286:187–201. [PubMed: 11943474]
54. Felsenstein KM, Goff SP. Expression of the gag-pol fusion protein of Moloney murine leukemia virus without gag protein does not induce virion formation or proteolytic processing. *J Virol*. 1988; 62:2179–2182. [PubMed: 2452901]
55. Parisien M, Major F. The MC-Fold and MC-Sym pipeline infers RNA structure from sequence data. *Nature*. 2008; 452:51–55. [PubMed: 18322526]
56. Lee J, Dethoff EA, Al-Hashimi HM. Invisible RNA state dynamically couples distant motifs. *Proc Natl Acad Sci USA*. 2014; 111:9485–9490. This study presents the detection of a second excited state in HIV-1 TAR RNA, providing significant insights into the intrinsic conformational plasticity encoded in RNA. [PubMed: 24979799]
57. Bannwarth S, Gatignol A. HIV-1 TAR RNA: the target of molecular interactions between the virus and its host. *Current HIV Res*. 2005; 3:61–71.
58. Latham MP, Brown DJ, McCallum SA, Pardi A. NMR methods for studying the structure and dynamics of RNA. *Chembiochem*. 2005; 6:1492–1505. [PubMed: 16138301]
59. Zhang Q, Kang M, Peterson RD, Feigon J. Comparison of solution and crystal structures of preQ1 riboswitch reveals calcium-induced changes in conformation and dynamics. *J Am Chem Soc*. 2011; 133:5190–5193. [PubMed: 21410253]
60. Matera AG, Wang Z. A day in the life of the spliceosome. *Nat Rev Mol Cell Biol*. 2014; 15:108–121. [PubMed: 24452469]
61. Madhani HD, Guthrie C. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell*. 1992; 71:803–817. [PubMed: 1423631]
62. Reiter NJ, Blad H, Abildgaard F, Butcher SE. Dynamics in the U6 RNA intramolecular stem-loop: a base flipping conformational change. *Biochemistry*. 2004; 43:13739–13747. [PubMed: 15504036]
63. Reiter NJ, Nikstad LJ, Allmann AM, Johnson RJ, Butcher SE. Structure of the U6 RNA intramolecular stem-loop harboring an S(P)-phosphorothioate modification. *RNA*. 2003; 9:533–542. [PubMed: 12702812]
64. Igumenova TI, Brath U, Akke M, Palmer AG 3rd. Characterization of chemical exchange using residual dipolar coupling. *J Am Chem Soc*. 2007; 129:13396–13397. [PubMed: 17929930]
65. Vallurupalli P, Hansen DF, Stollar E, Meirovitch E, Kay LE. Measurement of bond vector orientations in invisible excited states of proteins. *Proc Natl Acad Sci U S A*. 2007; 104:18473–18477. [PubMed: 18006656]

66. Clore GM, Iwahara J. Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. *Chem Rev.* 2009; 109:4108–4139. [PubMed: 19522502]
67. Frank AT, Horowitz S, Andricioaei I, Al-Hashimi HM. Utility of ¹H NMR chemical shifts in determining RNA structure and dynamics. *J Phys Chem B.* 2013; 117:2045–2052. [PubMed: 23320790]
68. Sripakdeevong P, Cevc M, Chang AT, Erat MC, Ziegeler M, Zhao Q, Fox GE, Gao X, Kennedy SD, Kierzek R, et al. Structure determination of noncanonical RNA motifs guided by (1)H NMR chemical shifts. *Nat Methods.* 2014; 11:413–416. [PubMed: 24584194]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

NMR characterization of excited conformational states in RNA. **(a)** A hierarchical free-energy landscape of RNA conformational transitions. *Top panel:* Tier-0 conformational transitions consist of large-scale changes in secondary structures such as pseudoknot formation from a hairpin. Due to high-energy barriers separating different secondary structures, conformational transition from a Tier-0 ground state (GS) to a Tier-0 excited state (ES) occurs at the timescale of tens-of-milliseconds to seconds and longer. *Middle panel:* Tier-1 conformational transitions consist of local base-pairing re-configurations such as base pair reshuffling within a hairpin. The energy barriers between Tier-1 states are smaller than those between Tier-0 states, and the resulting conformational transition from a Tier-1 GS to a Tier-1 ES occurs at the timescale of microseconds to tens-of-milliseconds. *Bottom panel:* Tier-2 conformational transitions consist of fine-tuned conformational changes such as base stacking dynamics and interhelical motions. With energy barriers of only a few $k_B T$, conformational transition from a Tier-2 GS to a Tier-2 ES occurs at the timescale of picoseconds to microseconds. **(b)** Solution-state NMR techniques for characterizing excited conformational states in RNA. *Left panel:* timescales of exchange processes and corresponding suitable NMR experiments (solid lines). Dashed lines indicate regions that are challenging to study by a given NMR experiment. *Right panel:* schematic representation of experimental profiles measured with different NMR techniques for characterizing excited states in RNA. NMR resonance for a sparsely populated and shortly lived excited state, which is not visible in a conventional Heteronuclear Single Quantum Coherence (HSQC) spectrum, is illustrated as red dashed circles for clarity.

Figure 2.

NMR characterization of Tier-0 excited conformational states. **(a)** The *Vibrio vulnificus* add adenine riboswitch in the absence of ligand undergoes a temperature-dependent conformational exchange between a ground state and a minor state at a rate of 1.31 s^{-1} at 25°C . The ground state is a binding-inactive translational-off state, and has a population ranging between $\sim 90\%$ at 10°C to $\sim 60\%$ at 40°C . The minor or excited state is a binding-active translational-off state, and has a population ranging between $\sim 10\%$ at 10°C to $\sim 40\%$ at 40°C . The minor/excited state is subsequently switched to the translational-on state in the presence of adenine. **(b)** The *Fusobacterium nucleatum* preQ1 riboswitch aptamer domain in the absence of ligand undergoes a conformational exchange between an unfolded ground state ($p_G \sim 90\%$) and a pseudoknot excited state ($p_E \sim 0\%$) at 25°C . A site-specific labeling strategy was applied on residues A3, A10, U20, and U32 to facilitate secondary structure confirmation of a proposed pseudoknot excited state of the ligand-free aptamer. As shown in dashed arrows, the pseudoknot state may be selected by preQ1 during adaptive recognition toward the ligand-bound active state. **(c)** The *Bacillus cereus* fluoride riboswitch aptamer in

the absence of ligand undergoes a conformational exchange between an unfolded ground state ($p_G = 90\%$) and a potential pseudoknot-like excited state ($p_E = 10\%$) at a rate of 112 s^{-1} at 30°C . This excited state is NMR ‘invisible’, and its detection and identification were obtained based on ^{13}C CEST NMR spectroscopy. As shown in dashed arrows, this potential pseudoknot excited state may also be selected by fluoride and magnesium during adaptive recognition toward the ligand-bound active state.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

NMR characterization of high-tier excited conformational states. (a–d) Tier-1 excited conformational states. (a) The MLV recoding signal has an overall pseudoknot secondary structure that undergoes a pH-dependent conformational exchange between a ground state ($p_G \sim 94\%$) and an excited state ($p_E \sim 6\%$) at physiological pH. The ground state has a loose pseudoknot conformation that inactivates the readthrough by the ribosome to produce only Gag protein. The excited state has a compact pseudoknot conformation with a base triple between protonated A17 and residues C23 and G53 as well as tertiary interactions between L2 loop and S1 helix. The compact pseudoknot excited state interacts with the ribosome to ensure the later to bypass the stop codon for producing Gag-Pol fusion protein. (b) The HIV-1 TAR RNA has an overall hairpin secondary structure that undergoes complex

conformational exchanges between one ground state ($p_G = 86.6\%$) and two excited states ($p_{E1} = 13\%$ and $p_{E2} = 0.4\%$). The ground state has a hexanucleotide loop that allows key residues C30, U31, G34, and A35 to interact with Tat and Cyclin T1. The apical loop of HIV-1 TAR undergoes a microsecond-timescale exchange toward the first excited state (ES1), which sequesters key loop residues to form an autoinhibited state that prevents transactivation of the HIV-1 genome. The two bulge residues and the entire upper helix undergoes a millisecond-timescale exchange toward the second excited state (ES2), which has a global register shift with the formation of four non-canonical base pairs. **(c)** The ribosomal A-site internal loop undergoes a conformational exchange between a ground state ($p_G = 97.5\%$) and an excited state ($p_E = 2.5\%$). The ground state has a flexible bulge of A1492 (A92) and A1493 (A93) that can flip out to interact with the codon–anticodon base pair between tRNA and mRNA for translational fidelity. The excited state flips out residue U95 and sequesters both A92 and A93 into a helical conformation, which impairs the decoding and disrupts formation of the B2a intersubunit bridge as highlighted by the green dashed line. **(d)** The HIV-1 SL1 undergoes complex conformational exchange between one ground state ($p_G = 89\%$) and two excited states ($p_{E1} = 9\%$ and $p_{E2} = 2\%$) that are centered on seven base pairs around the SL1 bulge. While the GS has a central-located internal bulge, ES1 and ES2 have upward and downward migrated bulges, respectively. This branched three-state reshuffling process generates a ‘moving zipper’ that provides a mechanism for ATP-independent HIV-1 SL1 isomerization. **(e)** A Tier-2 excited conformational state in the U6 spliceosomal RNA ISL. The U6 SL1 undergoes a conformational exchange between the ground state and the excited state that features a looped out residue U80 and a protonated residue A79 that forms a non-canonical base pair with C67. The looping out process of residue U80 may provide a pH-sensitive switch for controlling Mg^{2+} binding at the U6 ISL RNA.